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– ROZPRAWA DOKTORSKA –

**Synteza i badanie aktywności biologicznej pochodnych
związków pochodzenia naturalnego: ivermektyny i monenzyny**

*Synthesis and biological activity evaluation of derivatives of natural origin
compounds: ivermectin and monensin*

Praca wykonana

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– No, bo w naszym kraju – rzekła Alicja, ciągle jeszcze trochę zadyszana – na ogół byłoby się gdzie indziej, gdyby się bardzo długo i prędko biegło, tak jak myśmy biegły.

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Lewis Carroll, *Po drugiej stronie lustra*

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Badania dotyczące chemicznej modyfikacji iwermektyny mające na celu otrzymanie nowych pochodnych wykazujących aktywność przeciwnowotworową oraz przeciwpasożytniczą finansowane były przez Ministerstwo Nauki i Szkolnictwa Wyższego w ramach projektu Diamentowy Grant (nr 0159/DIA/2020/49), którego byłem kierownikiem.



**Ministerstwo Nauki
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1. Wykaz skrótów stosowanych w pracy

A549	–	linia komórkowa nowotworu płuc (ang. <i>lung cancer cell line</i>)
BALB-3T3	–	prawidłowe mysie fibroblasty (ang. <i>murine embryonic fibroblasts</i>)
BLM	–	czarna błona lipidowa (ang. <i>black-lipid membrane</i>)
CDI	–	karbonylodiimidazol (ang. <i>carbonyldiimidazole</i>)
CSCs	–	macierzyste komórki nowotworowe (ang. <i>cancer stem cells</i>)
DBU	–	1,8-diazabicyklo[5.4.0]undek-7-en
DCC	–	<i>N,N'</i> -dicykloheksylokarbodiimid (ang. <i>N,N'-dicyclohexylcarbodiimide</i>)
DIPEA	–	<i>N,N</i> -diizopropyletyloamina (ang. <i>N,N-diisopropylethylamine</i>)
DMF	–	<i>N,N</i> -dimetyloformamid (ang. <i>N,N-dimethylformamide</i>)
DMP	–	nadjodan Dessa-Martina (ang. <i>Dess-Martin periodinane</i>)
DMSO	–	dimetylosulfotlenek (ang. <i>dimethyl sulfoxide</i>)
DNA	–	kwas deoksyrybonukleinowy (ang. <i>deoxyribonucleic acid</i>)
ESI-MS	–	spektrometria mas z jonizacją metodą elektrorozpylania (ang. <i>electrospray ionization mass spectrometry</i>)
FT-IR	–	spektroskopia w podczerwieni z transformacją Fouriera (ang. <i>Fourier transform infrared spectroscopy</i>)
GABA	–	kwas γ -aminomasłowy (ang. <i>γ-aminobutyric acid</i>)
GI ₅₀	–	półmaksymalne zahamowanie wzrostu (ang. <i>half-maximal growth inhibition</i>)
GluCl	–	glutaminianowe kanały chlorkowe (ang. <i>glutamate-gated chloride ion channels</i>)
HaCaT	–	ludzkie keratynocyty (ang. <i>human keratinocytes</i>)
HATU	–	heksafluorofosforan azabenzotriazolotetrametylouranylowy (ang. <i>hexafluorophosphate azabenzotriazole tetramethyl uranium</i>)
HCT-116	–	linia komórkowa nowotworu jelita grubego (ang. <i>colon carcinoma cell line</i>)
HL-60	–	linia komórkowa białaczki promielocytowej (ang. <i>promyelocytic leukemia cell line</i>)
HOBt	–	<i>N</i> -hydroksybenzotriazol (ang. <i>N-hydroxybenzotriazole</i>)
HRMS	–	wysokorozdzielcza spektrometria mas (ang. <i>high-resolution mass spectrometry</i>)
Huh-7	–	linia komórkowa wątrobiaka (ang. <i>hepatoma cell line</i>)
IC ₅₀	–	półmaksymalne stężenie hamujące (ang. <i>half-maximal inhibitory concentration</i>)
IL-6	–	interleukina 6 (ang. <i>interleukin 6</i>)
IWR	–	iwermektyna (ang. <i>ivermectin</i>)
LoVo	–	linia komórkowa gruczolaka okrężnicy (ang. <i>colon adenocarcinoma</i>)
LoVo/DX	–	linia komórkowa gruczolaka okrężnicy oporna na działanie doksorubicyny (ang. <i>doxorubicin-resistant subline of LoVo</i>)

MCF-7	–	linia komórkowa nowotworu piersi (ang. <i>breast adenocarcinoma</i>)
MDA-MB-231	–	linia komórkowa potrójnie-ujemnego nowotworu piersi (ang. <i>triple-negative breast adenocarcinoma</i>)
MDR	–	oporność wielolekowa (ang. <i>multi-drug resistance</i>)
MON	–	monenzyna (ang. <i>monensin</i>)
NMR	–	spektroskopia magnetycznego rezonansu jądrowego (ang. <i>nuclear magnetic resonance</i>)
NTDs	–	zaniedbane choroby tropikalne (ang. <i>neglected tropical diseases</i>)
PC3	–	linia komórkowa przerzutowego nowotworu prostaty (ang. <i>metastatic prostate cancer cell line</i>)
<i>p</i> -TsOH	–	kwas <i>para</i> -toluenosulfonowy (ang. <i>para-toluenesulfonic acid</i>)
py	–	pirydyna (ang. <i>pyridine</i>)
ROS	–	reaktywne formy tlenu (ang. <i>reactive-oxygen species</i>)
SAR	–	zależność pomiędzy strukturą a aktywnością biologiczną (ang. <i>structure-activity relationship</i>)
scXRD	–	badania dyfrakcyjne monokryształów (ang. <i>single crystal X-ray diffraction</i>)
SD	–	odchylenie standardowe (ang. <i>standard deviation</i>)
SI	–	współczynnik selektywności (ang. <i>selectivity index</i>)
SW480	–	linia komórkowa pierwotnego nowotworu jelita grubego (ang. <i>primary colon cancer cells</i>)
SW620	–	linia komórkowa przerzutowego nowotworu jelita grubego (ang. <i>metastatic colon cancer cells</i>)
TBAB	–	bromek tetrabutylamoniowy (ang. <i>tetrabutylammonium bromide</i>)
TBAF	–	fluorek tetrabutylamoniowy (ang. <i>tetrabutylammonium fluoride</i>)
TBAI	–	jodek tetrabutylamoniowy (ang. <i>tetrabutylammonium iodide</i>)
TBDMSCl	–	chlerek <i>tert</i> -butylodimetylosililowy (ang. <i>tert-butyldimethylsilyl chloride</i>)
TEA	–	trietyloamina (ang. <i>triethylamine</i>)
THF	–	tetrahydrofuran (ang. <i>tetrahydrofuran</i>)
TLC	–	chromatografia cienkowarstwowa (ang. <i>thin-layer chromatography</i>)
tp	–	temperatura pokojowa (ang. <i>room temperature</i>)
TPP ⁺	–	kation trifenylofosfoniowy (ang. <i>triphenylphosphonium cation</i>)
V79	–	fibroblasty płuc chomika chińskiego (ang. <i>Chinese hamster lung fibroblasts</i>)
WHO	–	Światowa Organizacja Zdrowia (ang. <i>World Health Organization</i>)

2. Streszczenie w języku polskim

Celem niniejszej rozprawy doktorskiej były synteza oraz analiza spektroskopowa i spektrometryczna pochodnych związków pochodzenia naturalnego – iwermektyny i monenzyny. Ponadto, otrzymane analogi zostały zbadane pod kątem ich aktywności przeciwnowotworowej i/lub przeciwpasożytniczej wobec różnorodnych linii komórkowych ludzkich nowotworów oraz modelowych gatunków pasożytów chorobotwórczych (testy *in vitro*), co pozwoliło na określenie korelacji pomiędzy strukturą tych związków a ich aktywnością biologiczną.

Badania przeprowadzone w ramach niniejszej rozprawy doktorskiej są odpowiedzią na najważniejsze wyzwania współczesnej nauki i medycyny stawiane przez Światową Organizację Zdrowia (WHO). Zgodnie z prognozami WHO liczba nowych zachorowań na choroby nowotworowe będzie w następnych latach dynamicznie wzrastać, osiągając poziom około 30 milionów przypadków w 2040 roku.

Rozwój lekoopornych form pasożytów może z kolei w przyszłości znacząco utrudnić leczenie między innymi malarii – najbardziej śmiertelnej choroby pasożytniczej na świecie – oraz śpiączki afrykańskiej należącej do grupy tzw. zaniedbanych chorób tropikalnych. W świetle tych doniesień konieczne jest zatem poszukiwanie nowych kandydatów na leki, szczególnie do walki ze wspomnianymi powyżej chorobami.

Za obiekty moich badań wybrałem iwermektynę i monenzynę – związki pochodzenia naturalnego wykazujące szerokie spektrum właściwości biologicznych. Ich zastosowanie w medycynie i/lub weterynarii wynika z wyjątkowo wysokiej aktywności przeciwpasożytniczej. Ponadto, w przypadku obydwu związków udowodniono wysoką skuteczność w niszczeniu komórek nowotworowych obejmującą różne mechanizmy działania. Racjonalna modyfikacja struktury tych związków może doprowadzić do analogów o lepszym potencjale terapeutycznym (wyższej aktywności biologicznej i selektywności działania) niż związki wyjściowe.

Rozprawa doktorska obejmuje cykl siedmiu publikacji naukowych (I–VII) oraz komentarz do tych prac. W badaniach nad iwermektyną skupiłem się na modyfikacji chemicznej pozycji C13, która łączy fragment aglikonowy cząsteczki z komponentem cukrowym. W wyniku wieloetapowych syntez otrzymałem serię C13-*epi*-amidów iwermektyny oraz jej biokoniugatów z innymi związkami biologicznie aktywnymi.

Ponadto, odkryłem nową, nieznaną wcześniej reakcję przegrupowania układu heksahydrobenzofuranowego iwermektyny, która doprowadziła do otrzymania serii jej podwójnie modyfikowanych analogów.

W pracy eksperymentalnej nad modyfikacją chemiczną monenzyny skoncentrowałem się na modyfikacji jej w pozycjach C1 oraz C26. W efekcie otrzymałem biokoniugaty monenzyny z kationem trifenylofosfoniowym, tj. hybrydy mające celować w mitochondria komórek nowotworowych. Opracowanie metod syntezy C26-azydowej i C26-aminowej pochodnej monenzyny otworzyło z kolei zupełnie nowe możliwości syntetyczne, których efektem były C26-*N*-acylowane analogi oraz produkty makrocycliczne.

Analiza aktywności biologicznej pozwoliła zidentyfikować wysoce bioaktywne pochodne, które swoją skutecznością wielokrotnie przewyższyły związki wyjściowe. Przykładami mogą być nieprzegrupowany oraz przegrupowany amid 2-chloroacetylowy iwermektyny, które wykazały około 230-krotnie wyższą aktywność *in vitro* wobec pasożytów wywołujących śpiączkę afrykańską niż niemodyfikowana iwermektyna. Z kolei koniugat z artezunatem efektywnie hamował proliferację komórek nowotworowych użytych w testach oraz charakteryzował się brakiem cytotoksyczności wobec linii referencyjnej. Z drugiej strony, uretan *O*-fenyłowy monenzyny okazał się około 8-krotnie bardziej aktywny w zwalczaniu pasożytów wywołujących malarię niż związek wyjściowy, osiągając wartość parametru IC₅₀ niższą niż 1 nM.

Przeprowadzone przeze mnie badania udowodniły, że modyfikacja chemiczna struktury iwermektyny i monenzyny jest obiecującą metodą na poszukiwanie nowych, bioaktywnych związków o wysokim potencjale przeciwnowotworowym oraz przeciwpasożytniczym. Wiele z przeprowadzonych badań, w tym ocena właściwości przeciwnowotworowych pochodnych iwermektyny, opracowanie reakcji jej przegrupowania bądź syntezy C26-azydku i C26-aminy monenzyny, zostały przeprowadzone po raz pierwszy. Stawia to swego rodzaju „drogowskaz”, który nie tylko może wskazać naukowcom nowe ścieżki badawcze, ale też udowadnia, że modyfikacje chemiczne znanych związków pochodzenia naturalnego nadal może być źródłem obiecujących rezultatów badawczych, nieoczekiwanych odkryć i inspiracji.

3. Streszczenie w języku angielskim

The aim of this doctoral dissertation was the synthesis as well as spectroscopic and spectrometric analysis of derivatives of natural origin compounds – ivermectin and monensin. Additionally, the obtained analogs were examined for their anticancer and/or antiparasitic activity against various human cancer cell lines and model species of pathogenic parasites (*in vitro* assays), which allowed for the determination of the correlation between the structure of these compounds and their biological activity.

The research conducted within the framework of this doctoral dissertation is a response to the most important challenges faced by modern science and medicine, as identified by the World Health Organization (WHO). According to WHO projections, the number of new cancer cases will increase dynamically in the coming years, reaching around 30 million cases by 2040.

The development of drug-resistant forms of parasites may, in the future, significantly hinder the treatment of diseases such as malaria – the most deadly parasitic disease in the world – as well as African sleeping sickness, which belongs to the group of so-called neglected tropical diseases. In light of these reports, it is crucial to search for new drug candidates, particularly for combating the aforementioned diseases.

I selected ivermectin and monensin as the subjects of my research – naturally occurring compounds that exhibit a broad spectrum of biological properties. Their use in medicine and/or veterinary science is due to their exceptionally high antiparasitic activity. Furthermore, both compounds have been shown to be highly effective in destroying cancer cells, involving various mechanisms of action. Rational modification of the structure of these compounds could lead to analogs with better therapeutic potential (higher biological activity and selectivity) than the parent compounds.

This doctoral dissertation consists of a series of seven scientific publications (I–VII) and a commentary on these works. In the research on ivermectin, I focused on the chemical modification of the C13 position, which connects the aglycone fragment of the molecule with the sugar component. As a result of multi-step syntheses, I obtained a series of C13-*epi*-amides of ivermectin and its bioconjugates with other biologically active compounds. Additionally, I discovered a new, previously unknown rearrangement reaction

of the hexahydrobenzofuran system of ivermectin, which led to the synthesis of a series of its doubly modified analogs.

In the experimental work on the chemical modification of monensin, I focused on modifying it at the C1 and C26 positions. As a result, I obtained bioconjugates of monensin with the trifenylphosphonium cation, i.e., hybrids targeting the mitochondria of cancer cells. The development of synthesis methods for the C26-azide and C26-amino derivatives of monensin opened entirely new synthetic possibilities, leading to the C26-*N*-acylated analogs and macrocyclic products.

The analysis of biological activity allowed for the identification of highly bioactive analogs, which greatly surpassed the parent compounds in terms of effectiveness. Examples include the non-rearranged and rearranged 2-chloroacetyl amides of ivermectin which demonstrated approximately 230 times higher *in vitro* activity against parasites causing African sleeping sickness compared to unmodified ivermectin. On the other hand, the conjugate of this drug with artesunate effectively inhibited the proliferation of cancer cells used in the tests and exhibited a lack of cytotoxicity towards the reference cell line. Additionally, the *O*-phenyl urethane of monensin proved to be about 8 times more active against malaria-causing parasites than the parent compound, achieving an IC₅₀ value lower than 1 nM.

The research I conducted demonstrated that chemical modification of the structures of ivermectin and monensin is a promising approach for the discovery of new bioactive compounds with high anticancer and antiparasitic potential. Many of the studies carried out, such as the evaluation of the anticancer properties of ivermectin derivatives, the development of its rearrangement reaction, or the synthesis of C26-azido and C26-amino derivatives of monensin, were conducted for the first time. This serves as a kind of “signpost” that not only can guide scientists toward new research directions, but also proves that chemical modifications of known natural compounds can still be a source of promising research outcomes, unexpected discoveries, and inspiration.

4. Cele pracy

Głównym założeniem prowadzonych przeze mnie badań było przeprowadzenie chemicznej modyfikacji wybranych bioaktywnych związków pochodzenia naturalnego, co umożliwiłoby otrzymanie pochodnych o lepszym profilu terapeutycznym niż związki wyjściowe, tj. pochodnych o wyższej aktywności biologicznej. Celem niniejszej rozprawy doktorskiej było:

- (1) przeprowadzenie selektywnych modyfikacji iwermektyny i monenzyny;
- (2) szczegółowe zbadanie struktury i ocena czystości otrzymanych analogów przy użyciu metod spektroskopowych, spektrometrycznych oraz rentgenostrukturalnych;
- (3) określenie wpływu wprowadzonych modyfikacji na właściwości przeciwnowotworowe oraz przeciwpasożytnicze otrzymanych analogów. W tym celu zsyntetyzowane pochodne zostały poddane testom na aktywność przeciwproliferacyjną wobec różnorodnych linii komórkowych ludzkich nowotworów oraz aktywność przeciwpasożytniczą wobec modelowych gatunków pasożytów chorobotwórczych (testy *in vitro*). Ponadto, badania zostały przeprowadzone również na zdrowych komórkach w celu określenia cytotoksyczności i selektywności działania otrzymanych związków;
- (4) określenie zależności pomiędzy strukturą a aktywnością biologiczną (ang. *structure-activity relationship, SAR*) nowych pochodnych iwermektyny i monenzyny.

Ze względu na interdyscyplinarny charakter projektu, realizacja założonych celów była możliwa dzięki nawiązaniu współpracy z naukowcami z wiodących krajowych i zagranicznych ośrodków naukowo-badawczych, w szczególności z:

- a) dr. Dietmarem Steverdingiem z Bob Champion Research & Education Building, Norwich Medical School, University of East Anglia, Wielka Brytania;
- b) prof. Miguelem Prudêncio, dr Dianą Fontinhą, dr Sofią Santanę, Eyobem Worknehem, MSc i Bárbarą Teixeirą, MSc z Instituto de Medicina Molecular João Lobo Antunes, Faculdade de Medicina, Universidade de Lisboa & Gulbenkian Institute for Molecular Medicine, Avenida Prof. Egas Moniz, Portugalia;
- c) prof. Martą Strugą, dr Dagmarą Otto-Ślusarczyk, dr Magdaleną Mielczarek-Putą, dr Małgorzatą Podsiad i Gwan Yong Limem z Katedry i Zakładu

Biochemii, Wydziału Lekarskiego Warszawskiego Uniwersytetu Medycznego;

- d) prof. Piotrem Bednarczykiem i mgr. Jakubem Hoserem z Katedry Fizyki i Biofizyki, Instytutu Biologii, Szkoły Głównej Gospodarstwa Wiejskiego w Warszawie;
- e) prof. Joanną Wietrzyk i dr Dąmą Kłopotowską z Instytutu Immunologii i Terapii Doświadczalnej Polskiej Akademii Nauk im. Ludwika Hirszfelda we Wrocławiu;
- f) prof. Janem Janczakiem z Instytutu Niskich Temperatur i Badań Strukturalnych Polskiej Akademii Nauk we Wrocławiu;
- g) dr. Szymonem Sobczakiem z Zakładu Chemii Materiałów, Wydziału Chemii Uniwersytetu im. Adama Mickiewicza w Poznaniu.

Realizacja powyższych celów badawczych (1–4) doprowadziła do otrzymania bogatej biblioteki nowych pochodnych iwermektyny oraz monenzyny, w obrębie której możliwe było zidentyfikowanie ich wysoce bioaktywnych i selektywnych analogów o wysokim potencjale terapeutycznym (tzw. „hit compounds”). Uzyskany materiał badawczy powinien stanowić swoisty „drogowskaz” umożliwiający naukowcom bardziej racjonalne projektowanie nowych, bioaktywnych związków w oparciu o szkielety iwermektyny i monenzyny, które w przyszłości mogą zostać potencjalnymi kandydatami na nowe leki przeciwnowotworowe i/lub przeciw pasożytnicze.

5. Wstęp

Choroby nowotworowe to niezwykle poważny problem cywilizacyjny, który dotyka wszystkie grupy społeczne i wiekowe, stanowiąc jedną z głównych przyczyn zgonów we współczesnym świecie [1,2]. Pojęciem „nowotwór” określa się grupę chorób, które charakteryzują się utratą kontroli organizmu nad naturalnym procesem wzrostu i podziału komórkowego, prowadząc do powstania tzw. guza pierwotnego, który niszczy sąsiednie tkanki [3]. Następnie dochodzi do jego dalszego rozprzestrzeniania na inne tkanki i narządy, prowadząc do tzw. przerzutów [3]. Według danych Światowej Organizacji Zdrowia (ang. *World Health Organization, WHO*) w 2020 roku odnotowano blisko 10 milionów zgonów spowodowanych chorobami nowotworowymi oraz ponad 19 milionów nowych przypadków zachorowań [1,2]. Zgodnie z prognozami opublikowanymi przez WHO, wartości te będą dynamicznie wzrastać w kolejnych latach, osiągając poziom około 30 milionów zachorowań w 2040 roku, co będzie poważnym obciążeniem dla światowego systemu ochrony zdrowia [1,2]. Przytoczone dane jednoznacznie potwierdzają, że walka z tą grupą różnorodnych i niezwykle trudnych do zwalczenia chorób jest jednym z najważniejszych wyzwań współczesnej nauki i medycyny. Powszechnie stosowane metody leczenia chorób nowotworowych uwzględniają radioterapię, medycynę nuklearną, leczenie chirurgiczne lub systemowe (w tym głównie chemioterapię). Wiele ze współcześnie stosowanych chemioterapeutyków, np. cisplatyna lub paklitaksel, charakteryzuje się niską selektywnością działania, niszcząc nie tylko komórki nowotworowe, ale również zdrowe, co powoduje szereg niebezpiecznych dla pacjenta skutków ubocznych [4]. Koniecznością jest więc poszukiwanie nowych chemioterapeutyków, które poza wysoką aktywnością przeciwnowotworową, będą działać selektywnie, co zwiększy nie tylko skuteczność, ale i komfort leczenia onkologicznego.

Oprócz chorób nowotworowych, ogromne zagrożenie dla współczesnego świata stanowią epidemie chorób pasożytniczych. Jedną z nich – malaria, zgodnie z danymi WHO, odpowiadała za ponad 600 tysięcy zgonów w 2023 roku, co czyni ją najbardziej śmiertelnością chorobą pasożytniczą na świecie [5]. Jest ona wywoływana przez pasożyty *Plasmodium* przenoszone w formie sporozoitów przez zakażone samice komarów *Anopheles* [5,6]. Po ukąszeniu przez zakażonego owada sporozycyty infekują hepatocyty ssaka, w których dochodzi do schizogonii i wytworzenia licznych merozoitów, które po uwolnieniu do krwiobiegu cyklicznie infekują erytrocyty, wywołując objawy malarii, takie

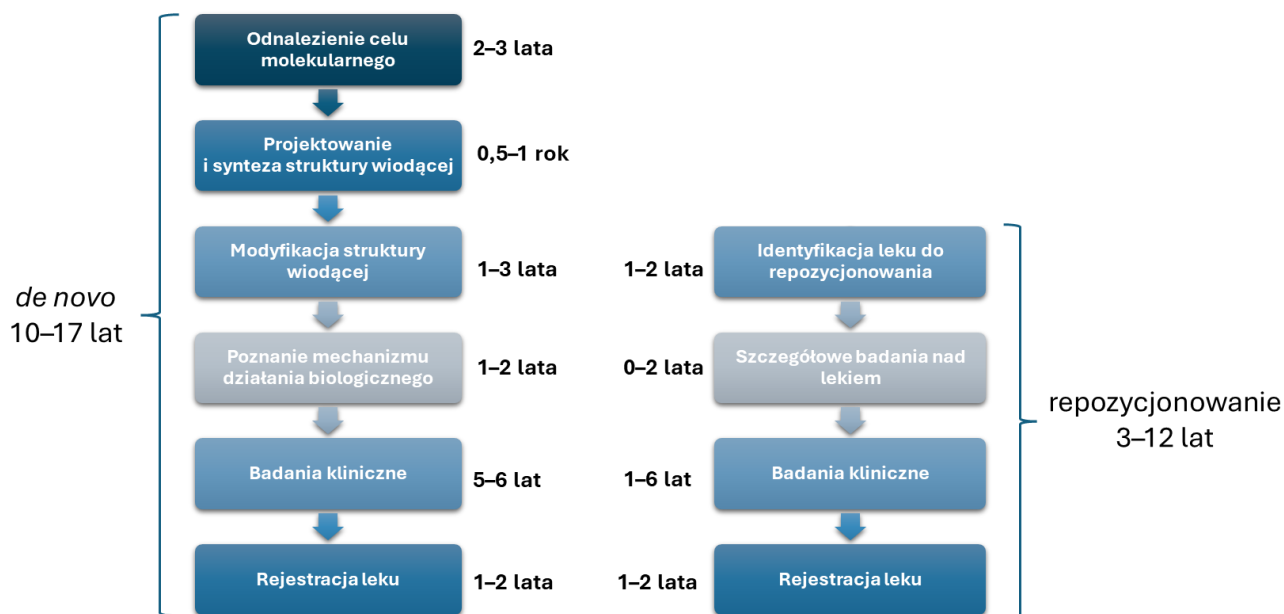
jak: gorączka i ból głowy. Choroba może szybko postępować, prowadząc do ciężkich powikłań, a nawet śmierci chorego [5,7,8]. Znanych jest wiele metod zwalczania i przeciwdziałania malarii. Do najważniejszych należy wprowadzenie szczepionek RTS,S/AS01 i R21/Matrix-M, jak również stosowanie leczenia chemicznego z użyciem chlorochiny, prymachiny oraz artemizyny [5,9]. Jednakże, ze względu na rosnący problem lekooporności, poszukiwanie nowych substancji skutecznych w walce z malarią pozostaje niezwykle istotnym wyzwaniem naukowym [6,7,10].

Do chorób pasożytniczych należą również tzw. zaniedbane choroby tropikalne (ang. *neglected tropical diseases, NTDs*). Jest to grupa około 20 chorób, które dotyczą głównie ludzi żyjących w biednych regionach tropikalnych i subtropikalnych, jednak szacuje się, że NTDs stanowią zagrożenie zdrowotne i ekonomiczne dla około miliarda ludzi na całym świecie [11]. Jednym z głównych przedstawicieli NTDs jest trypanosomatoza afrykańska, zwana też śpiączką afrykańską (jeśli dotyczy ludzi) lub nagana (jeśli dotyczy zwierząt), która wywoływana jest przez pasożyty z rodzaju *Trypanosoma* [12–15]. Do zakażenia dochodzi w momencie ukąszenia przez zainfekowaną muchę tse-tse (*Glossina* sp.) będącą wektorem pasożyta [12–15]. W przebiegu choroby można wyróżnić dwa stadia: fazę hemolimfatyczną, w której pasożyty dostają się do krwi i układu limfatycznego człowieka, a także fazę neurologiczną, w której pasożyty są obecne w płynie mózgowo-rdzeniowym osoby chorej [12–15]. Nieleczona śpiączka afrykańska może prowadzić do zaburzeń sensorycznych, śpiączki, a ostatecznie do śmierci [12–15]. Dzięki skutecznym działaniom podjętym przez WHO, liczba przypadków zachorowań na tę chorobę w ostatnich latach uległa znacznemu zmniejszeniu, jednak ze względu na niewielką liczbę leków oraz rosnący problem lekooporności, konieczne jest poszukiwanie nowych substancji skutecznych w walce z pasożytami z rodzaju *Trypanosoma* [11,12,16]. Ze względu na stosunkowo niską zachorowalność tego typu działania prewencyjne są póki co mało opłacalne dla przemysłu farmaceutycznego.

Dlatego niezwykle ważna misja poszukiwania nowych oraz skutecznych leków przeciw pasożytniczych spada na świat akademicki. Mimo, że dla nas – Europejczyków – problem ten wydaje się być mało istotny, należy pamiętać, że ze względu na zmiany klimatu, warunki do rozwoju malarii i innych chorób wektorowych w Europie stają się coraz lepsze [17,18]. Jest to problem na tyle naglący, że został bezpośrednio ujęty w Celach Zrównoważonego Rozwoju, tj., aby „(...) do 2030 roku wyeliminować epidemię AIDS,

gruźlicy, malarii i zaniedbanych chorób tropikalnych oraz zwalczyć wirusowe zapalenie wątroby, choroby przenoszone przez wodę oraz inne choroby zakaźne” [19].

Niestety, wprowadzenie nowego leku do użytku klinicznego jest procesem nie tylko bardzo kosztownym, ale przy tym niezwykle czaso- i pracochłonnym. Dane statystyczne jasno pokazują, że zaprojektowanie i synteza leku o aktywności przeciwnowotworowej *de novo* (od początku) to koszt około 650 milionów dolarów amerykańskich [20]. Na ten proces składa się wiele etapów, takich jak: identyfikacja i optymalizacja struktury wiodącej, badanie aktywności biologicznej i toksyczności, badania przedkliniczne oraz kliniczne. Pomimo ogromu włożonej pracy, bardzo często proces ten nie przynosi zadowalających rezultatów [21]. Obiecującą alternatywą może być tzw. strategia repozycjonowania, która polega na poszukiwaniu pożądanej aktywności biologicznej wśród leków, które zostały już wprowadzone do użytku. Dzięki temu czas potrzebny do zarejestrowania nowej substancji aktywnej ulega znacznemu skróceniu, a cały proces jest zdecydowanie bardziej korzystny ze względów ekonomicznych ([Rysunek 1](#)) [21–23].

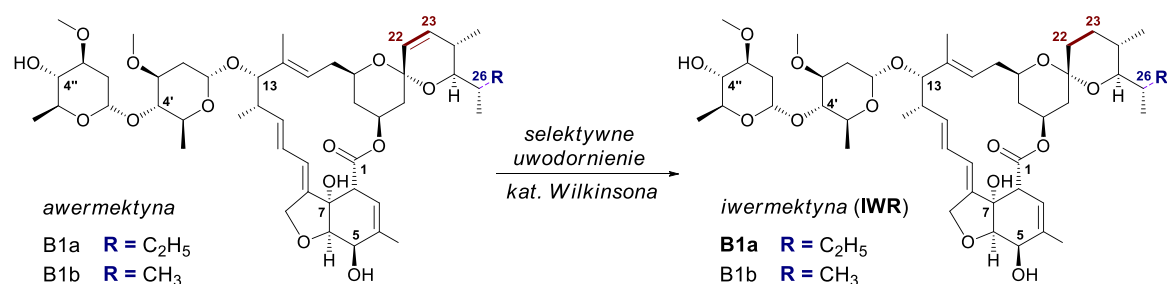


Rysunek 1. Porównanie strategii poszukiwania leków *de novo* z metodą repozycjonowania. Całkowity czas trwania pierwszego procesu wynosi od 10 do 17 lat, natomiast w drugim przypadku okres ten ulega znacznemu skróceniu i wynosi od 3 do 12 lat [21–23].

Jako że repozycjonowany lek jest klinicznie przebadany, znany jest jego mechanizm działania, cel molekularny, właściwości farmakokinetyczne oraz farmakodynamiczne [24]. Wśród leków, które początkowo znalazły zastosowanie w leczeniu konkretnych chorób pasożytniczych u ludzi i/lub zwierząt, a obecnie są

rozpatrywane jako niezwykle obiecujący kandydaci na nowe leki onkologiczne i/lub inne choroby pasożytnicze, należy wyróżnić dwa związki pochodzenia naturalnego, które stanowiły główne obiekty moich badań – ivermektynę oraz monenzynę.

Iwermektyna (**IWR**, [Rysunek 2](#)) to 16-członowy lakton będący 22,23-dihydro pochodną awermektyny, która została po raz pierwszy wyizolowana w Japonii w 1967 roku ze szczepu *Streptomyces avermitilis* [25]. **IWR** wykazuje szerokie spektrum aktywności przeciwpasożytniczej i jest stosowana w leczeniu wielu chorób, takich jak: ślepotą rzeczną (onchocerkzoza), słońowaczna, węgoreczka oraz wszawica [26]. Związek ten dopuszczono do stosowania u ludzi w 1987 roku, co znacznie poprawiło jakość życia między innymi w krajach Afryki Subsaharyjskiej i Indiach, umożliwiając leczenie chorób, które wcześniej niosły pewną śmierć [27]. Stosowana w leczeniu **IWR** to mieszanina dwóch związków: **IWR B1a**, która w pozycji C26 posiada grupę etylową, a także **IWR B1b**, która w pozycji C26 posiada grupę metylową ([Rysunek 2](#)). Mieszanina ta zawiera przynajmniej 80% **IWR B1a** oraz maksymalnie 20% **IWR B1b** [28].



[Rysunek 2](#). Struktura awermektyny oraz ivermektyny (**IWR**).

Za odkrycie i badania nad **IWR** Satoshi Ōmura oraz William Campbell zostali w 2015 roku uhonorowani Nagrodą Nobla w dziedzinie fizjologii/medycyny, a konkretnie „(...) za odkrycia dotyczące nowej terapii infekcji spowodowanych przez pasożytnicze nicienie” [29]. **IWR**, dzięki swojej skuteczności, wszechstronności i bezpieczeństwu stosowania, tak znacząco wpłynęła na świat nauki i medycyny, że wielu badaczy określa ją mianem „wonder drug”, przyrównując ten lek do aspiryny czy penicyliny [30].

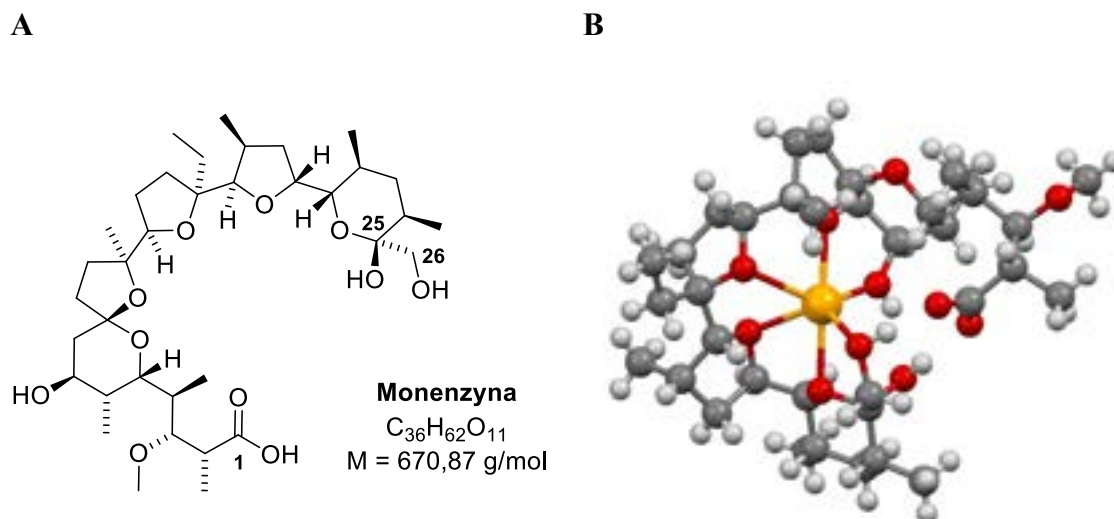
Mechanizm aktywności przeciwpasożytniczej **IWR** jest związany z jej zdolnością do wiązania się z centrum allosterycznym glutaminianowych kanałów chlorkowych (ang. *glutamate-gated chloride ion channels, GluCl*) w błonach mięśni gardła, nerwach ruchowych, żeńskich drogach rodnych oraz porach wydalniczych/wydzielniczych nicieni, a także mięśni oraz nerwów owadów i skorupiaków. Prowadzi to do hiperpolaryzacji,

paraliżu, a następnie śmierci pasożyta [30–32]. Ponadto, **IWR** jest też agonistą neurotransmitera – kwasu γ -aminomasłowego (GABA), przez co zakłóca transmisję synaptyczną w ośrodkowym układzie nerwowym pasożytów [30–32]. Co ważne, **IWR** nie przedostaje się przez barierę krew-mózg, w związku z czym efekt ten nie pojawia się u ssaków (w tym u ludzi). **IWR** jest zatem związkiem bezpiecznym. Większość skutków ubocznych związanych z jej stosowaniem, tj. świąd, biegunka i nudności, wynika z reakcji organizmu na śmierć pasożytów, a nie z działania samego leku. Dolegliwości te są na ogół łagodne i ustępują po leczeniu objawowym [33].

Oprócz działania przeciwpasożytniczego, w literaturze naukowej pojawia się coraz więcej doniesień opisujących aktywność przeciwnowotworową **IWR** wobec różnego rodzaju nowotworów, w tym: raka jelita grubego, raka piersi, glejaka, białaczki, czerniaka oraz raka prostaty [22,34]. Mechanizm biologicznego działania **IWR** wobec komórek nowotworowych jest bardzo różnorodny i dotyczy wielu procesów biochemicznych zachodzących w tychże komórkach. **IWR** wykazuje zdolność do zahamowania syntezy białek odpowiedzialnych za występowanie oporności wielolekowej (ang. *multi-drug resistance*, *MDR*) oraz szlaku sygnałowego AKT/mTOR, który jest głównym regulatorem rozwoju raka jajnika [28,34]. Ponadto, działanie **IWR** związane jest z degradacją PAK-1, głównej kinazy odpowiedzialnej za proces nowotworzenia, jak i ze zwiększeniem poziomu reaktywnych form tlenu (ang. *reactive-oxygen species*, *ROS*) wewnątrz komórek guza, co prowadzi do pojawienia się stresu oksydacyjnego, uszkodzenia DNA, a w konsekwencji do śmierci komórek nowotworowych [28,34]. Należy podkreślić, że po zastosowaniu **IWR** odnotowano znaczące zmniejszenie liczby macierzystych komórek nowotworowych (ang. *cancer stem cells*, *CSCs*), czyli niewielkiej subpopulacji komórek (5–10% masy guza), których obecność związana jest z progresem, przerzutowaniem oraz nawrotami choroby nowotworowej [28,34].

Szczegółowy przegląd literatury naukowej omawiającej ostatnie 40 lat stosowania **IWR** jako niezwykle skutecznego leku przeciwpasożytniczego w medycynie oraz weterynarii, jak również użycia tego leku w terapiach eksperymentalnych lub poza wskazaniem (ang. *off-label*) można znaleźć w pracy przeglądowej: **Michał Sulik**, Michał Antoszczak, Adam Huczyński, Dietmar Steverding, „*Antiparasitic activity of ivermectin: Four decades of research into a “wonder drug”*”, *European Journal of Medicinal Chemistry* 261 (2023) 115838. Praca ta została dołączona do cyklu publikacji stanowiących niniejszą rozprawę doktorską jako **Publikacja I**.

Drugim obiektem moich badań była monenzyna (**MON**, [Rysunek 3A](#)) – związek chemiczny pochodzenia naturalnego, który należy do grupy polieteryowych antybiotyków jonoforowych. **MON** została po raz pierwszy wyizolowana w 1967 roku ze szczepu *Streptomyces cinnamomensis*, stanowiąc pierwszy jonofor, którego strukturę dokładnie opisano i wyjaśniono [35]. **MON** przyjmuje strukturę pseudocykliczną, która wynika z obecności wewnątrzcząsteczkowych wiązań wodorowych typu „głowa do ogona” pomiędzy grupą karboksylową a grupami hydroksylowymi znajdującymi się po przeciwnej stronie cząsteczki [36]. Atomy tlenu tworzą hydrofilową kieszeń zdolną do koordynowania kationów metali, podczas gdy zewnętrzna część jonoforu ma charakter hydrofobowy, co ułatwia dyfuzję przez błony komórkowe ([Rysunek 3B](#)) [37]. Uwolnienie kationu w środowisku wewnątrzkomórkowym prowadzi do zmiany gradientu stężeń jonów i pH, a w konsekwencji do uszkodzenia mitochondriów i śmierci komórki [38].



[Rysunek 3](#). (A) struktura chemiczna monenzyny (**MON**), (B) struktura soli sodowej monenzyny (kolor pomarańczowy – kation sodu; kolor czerwony – atomy tlenu; kolor szary – szkielet węglowodorowy).

Zdolność **MON** do kompleksowania kationów metali (głównie kationu sodu) oraz przenoszenia powstałego kompleksu przez błony biologiczne nadaje temu jonoforowi szerokie spektrum aktywności biologicznych (właściwości przeciwbakteryjnych, przeciw pasożytniczych, przeciwgrzybiczych oraz przeciwnowotworowych) [38]. Transport kationu realizowany przez niemodyfikowaną **MON** oraz inne jonofory odbywa się na dwa sposoby. Pierwszy z nich to transport elektroneutralny, w którym potencjał transbłonowy nie ulega zmianie [39,40]. Zakłada on deprotonowanie grupy karboksylowej jonoforu, więc może przebiegać jedynie w środowisku zasadowym [39,40]. Warto jednak pamiętać, że ze względu na specyficzny metabolizm glukozy realizowany przez komórki

nowotworowe (efekt Warburga), ich środowisko komórkowe jest zakwaszone przez powstający kwas mlekowy [41]. W tych warunkach niemożliwe jest deprotonowanie grupy karboksylowej jonoforu, a transport przebiega zgodnie z mechanizmem elektrogennym – ze zmianą potencjału transbłonowego [39,40]. Ponadto dowiedziono, że pochodne antybiotyków jonoforowych z zablokowaną grupą karboksylową (np. estry lub amidy) mogą efektywnie realizować transport kationów przez błony biologiczne. Odbywa się to na drodze trzeciego mechanizmu transportu – transportu biomimetycznego [39,40].

Ze względu na swoją skuteczność w zwalczaniu pasożytów, **MON** znalazła zastosowanie w weterynarii jako niehormonalny stymulator wzrostu dodawany do pasz (Coxidin®) w celu zwalczania kokcydiozy – choroby zwierząt wywoływanej przez pierwotniaki rodzaju *Eimeria* [42,43]. Poza właściwościami kokcydiostatycznymi, **MON** dowiodła swojej skuteczności w zwalczaniu różnorodnych pasożytów odpowiedzialnych między innymi za NTDs (trypanosomatoza afrykańska, leishmanioza), toksoplazmozę czy malarię [44–49]. Wykazano, że jonofor hamuje wzrost świdrowców z gatunku *T. brucei* w stężeniach mikromolowych, wykazując przy tym niską cytotoksyczność wobec referencyjnej linii komórkowej oraz umiarkowaną selektywność działania [45]. Ponadto udowodniono, że **MON** wykazuje aktywność w stężeniach nanomolowych wobec zarodźców z gatunku *P. falciparum* opornych na działanie chlorochiny, będąc przy tym skuteczniejszą niż inne leki stosowane powszechnie przeciwko malarii (amodiachina, artemeter, artemizynina, artesunat, pirymetamina, trimetoprim lub chinina) [50,51].

Oprócz wysokiej skuteczności w zwalczaniu różnorodnych pasożytów, dowiedziono, że **MON** hamuje proliferację wielu rodzajów komórek nowotworowych – między innymi chłoniaka, szpiczaka oraz komórek nowotworu okrężnicy – poprzez zatrzymanie cyklu komórkowego i doprowadzenie do utraty mitochondrialnego potencjału błonowego [52–54]. Badania dowiodły również skuteczności **MON** w przełamywaniu oporności wielolekowej komórek nowotworowych [55,56]. W ostatnich latach przeprowadzono badania przesiewowe mające na celu znalezienie potencjalnych leków skutecznych w walce z nowotworem prostaty. Zbadano efekt niemal 5000 znanych leków i związków lekopodobnych. Jedynie cztery z nich, w tym **MON**, selektywnie hamowały wzrost komórek nowotworowych prostaty w stężeniach nanomolowych, nie wykazując przy tym znaczącego wpływu na wzrost zdrowych komórek nabłonka prostaty [57].

Oba omawiane związki – **IWR** i **MON** – zostały zarejestrowane jako leki lub preparaty weterynaryjne w zwalczaniu różnorodnych chorób pasożytniczych. W tym miejscu należy dodać, że wiele związków chemicznych aktywnych przeciw pasożytniczo wykazuje także dużą skuteczność w zwalczaniu komórek nowotworowych [58]. Okazało się to prawdą również w przypadku **IWR** i **MON**, które wykazują aktywność przeciwnowotworową na drodze różnych mechanizmów molekularnego działania. W badaniach własnych potraktowałem oba związki jako struktury wiodące będące punktem startowym w poszukiwaniu nowych leków o wysokiej aktywności przeciwnowotworowej. Nie należy jednak zapominać o pierwotnym zastosowaniu obu związków, dlatego wiele z nowo otrzymanych pochodnych zostało również zbadanych pod kątem ich skuteczności w zwalczaniu pasożytów.

6. Badania własne

Podstawą przygotowania niniejszej rozprawy doktorskiej był cykl siedmiu artykułów naukowych (jednej pracy przeglądowej oraz sześciu prac twórczych), w których opisałem wyniki przeprowadzonych przeze mnie badań. Kopie niżej wymienionych artykułów, wraz z suplementami oraz oświadczeniami współautorów, dołączono do rozprawy (Rozdział 11 i 12).

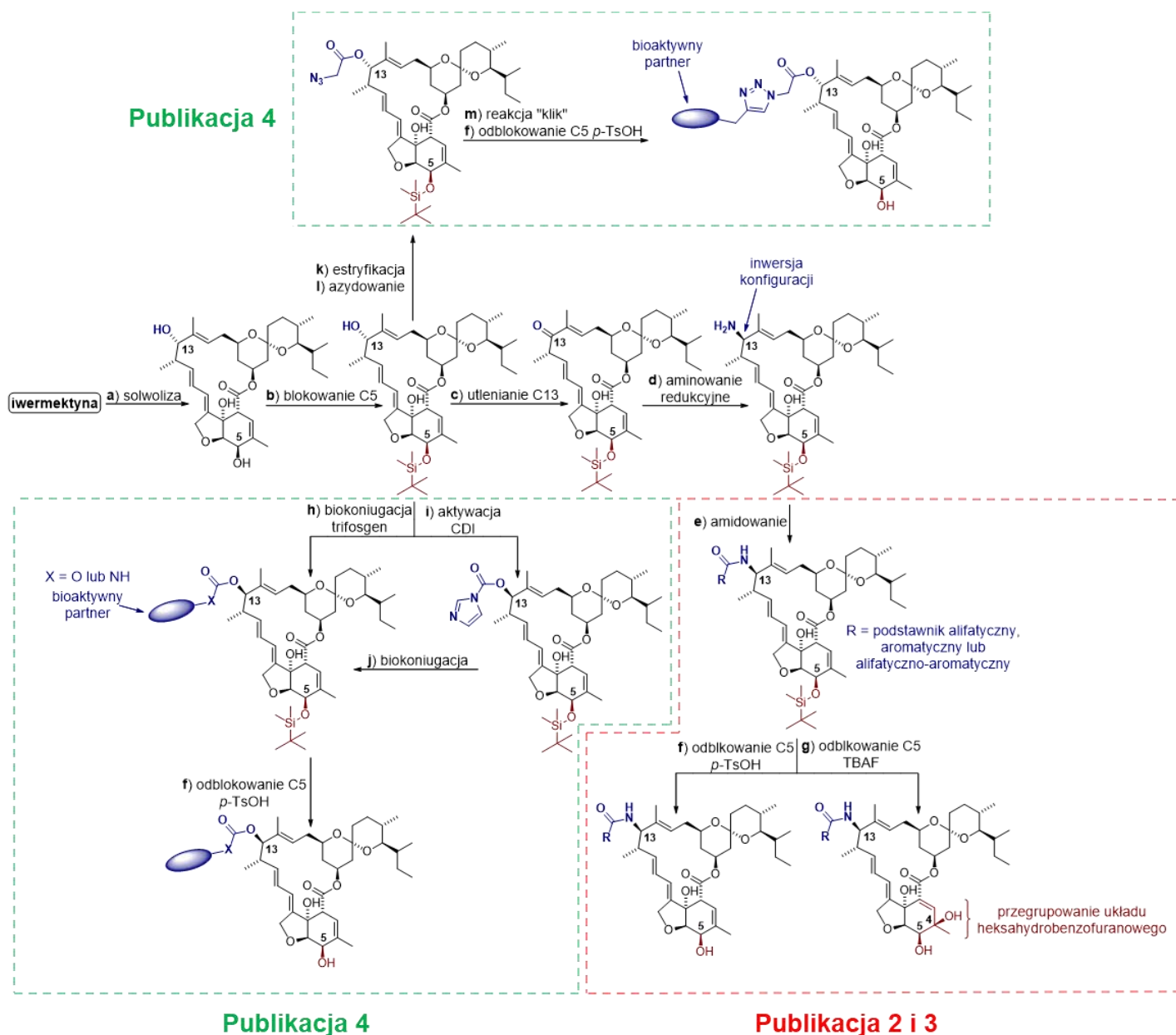
- I. **Michał Sulik**, Michał Antoszczak, Adam Huczyński, Dietmar Steverding, „*Antiparasitic activity of ivermectin: Four decades of research into a “wonder drug”*”, *European Journal of Medicinal Chemistry* 261 (2023) 115838.
- II. **Michał Sulik**, Diana Fontinha, Dietmar Steverding, Szymon Sobczak, Michał Antoszczak, Miguel Prudêncio, Adam Huczyński, „*Unexpected rearrangement of ivermectin in the synthesis of new derivatives with trypanocidal and antiplasmodial activities*”, *European Journal of Medicinal Chemistry* 263 (2024) 115951.
- III. **Michał Sulik**, Dagmara Otto-Ślusarczyk, Michał Antoszczak, Marta Struga, Adam Huczyński, „*Ivermectin and its synthetic derivatives – A new class of anticancer agents*”, *European Journal of Medicinal Chemistry Reports* 12 (2024) 100176.
- IV. **Michał Sulik**, Dagmara Otto-Ślusarczyk, Dietmar Steverding, Marta Struga, Adam Huczyński, „*Antiproliferative and trypanocidal activity of ivermectin bioconjugates*”, *ACS Omega* (2025), DOI: 10.1021/acsomega.5c02998.
- V. **Michał Sulik**, Marta Jędrzejczyk, Magdalena Mielczarek-Putra, Gwan Yong Lim, Małgorzata Podsiad, Jakub Hoser, Piotr Bednarczyk, Marta Struga, Adam Huczyński, „*Mitochondria-targeting phosphonium salts of monensin A as the source of novel antiproliferative agents*”, *European Journal of Medicinal Chemistry Reports* 14 (2025) 100275.
- VI. **Michał Sulik**, Robert Graniczny, Jan Janczak, Dagmara Kłopotowska, Joanna Wietrzyk, Adam Huczyński, „*From Pseudocyclic to Macrocyclic Ionophores: Strategies toward the Synthesis of Cyclic Monensin Derivatives*”, *Journal of Organic Chemistry* 90, 3, (2025) 1344–1353.
- VII. **Michał Sulik**, Eyob A. Workneh, Sofia Santana, Bárbara Teixeira, Miguel Prudêncio, Jan Janczak, Adam Huczyński, „*Chemical modification of monensin as a source of potent antiplasmodial agents*”, *Bioorganic & Medicinal Chemistry* 123 (2025) 118177.

Publikacja **I** to praca przeglądowa, w której omówiono ostatnie 40 lat stosowania iwermektyny jako niezwykle skutecznego leku przeciw pasożytniczego w medycynie oraz weterynarii, jak również użycia tego środka w terapiach eksperymentalnych lub poza wskazaniem (ang. *off-label*). Praca ta stanowi swoisty wstęp teoretyczny niniejszej rozprawy doktorskiej.

Publikacje **II–IV** to prace twórcze opisujące badania nad chemiczną modyfikacją struktury iwermektyny (formy B1a) oraz wpływem tych modyfikacji na aktywność biologiczną (przeciw pasożytniczą oraz przeciwnowotworową) otrzymanych po raz pierwszy pochodnych. Publikacje **V–VII** to prace twórcze, w których omówione zostały badania nad chemiczną modyfikacją struktury monenzyny oraz wpływem tych modyfikacji na aktywność biologiczną (przeciw pasożytniczą oraz przeciwnowotworową), jak również właściwości strukturalne i kompleksotwórcze otrzymanych pochodnych.

Dla zachowania porządku i przejrzystości rozprawy, opisy badań nad iwermektyną oraz monenzyną zostały zamieszczone w osobnych podrozdziałach. Biorąc pod uwagę obszerne serie otrzymanych pochodnych oraz mnogość zastosowanych procedur syntetycznych, na początku każdego podrozdziału została umieszczona schematyczna mapa przedstawiająca przekształcenia, jakie przeprowadzono w obrębie danego związku chemicznego. Na każdym z tych schematów wyróżniono kolorową ramką obszary odnoszące się do konkretnej publikacji. W trakcie analizy wyników badań zastosowano nowe oznaczenia związków X-Y, gdzie X to cyfra rzymska odnosząca się do numeru publikacji, a Y to cyfra arabska odnosząca się do oryginalnego oznaczenia związku w publikacji. Oznacza to, że związek nr **6a** w publikacji **II** został oznaczony symbolem **II-6a**.

6.1. Modyfikacja chemiczna iwermektyny



Schemat 1. Mapa syntez przeprowadzonych w celu chemicznej modyfikacji iwermektyny. *Warunki i reagenty:* (a) H_2SO_4 , CH_3OH , 0°C do tp; (b) TBDMSCl, imidazol, CH_2Cl_2 , tp; (c) DMP, CH_2Cl_2 , tp; (d) NH_4OAc , NaBH_3CN , CH_3OH , 60°C do tp; (e) chlorek kwasowy, TEA, CH_2Cl_2 , 0°C do tp, lub kwas karboksylowy, HATU, DMF, tp; (f) $p\text{-TsOH}$, CH_3OH , 0°C do tp; (g) TBAF, THF, tp; (h) partner do biokoniugacji, TEA, trifosgen, THF, 0°C do tp; (i) CDI, toluen, tp; (j) partner do biokoniugacji, DBU, DMF, 90°C ; (k) chlorek chloroacetyl, py, CH_2Cl_2 , 0°C do tp; (l) NaN_3 , DMF, 60°C ; (m) propargilowy partner do biokoniugacji, DIPEA, CuI, CH_3CN , N_2 , tp.

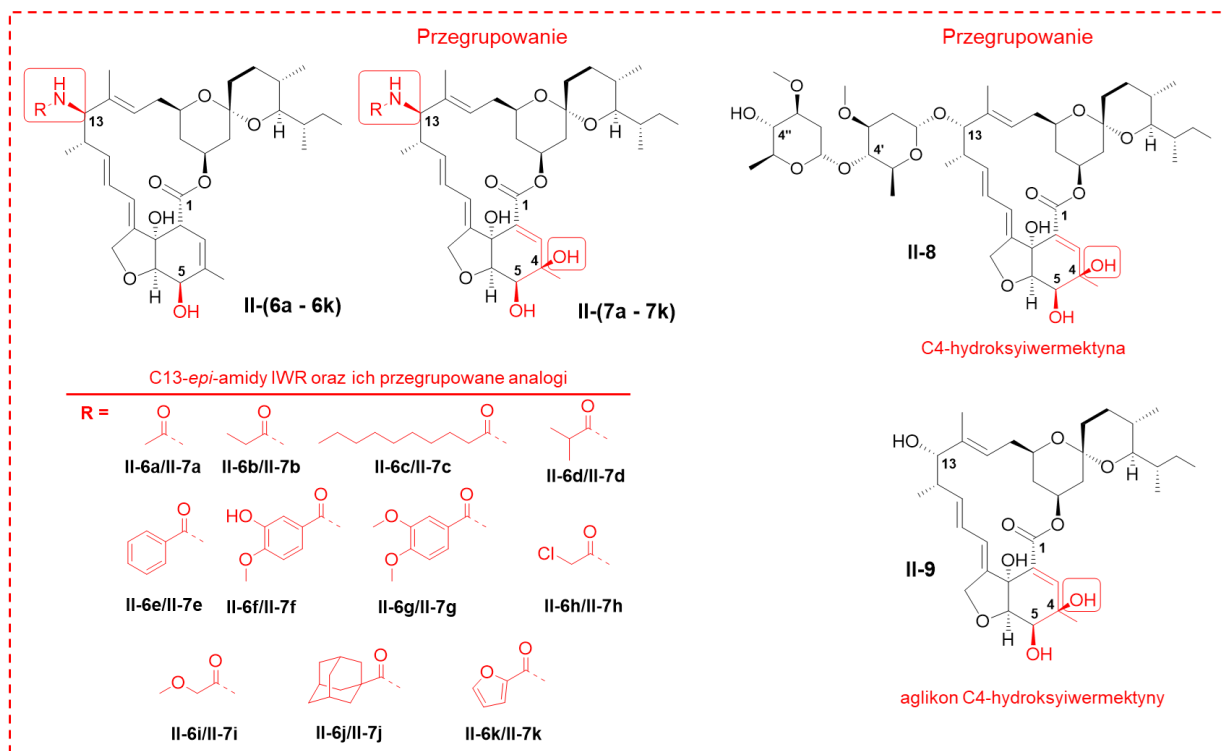
Większość znanych w literaturze przekształceń chemicznych iwermektyny (**IWR**) lub awermektyny dotyczy modyfikacji ich reszty cukrowej (zwłaszcza pozycji C4' oraz grupy hydroksylowej w pozycji C4'') lub grupy hydroksylowej w pozycji C5 [59–73]. Co ważne, przeprowadzone badania biologiczne wykazały, że C4''-podstawione pochodne **IWR** charakteryzowały się wyższą aktywnością biologiczną oraz lepszymi właściwościami farmakokinetycznymi od związku wyjściowego [59,70–73]. Dowodzi to jednoznacznie, że racjonalna modyfikacja **IWR** może doprowadzić do pochodnych o większym potencjale terapeutycznym od struktury natywnej. Z drugiej strony, udowodniono, że grupa hydroksylowa w pozycji C5 jest kluczowa dla zachowania aktywności przeciwpasożytniczej, co oznacza, że racjonalna modyfikacja **IWR** nie powinna obejmować przekształceń tej grupy funkcyjnej [74]. Szczególnie intrygująca w tym kontekście wydała mi się rola pozycji C13, która łączy aglikon **IWR** z komponentem cukrowym. Okazało się, że w literaturze naukowej jest niewiele doniesień traktujących o chemicznej modyfikacji pozycji C13 i jej wpływowi na aktywność otrzymanych w ten sposób analogów. W związku z tym w ramach pracy doktorskiej skupiłem się na selektywnej funkcjonalizacji pozycji C13 **IWR**.

Na [Schemacie 1](#) przedstawiono mapę przekształceń chemicznych **IWR**, które doprowadziły do otrzymania jej nowych pochodnych opisanych w publikacjach **II–IV**. W sumie otrzymałem 34 nowe pochodne **IWR**, które zbadano pod kątem aktywności przeciwnowotworowej oraz przeciwpasożytniczej we współpracy z biologami z wiodących krajowych i zagranicznych ośrodków naukowych. Dane z badań spektroskopowych wraz z kopiami widm NMR otrzymanych po raz pierwszy związków znajdują się w załączonych do niniejszej rozprawy publikacjach i/lub materiałach uzupełniających. Zamieszczono w nich również dane z badań aktywności biologicznej.

Badania opisane w publikacjach **II** oraz **III** polegały na syntezie serii 22 nowych C13-*epi*-amidowych pochodnych **IWR**, które otrzymałem na drodze amidowania prekursora C13-*epi*-aminowego zsyntetyzowanego uprzednio w czteroetapowej procedurze ([Schemat 1](#), [Rysunek 4](#)), a także 2 pochodnych będących produktami przegrupowania układu heksahydrobenzofuranowego **IWR** i jej aglikonu. W obu pracach badania biologiczne przeprowadzono na tych samych pochodnych **IWR**, jednak publikacja **II** dotyczy badań właściwości przeciwpasożytniczych (przeciwmalarycznych oraz przeciwtrypanosomowych), publikacja **III** zaś właściwości przeciwnowotworowych. Ze

względem na odmienny system numeracji zastosowany w obu pracach, dla większego porządku w omawianiu wyników zostałem przy systemie numeracji z publikacji **II**.

Publikacje II i III



Rysunek 4. Struktury pochodnych iwermektyny opisane w publikacjach **II** i **III**.

Jako że zaplanowane modyfikacje dotyczyły pozycji C13 **IWR**, w pierwszym kroku konieczne było usunięcie podstawnika cukrowego, którego dokonałem w warunkach powolnego wkraplania stężonego kwasu siarkowego(VI) do roztworu **IWR** w metanolu (**Schemat 1**). Otrzymałem w ten sposób aglikon, który zawierał w swojej strukturze dwie 2° grupy hydroksylowe wrażliwe na dalsze modyfikacje – w pozycjach C5 oraz C13. Jak zaznaczono wcześniej, grupa hydroksylowa w pozycji C5 jest niezbędna do zachowania aktywności przeciwpasożytnej, w związku z czym konieczne było wprowadzenie grupy ochronnej. Dużo wyższa reaktywność grupy hydroksylowej w pozycji C5 niż tej w pozycji C13 pozwoliła mi na przeprowadzenie reakcji z chlorkiem *tert*-butylodimetylosililowym w sposób całkowicie regioselektywny (**Schemat 1**). Kolejnym krokiem było przeprowadzenie reakcji utlenienia grupy hydroksylowej w pozycji C13. Biorąc jednak pod uwagę jej stosunkowo niską reaktywność, konieczne okazało się znalezienie skutecznych reagentów utleniających oraz zoptymalizowanie warunków prowadzenia reakcji, co ostatecznie pozwoliło mi zidentyfikować nadjodan Dessa-Martina (DMP) jako najefektywniejszy utleniacz (**Schemat 1**). Otrzymany w ten sposób C13-keton **IWR**

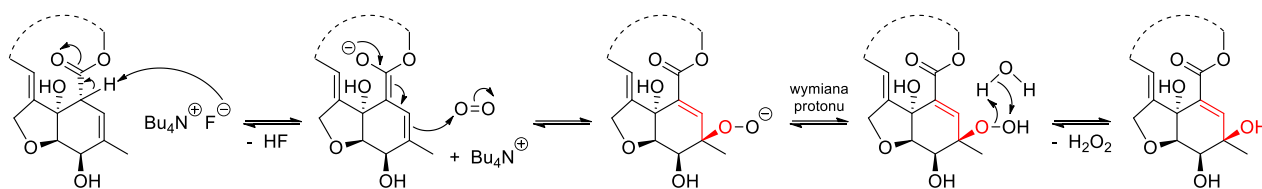
poddałem następnie diastereoselektywnemu aminowaniu redukcyjnemu przy użyciu octanu amonu oraz cyjanoborowodoru sodu, opierając się na opisanej w literaturze procedurze [75]. Umiarkowana wydajność reakcji (około 30%) skłoniła mnie jednak do modyfikacji warunków jej prowadzenia. Ogrzewanie mieszaniny reakcyjnej korzystnie wpłynęło na efektywność aminowania redukcyjnego, dzięki czemu oczekiwany produkt wyizolowałem z 51% wydajnością ([Schemat 1](#)). Co ważne, w wyniku reakcji dochodziło do inwersji konfiguracji na asymetrycznym atomie węgla C13. Stereochemię potwierdzono krystalograficznie.

Mając dostęp do aminowego prekursora **IWR**, możliwe było otrzymanie serii amidów tego związku przy wykorzystaniu komercyjnie dostępnych chlorków kwasowych lub kwasów karboksylowych ([Schemat 1](#)). Zastosowanie podstawników o różnej wielkości, budowie (liniowe, rozgałęzione) i charakterze chemicznym (alifatyczne, aromatyczne, alifatyczno-aromatyczne) pozwoliło mi na otrzymanie bogatej biblioteki C13-*epi*-amidów ([Rysunek 4](#)).

Ostatnim etapem syntezy było usunięcie grupy ochronnej w pozycji C5, co doprowadziło do kluczowego odkrycia – zauważyłem, że zastosowanie TBAF jako czynnika usuwającego grupy silylowe prowadzi do nietypowego przegrupowania pierścienia heksahydrobenzofuranowego **IWR** obejmującego migrację wiązania podwójnego oraz stereospecyficzne hydroksylowanie pozycji C4, co potwierdzono krystalograficznie ([Schemat 1](#)). W celu otrzymania pochodnych pojedynczo modyfikowanych (z zachowaniem natywnego układu pierścienia **IWR**) zastosowałem zmodyfikowaną procedurę usuwania grup ochronnych z wykorzystaniem kwasu *para*-toluenosulfonowego ([Schemat 1](#)). Dzięki nowo opracowanej reakcji przegrupowania możliwe było otrzymanie dwóch grup analogów – zaplanowanych początkowo C13-*epi*-amidów **IWR** (**II-(6a-6k)**) z wydajnością 20–43% po dwóch etapach (amidowania i zdjęcia grupy ochronnej w pozycji C5) oraz ich analogów ze zmodyfikowanym pierścieniem heksahydrobenzofuranowym (**II-(7a-7k)**, [Rysunek 4](#)) z wydajnością 16–35% po dwóch etapach. Ponadto, opracowanie optymalnych warunków dla reakcji przegrupowania pozwoliło mi na otrzymanie dwóch dodatkowych związków – przegrupowanych analogów **IWR** (**II-8**) oraz jej aglikonu (**II-9**).

W celu wyjaśnienia potencjalnego mechanizmu tego nieoczekiwanego przegrupowania, dokonałem szczegółowego przeglądu literatury naukowej i natrafiłem na

artykuł traktujący o hydroksylujących właściwościach TBAF [76]. Autorzy w zaproponowanym przez siebie mechanizmie podkreślili kluczową rolę tlenu atmosferycznego jako czynnika elektrofilowego i źródła grupy hydroksylowej. W celu optymalizacji warunków reakcji sprawdziłem wpływ różnego rodzaju zmiennych, w tym zmiany rozpuszczalnika (m.in. CHCl_3 , CH_2Cl_2 , CH_3OH) oraz inicjatora reakcji (TBAB, TBAI, KF), jednak do przegrupowania dochodziło jedynie przy zastosowaniu TBAF w THF. Postęp reakcji monitorowałem za pomocą metody TLC, ale również na podstawie zmiany koloru mieszaniny reakcyjnej, która z czasem przybierała intensywnie czerwone zabarwienie. W celu potwierdzenia roli tlenu, przeprowadziłem reakcję w otwartej kolbie (ang. *open vessel*) oraz w atmosferze gazu obojętnego (argonu). Do zmiany zabarwienia dochodziło jedynie w wariancie „open vessel”. Ponadto, dodatek nadtlenu wodoru do mieszaniny reakcyjnej hamował postęp reakcji. Biorąc pod uwagę wszystkie obserwacje, zaproponowano potencjalny mechanizm przegrupowania (Schemat 2).



Schemat 2. Proponowany mechanizm przegrupowania pierścienia heksahydrobenzofuranowego iwermektyny.

Jednoznacznym potwierdzeniem struktury przegrupowanych analogów było wykonanie analizy rentgenograficznej krystalicznych pochodnych **IWR**. Acetamid **II-6a** oraz jego przegrupowany analog **II-7a** zostały scharakteryzowane za pomocą metody scXRD (ang. *single crystal X-ray diffraction*) we współpracy z dr. Szymonem Sobczakiem z Zakładu Chemii Materiałów Wydziału Chemii Uniwersytetu im. Adama Mickiewicza w Poznaniu. Przegrupowanie pierścienia heksahydrobenzofuranowego nie wpłynęło znacząco na zmianę konformacji związku **II-7a** w porównaniu do nieprzegrupowanego odpowiednika **II-6a**. Oba związki krystalizowały w odmiennych grupach przestrzennych – związek **II-6a** w grupie $P2_1$ układu jednoskośnego, a solwat związku **II-7a** z acetonem w grupie $P2_12_12$ układu rombowego. Ponadto, analiza scXRD pozwoliła potwierdzić, że w wyniku aminowania redukcyjnego dochodzi do inwersji konfiguracji na asymetrycznym atomie węgla C13. Poza analizą scXRD, wszystkie otrzymane analogi zostały w pełni scharakteryzowane za pomocą metod spektroskopowych (^1H NMR, ^{13}C NMR) oraz spektrometrycznych (HRMS). Najważniejszy sygnał analityczny w widmie ^{13}C NMR

pochodnych **IWR** pochodził od nowo wprowadzonej grupy amidowej. W zależności od wprowadzonego podstawnika amidowego pojawiał się on w dość szerokim przedziale 177,6–157,9 ppm. Przegrupowanie układu heksahydrobenzofuranowego powodowało ponadto przesunięcie sygnału pochodzącego od grupy laktonowej w pozycji C1 w kierunku niższych wartości ppm. Z kolei w widmach otrzymanych z analizy HRMS główny pik pochodził od kompleksów produktów z kationem sodu, tj. $[M+Na]^+$.

Wszystkie otrzymane pochodne zostały zbadane pod kątem aktywności przeciwpasożytniczej *in vitro* na modelu trypanosomatozy afrykańskiej we współpracy międzynarodowej z dr. Dietmarem Steverdingiem z Norwich Medical School, University of East Anglia, Norwich, Wielka Brytania. Działanie trypanobójcze i cytotoksyczne określono odpowiednio przy użyciu czynnika wywołującego chorobę – pasożyta *Trypanosoma brucei brucei*, a także referencyjnych komórek ludzkiej białaczki szpikowej HL-60. Większość otrzymanych pochodnych wykazała wyższą aktywność oraz lepszą selektywność działania wobec pasożyta niż niemodyfikowana chemicznie **IWR**. Co ważne, przegrupowany oraz nieprzegrupowany amid 2-chloroacetylowy (odpowiednio **II-7h** i **II-6h**) wykazały około 230-krotnie wyższą aktywność *in vitro* niż związek wyjściowy. Była to aktywność porównywalna do tych wykazywanych przez suraminę oraz bromek etydyiny – leki powszechnie stosowane w leczeniu odpowiednio śpiączki afrykańskiej (u ludzi) oraz nagany (u zwierząt). Oznacza to, że otrzymane przeze mnie pochodne **IWR** mogą być rozpatrywane jako potencjalni kandydaci na środki do walki z chorobami wywoływanymi przez *T. b. brucei*.

Wybrane związki zostały również zbadane pod kątem ich aktywności przeciwmalarycznej wobec wątrobowego stadium *Plasmodium berghei* we współpracy międzynarodowej z prof. Miguelem Prudêncio z Instituto de Medicina Molecular João Lobo Antunes, Faculdade de Medicina, Universidade de Lisboa & Gulbenkian Institute for Molecular Medicine, Portugalia. Badania dotyczyły 6 związków – **IWR**, jej aglikonu oraz amidu acetylowego **II-6a**, a także ich przegrupowanych analogów (odpowiednio **II-8**, **II-9** oraz **II-7a**). Pojedynczo modyfikowane pochodne (aglikon **IWR** oraz **II-6a**) wykazały niższą aktywność przeciwmalaryczną niż związek wyjściowy, natomiast amid acetylowy przegrupowanej **IWR** (związek **II-7a**) okazał się nie tylko 4-krotnie bardziej aktywny niż nieprzegrupowany odpowiednik **II-6a**, ale również 2,5-krotnie bardziej aktywny wobec *P. berghei* niż niemodyfikowana chemicznie **IWR**. Wskazuje to jednoznacznie, że opracowana przeze mnie reakcja przegrupowania może być pomocnym narzędziem

w poszukiwaniu nowych bioaktywnych związków opartych na strukturze **IWR** o szerokim spektrum aktywności przeciwpasożytniczych.

Wysoki potencjał **IWR** w zwalczaniu komórek nowotworowych został omówiony we wstępie do niniejszej rozprawy doktorskiej. W literaturze naukowej brakowało natomiast jakichkolwiek doniesień na temat aktywności przeciwnowotworowej pochodnych tego leku. W związku z tym, otrzymane pochodne **IWR** zostały po raz pierwszy szczegółowo zbadane pod kątem ich właściwości przeciwnowotworowych we współpracy krajowej z zespołem prof. Marty Strugi z Katedry i Zakładu Biochemii Wydziału Lekarskiego Warszawskiego Uniwersytetu Medycznego. Właściwości cytotoksyczne wszystkich związków określono w badaniach na liniach nowotworowych prostaty (PC3), piersi (MDA-MB-123), płuc (A549), jelita grubego (HCT-116) oraz normalnej linii ludzkich keratynocytów (HaCaT). W wyniku przeprowadzonych testów po raz pierwszy dowiedliśmy, że wybrane pochodne wykazują wyższą aktywność przeciwproliferacyjną i/lub selektywność działania niż chemicznie niemodyfikowana **IWR**. Przełomowym odkryciem w tym kontekście było zwiększenie aktywności **IWR** oraz wybranych pochodnych w wyniku przegrupowania układu heksahydrobenzofuranowego. Wyniki aktywności przeciwproliferacyjnej tych związków na poszczególnych liniach komórkowych zostały przedstawione w Tabeli 1. Bardziej szczegółowy opis właściwości przeciwproliferacyjnych otrzymanych pochodnych znajduje się w publikacji III.

Pary związków, w których produkt przegrupowany wykazywał znacząco lepszą aktywność biologiczną lub niższą cytotoksyczność niż jego nieprzegrupowany analog zostały zaznaczone na czerwono (Tabela 1). Przedstawione wyniki badań pokazują, że za pomocą racjonalnej modyfikacji struktury **IWR** można wpływać na aktywność pochodnych wobec określonych linii komórkowych. Co istotne, przegrupowana **IWR** (związek II-8), oprócz wyższej aktywności przeciwproliferacyjnej ($IC_{50, PC3} = 7,4 \mu M$; $IC_{50, MDA-MB-231} = 8,4 \mu M$), wykazuje też niższą cytotoksyczność wobec komórek prawidłowych ($IC_{50, HaCaT} = 22,8 \mu M$), dzięki czemu wyznaczony współczynnik selektywności dla tego związku jest ponad dwukrotnie wyższy niż dla związku wyjściowego (Tabela 1). Żaden z otrzymanych związków nie okazał się bardziej aktywny niż doksorubicyna – lek przeciwnowotworowy użyty jako odnośnik, jednak wszystkie otrzymane analogi wykazały niższą cytotoksyczność wobec linii HaCaT niż ten lek. Ponadto, współczynniki selektywności dla większości otrzymanych analogów okazały się wyższe niż w przypadku doksorubicyny. Wyniki omawianych badań stanowiły podstawę

zgłoszenia wynalazku w Urzędzie Patentowym RP (zgłoszenie nr P.444419 pt. „Nowe pochodne iwermektyny, sposób ich otrzymywania oraz zastosowanie do leczenia onkologicznego”).

Tabela 1. Aktywność przeciwproliferacyjna wybranych pochodnych iwermektyny. Dane podano jako wartość parametru IC_{50} [μM] (średnia $\pm$ SD). SI = IC_{50} dla komórek prawidłowych/ IC_{50} dla komórek nowotworowych.

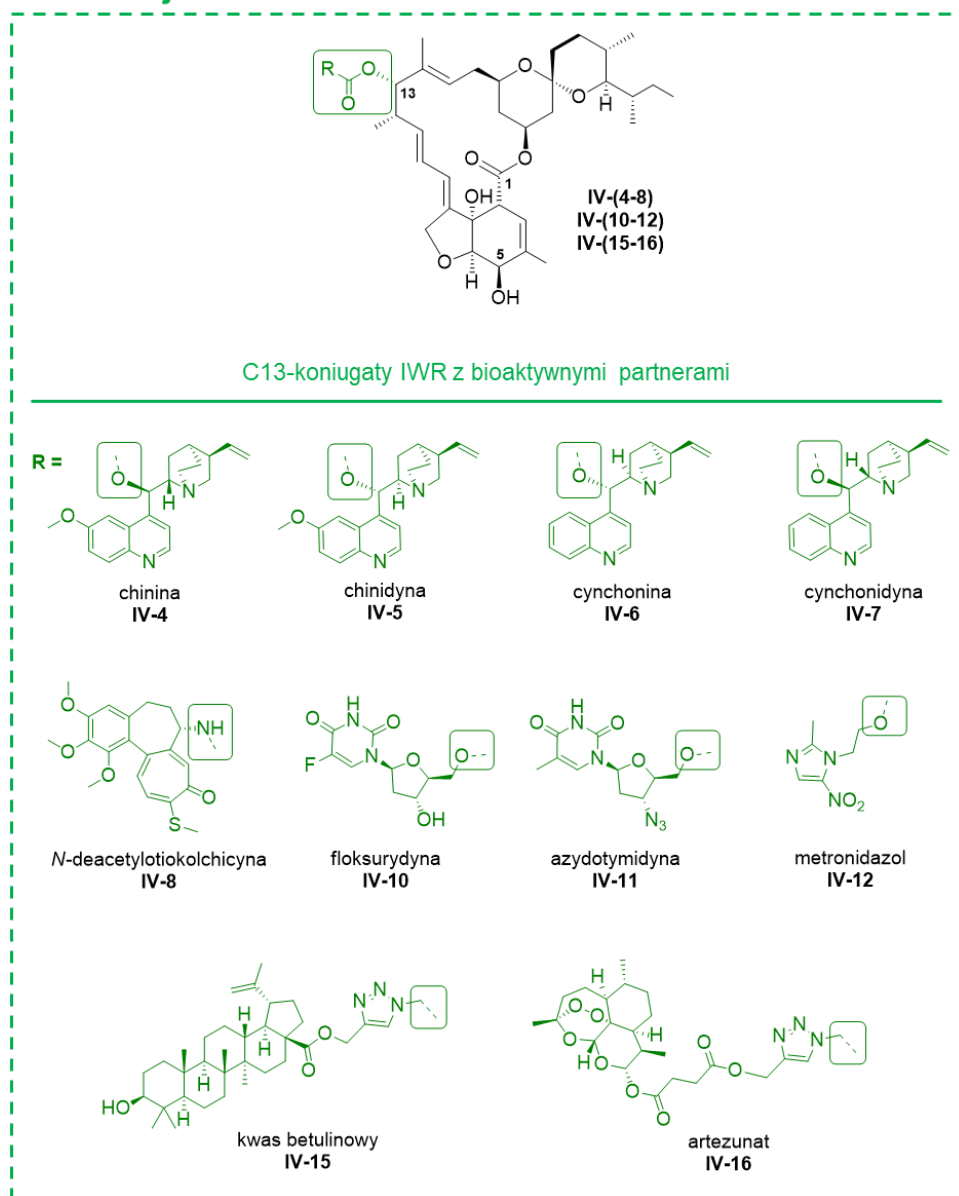
Związek	Nowotworowe linie komórkowe				Komórki prawidłowe
	PC3		MDA-MB-231		HaCaT
	IC_{50} [μM]	SI	IC_{50} [μM]	SI	IC_{50} [μM]
IWR	8,5 $\pm$ 0,2	1,3	8,8 $\pm$ 0,3	1,2	10,8 $\pm$ 3,1
II-8	7,4 $\pm$ 0,2	3,0	8,4 $\pm$ 0,3	2,7	22,8 $\pm$ 0,3
aglikon IWR	17,1 $\pm$ 1,2	1,0	12,1 $\pm$ 0,1	1,4	16,9 $\pm$ 8,1
II-9	9,3 $\pm$ 0,1	1,6	14,8 $\pm$ 0,9	1,0	15,3 $\pm$ 0,6
II-6g	8,1 $\pm$ 1,1	1,3	8,2 $\pm$ 0,3	1,3	10,8 $\pm$ 2,6
II-7g	7,2 $\pm$ 0,4	1,3	7,3 $\pm$ 0,5	1,3	9,3 $\pm$ 0,6
II-6h	17,6 $\pm$ 3,4	1,6	9,5 $\pm$ 1,7	3,0	28,9 $\pm$ 1,3
II-7h	8,8 $\pm$ 0,6	3,2	8,8 $\pm$ 0,2	3,2	27,9 $\pm$ 1,8
II-6j	9,1 $\pm$ 0,8	0,7	10,3 $\pm$ 3,5	0,6	6,4 $\pm$ 0,5
II-7j	9,6 $\pm$ 0,3	2,3	15,4 $\pm$ 5,1	1,5	22,8 $\pm$ 2,7
II-6k	7,8 $\pm$ 0,7	1,0	28,5 $\pm$ 0,6	0,3	8,2 $\pm$ 1,9
II-7k	8,9 $\pm$ 0,1	1,7	8,9 $\pm$ 0,1	1,7	15,1 $\pm$ 2,1
doksorubicyna	0,59 $\pm$ 0,02	0,14	1,83 $\pm$ 0,1	0,15	0,29 $\pm$ 0,1

Oprócz analizy właściwości przeciwproliferacyjnych nowych pochodnych **IWR**, przeprowadzono szczegółowe badania dotyczące mechanizmu ich działania biologicznego. Uwzględniały one analizę wczesnej i późnej apoptozy, cyklu komórkowego, poziomu interleukiny 6 oraz stresu oksydacyjnego. Badania wykazały, że wybrane pochodne **IWR** zwiększały populację komórek nowotworowych PC3, MDA-MB-231 oraz A549 w fazach subG1 oraz G0/G1, a jednocześnie zmniejszały populację komórek w fazach S oraz G2/M, podczas gdy w przypadku komórek HCT-116 zatrzymanie cyklu komórkowego zwiększało się w fazie S/G2/M. Mechanizm leżący u podstaw działania przeciwnowotworowego badanych związków był zróżnicowany w zależności od badanej linii komórek nowotworowych i uwzględniał m.in. wytwarzanie reaktywnych form tlenu (ROS) oraz peroksydację lipidów. Wykazano, że pochodne **IWR** mogą hamować wzrost komórek nowotworowych i indukować apoptozę przez zależną od mitochondriów produkcję ROS, jak również działać jako inhibitory interleukiny 6.

W ostatnich latach Singh ze współpracownikami przeprowadzili badania polegające na zastąpieniu podstawnika cukrowego w strukturze **IWR** innymi związkami wykazującymi aktywność biologiczną (np. pochodnymi aminochinoliny lub ferrocenu) [77,78]. Tego typu hybrydy wykazały wysoką aktywność *in vitro* wobec pasożytów *Plasmodium* wywołujących malarię [77,78]. Do ich syntezy zastosowano łączniki o zróżnicowanym charakterze chemicznym i stabilności (ugrupowania węglanowe, uretanowe oraz 1,2,3-triazolowe) [77,78]. Strategia projektowania związków chemicznych, w której łączymy ze sobą dwie lub więcej bioaktywnych cząsteczek nosi nazwę biokoniugacji [79,80]. Jest to bardzo często stosowana metoda syntezy nowych analogów związków pochodzenia naturalnego, które mogą wykazywać wyższą skuteczność działania biologicznego niż ich komponenty wyjściowe [79,80].

Zainspirowany korzyściami wynikającymi z możliwości zastąpienia komponentu cukrowego **IWR** innymi ugrupowaniami chemicznymi oraz wynikami właściwości przeciwnowotworowych i przeciwpasożytniczych C13 pochodnych **IWR** omówionych w publikacjach **II** i **III**, postanowiłem przeprowadzić syntezę nowych hybryd tego związku z bioaktywnymi partnerami wykazującymi wysoką aktywność biologiczną. Do tych partnerów należą alkaloidy kory chinowej (chinina, chinidyna, cynchonina, cynchonidyna). Niektóre z nich stosowane były jako leki przeciwmalaryczne i przeciwarytmiczne, a w ostatnim czasie udokumentowano również ich skuteczność w przełamywaniu oporności wielolekowej komórek nowotworowych [81,82]. Wybrałem także dwa analogi nukleozydów (floksurydynę, azydotymidynę), które są stosowane jako leki przeciwnowotworowe [83,84]. W pracach syntetycznych uwzględniłem również *N*-deacetylotiokolchicynę, kwas betulinowy, artezunat i metronidazol, które są znane ze swojej wysokiej aktywności biologicznej (przeciwnowotworowej i/lub przeciwdrobnoustrojowej) [85–89]. Dzięki zastosowaniu różnych podejść syntetycznych otrzymałem serię 10 biokoniugatów **IWR**, które zostały następnie zbadane pod kątem ich aktywności *in vitro* wobec różnorodnych komórek nowotworowych oraz komórek pasożyta *T. b. brucei* ([Schemat 1](#), [Rysunek 5](#)). Wyniki omawianych badań są zasadniczą częścią publikacji **IV**.

Publikacja IV



Rysunek 5. Struktury pochodnych iwermektyny opisane w publikacji IV.

Substratem wyjściowym do syntezy wszystkich biokoniugatów był blokowany w pozycji C5 aglikon **IWR**, którego synteza została omówiona (Schemat 1), jednakże do otrzymania niektórych końcowych produktów biokoniugacji konieczne było przeprowadzenie dodatkowych modyfikacji w pozycji C13 (Schemat 1). Większość wymienionych partnerów, ze względu na kompatybilność grup funkcyjnych, użyłem w swojej pierwotnej formie, jednak w trzech przypadkach konieczne było ich wstępne przygotowanie do biokoniugacji. Dla przykładu, *N*-deacetylotiokolchicinę otrzymałem w wyniku dwuetapowej modyfikacji kolchicyny obejmującej tiolowanie oraz hydrolizę

ugrupowania amidowego. Z kolei w przypadku kwasu betulinowego oraz artezunatu konieczne było otrzymanie ich estrów propargilowych.

Syntezę biokoniugatów zaplanowałem przy użyciu trzech metod. Pierwsza z nich zakładała użycie trifosgeny w obecności trietyloaminy, co pozwoliło mi otrzymać hybrydy **IWR** z alkaloidami kory chinowej oraz *N*-deacetylotiokolchiciną, w których blokowany aglikon połączyłem z wybranym partnerem za pomocą ugrupowania węglanowego lub uretanowego ([Schemat 1](#)). Metoda ta okazała się nieskuteczna w syntezie koniugatów **IWR** z analogami nukleozydów oraz metronidazolem. Aby pokonać ten syntetyczny problem, otrzymałem aktywowany prekursor **IWR** w reakcji blokowanego aglikonu z karbonyldiimidazolem (CDI, [Schemat 1](#)), który następnie skoniugowałem z wybranymi partnerami. Wszystkie otrzymane hybrydy poddałem następnie reakcji z kwasem *para*-toluenosulfonowym w celu usunięcia grupy silylowej w pozycji C5, co pozwoliło otrzymać końcowe produkty **IV-(4-8)** oraz **IV-(10-12)** z wydajnością 14-30% po dwóch etapach ([Rysunek 5](#)).

Ostatnia metoda syntezy biokoniugatów obejmowała zastosowanie reakcji „klik”, tj. 1,3-dipolarnej cykloadycji Huisgena katalizowanej jonami Cu(I), do połączenia **IWR** z kwasem betulinowym lub artezunatem za pomocą pierścienia 1,2,3-triazolowego. W tym celu, poza otrzymaniem partnerów propargilowych, konieczna była synteza azydowego prekursora **IWR**. Niestety, pomimo sprawdzenia najróżniejszych metod i warunków prowadzenia reakcji (substytucji, Appela, Mitsunobu), niska reaktywność pozycji C13 uniemożliwiła bezpośrednie wprowadzenie grupy azydowej w miejsce grupy hydroksylowej. Postanowiłem zatem otrzymać prekursor azydowy przy użyciu znanej literaturowo metody modyfikacji estrowej pochodnej **IWR** [77]. W tym celu blokowany aglikon **IWR** poddałem reakcji estryfikacji z chlorkiem chloroacetyl, a następnie przeprowadziłem reakcję substytucji nukleofilowej z azydkiem sodu ([Schemat 1](#)). Niestety, zbliżona polarność estrowego substratu i azydowego produktu znacząco utrudniała oczyszczenie azydku **IWR** metodami chromatograficznymi. Dlatego wstępnie oczyszczony produkt azydowania użyłem bezpośrednio w reakcji cykloadycji Huisgena zgodnie z protokołem Meldala, stosując jodek miedzi(I) jako źródło kationów Cu(I) ([Schemat 1](#)) [90]. W następnym kroku w trakcie usuwania silylowej grupy ochronnej w pozycji C5 za pomocą kwasu *para*-toluenosulfonowego zauważyłem, że w tych warunkach dochodzi także do rozkładu artezunatu. Chcąc rozwiązać ten problem syntetyczny, zdecydowałem się odwrócić sekwencję modyfikacji – najpierw

przeprowadziłem reakcję odblokowania pozycji C5 azydku **IWR**, a następnie reakcję typu „klik”. Dzięki opisanym procedurom z sukcesem otrzymałem dwie hybrydy triazolowe – **IV-15** oraz **IV-16** – z wydajnością 33-63% ([Rysunek 5](#)).

Wszystkie otrzymane biokoniugaty zostały w pełni scharakteryzowane za pomocą metod spektroskopowych (^1H NMR, ^{13}C NMR) oraz spektrometrycznych (ESI MS). Ze względu na obszerną serię użytych do biokoniugacji partnerów należących do różnych grup związków biologicznie czynnych, niezwykle pomocna w identyfikacji hybryd okazała się spektrometria mas – w widmach ESI MS główne sygnały pochodziły od kompleksów produktów z kationem sodu $[\text{M}+\text{Na}]^+$ i/lub protonem $[\text{M}+\text{H}]^+$. Dzięki analizie NMR potwierdziłem obecność sygnałów diagnostycznych w widmach otrzymanych biokoniugatów, między innymi w widmach ^{13}C NMR sygnały od grupy węglanowej pojawiały się w wąskim zakresie 154,6–153,9 ppm, sygnał od grupy uretanowej zaś przy 158,4 ppm. Z kolei w widmach ^1H NMR bardzo charakterystycznymi sygnałami były singlety pochodzące od atomów wodoru w pierścieniach 1,2,3-triazolowych produktów typu „klik” lub układzie imidazolowym obecnym w strukturze hybrydy z metronidazolem, które obserwowałem w zakresie 7,88–7,78 ppm.

Wszystkie otrzymane hybrydy, partnerzy do biokoniugacji oraz niemodyfikowana chemicznie **IWR** zostały zbadane pod kątem ich aktywności przeciw pasożytniczej (wobec pasożytów *T. b. brucei*) oraz przeciwproliferacyjnej (wobec nowotworowych linii komórek nowotworowych identycznych z tymi z publikacji **III**, tj. PC3, MDA-MB-231, A549 oraz HCT-116). Jako linie referencyjne posłużyły komórki HL-60 (w badaniach przeciw pasożytniczych) oraz HaCaT (w badaniach przeciwproliferacyjnych). Badania biologiczne przeprowadzono w ramach współpracy krajowej z zespołem prof. Marty Strugi z Katedry i Zakładu Biochemii Wydziału Lekarskiego Warszawskiego Uniwersytetu Medycznego oraz współpracy międzynarodowej z dr. Dietmarem Steverdingiem z Norwich Medical School, University of East Anglia, Norwich, Wielka Brytania.

Większość otrzymanych biokoniugatów wykazała wyższą aktywność niż niemodyfikowana **IWR** w zwalczaniu pasożytów *T. b. brucei*, a wartości GI_{50} wahały się w przedziale od 1,3 do 2,4 μM . Wyjątkiem okazał się koniugat **IWR** z kwasem betulinowym (**IV-15**), który wykazał niższą aktywność ($\text{GI}_{50} = 21,9 \mu\text{M}$) niż jego prekursorzy ($\text{GI}_{50, \text{IWR}} = 2,9 \mu\text{M}$ oraz $\text{GI}_{50, \text{kwas betulinowy}} = 15,4 \mu\text{M}$). Dla biokoniugatów **IV-4**, **IV-5**, **IV-6**, **IV-7** i **IV-10** aktywność trypanobójcza znajdowała się w zakresie

pomiędzy aktywnością **IWR** a odpowiednim partnerem użytym do biokoniugacji. Współczynniki selektywności dla wszystkich badanych biokoniugatów były umiarkowanie wysokie, nie przekraczając wartości 100. Jedynie związki **IV-5**, **IV-7** oraz **IV-16** wykazały wyższe wartości tego parametru niż wyjściowa **IWR**.

Biokoniugacja **IWR** z alkaloidami kory chinowej, nukleozydami lub metronidazolem okazała się skuteczną metodą zwiększenia aktywności przeciwaproliferacyjnej **IWR** wobec linii komórkowej raka prostaty (PC3), która była najbardziej oporna na działanie niemodyfikowanego leku i poszczególnych partnerów użytych do biokoniugacji (np. $IC_{50, IWR} = 33,9 \mu M$; $IC_{50, \text{cynchonina}} = 74,3 \mu M$; $IC_{50, IV-6} = 4,1 \mu M$). Z kolei koniugat **IWR** z *N*-deacetylotiokolchicyną (**IV-8**) wykazał co prawda aktywność przeciwaproliferacyjną zbliżoną do **IWR**, jednak jego brak cytotoksyczności wobec referencyjnej linii komórkowej HaCaT ($IC_{50} > 100 \mu M$) wskazuje na jego wysoką selektywność przeciwnowotworowego działania.

Należy podkreślić, że wszystkie otrzymane biokoniugaty **IWR** charakteryzowały się wartościami współczynników selektywności wyższymi niż 1, ukazując ich preferencyjne działanie na komórki nowotworowe. Jest to przewaga otrzymanych hybryd nad powszechnie stosowanym lekiem onkologicznym – doksorubicyną, która okazała się wysoce cytotoksyczna wobec linii referencyjnej HaCaT.

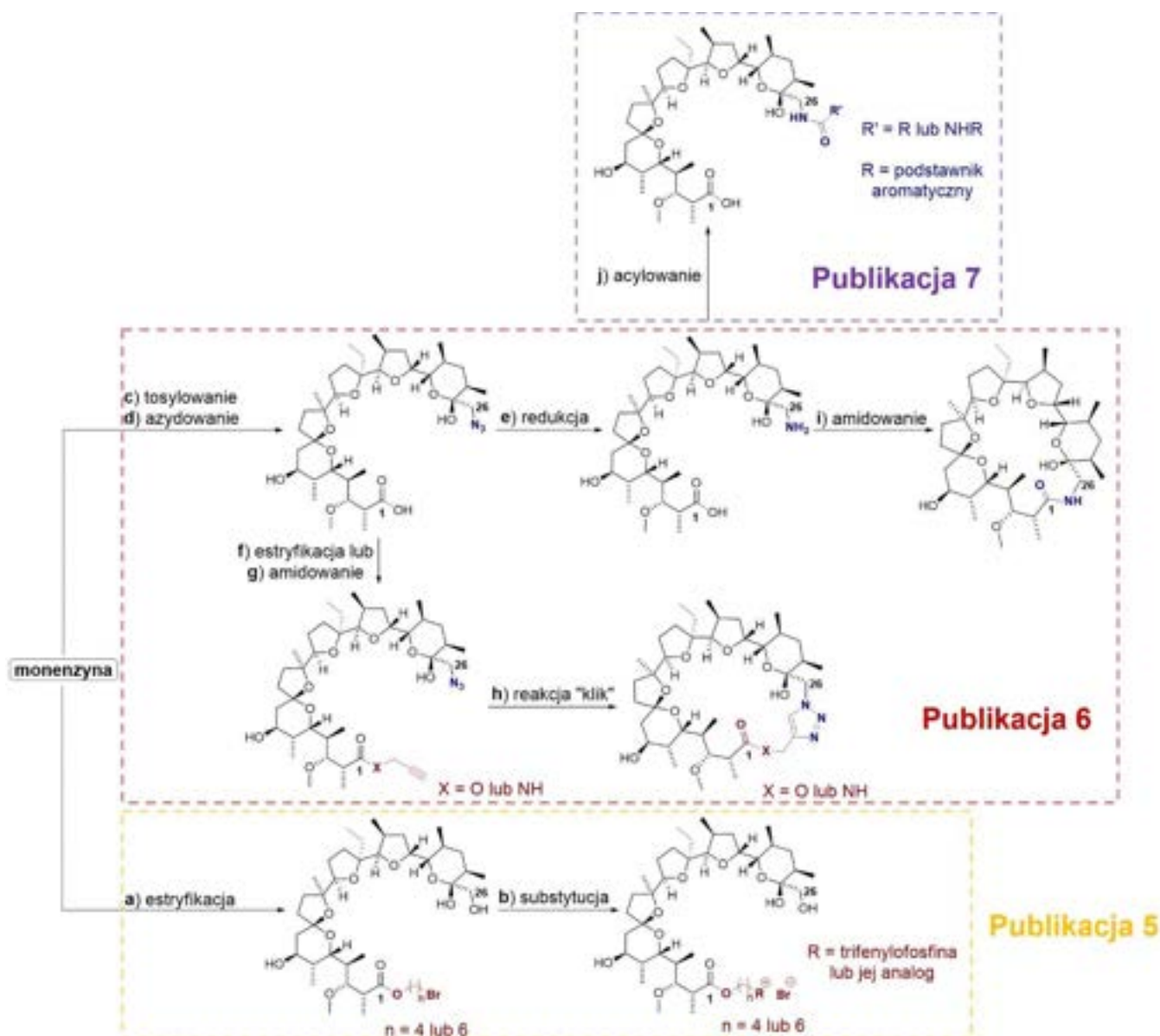
Zarówno w kontekście aktywności przeciwpasożytniczej, jak i przeciwaproliferacyjnej na wyróżnienie zasługuje związek **IV-16** będący biokoniugatem **IWR** i artezunatu. Wartość parametru GI_{50} dla tego związku wyniosła $0,39 \mu M$, czyli odpowiednio 7-krotnie i 9-krotnie mniej niż dla **IWR** i artezunatu. Współczynnik selektywności dla tej hybrydy wobec pasożytów *T. b. brucei* okazał się najwyższy ze wszystkich testowanych biokoniugatów, osiągając wartość około 46. Co ważne, w celu potwierdzenia, że wysoka aktywność biologiczna związku **IV-16** jest wynikiem przeprowadzonej biokoniugacji, oznaczono także aktywność trypanobójczą równomolowej mieszaniny **IWR** i artezunatu. Aktywność ta była 23-krotnie słabsza ($GI_{50} = 9,0 \mu M$) niż dla związku **IV-16**, co sugeruje synergistyczne działanie jako połączona wiązaniem kowalencyjnym hybryda. Związek **IV-16** wykazał ponadto silne działanie w badaniach przeciwaproliferacyjnych. Oprócz najwyższej aktywności spośród testowanych biokoniugatów ($IC_{50} = 3,8 \mu M$ wobec linii A549 oraz $IC_{50} = 4,5 \mu M$ wobec linii HCT-116), związek ten nie wykazał cytotoksyczności wobec linii referencyjnej

HaCaT ($IC_{50} > 100 \mu M$), co dowiodło jego wysokiej selektywności działania ($SI > 26,3$ wobec linii A549 oraz $SI > 22,2$ wobec linii HCT-116). Aktywność przeciwproliferacyjna równomolowej mieszaniny **IWR** i artezunatu była różna dla rozpatrywanych linii komórkowych. Związek **IV-16** okazał się bardziej aktywny wobec linii MDA-MB-231, natomiast mieszanina obu leków okazała się zdecydowanie bardziej aktywna wobec linii PC3. Aktywność przeciwproliferacyjna wobec linii A549 oraz HCT-116 dla hybrydy oraz mieszaniny była zbliżona. Należy jednak podkreślić, że związek **IV-16** wykazuje wielokrotnie niższą cytotoksyczność wobec linii HaCaT niż mieszanina **IWR** i artezunatu. Hybryda **IV-16** powinna zatem służyć jako struktura wiodąca do dalszych badań w kontekście poszukiwania nowych kandydatów na środki przeciwnowotworowe i/lub przeciwpasożytnicze. Dane dotyczące aktywności biologicznej związku **IV-16** znajdują się w Tabeli 2 (czerwona ramka), natomiast szczegółowy opis badań biologicznych otrzymanych biokoniugatów znajduje się w publikacji IV.

Tabela 2. Aktywność przeciwtrypanosomowa oraz przeciwproliferacyjna iwermektyny (**IWR**), artezunatu oraz ich koniugatu (**IV-16**). Uwzględniono również aktywność równomolowej mieszaniny **IWR** i artezunatu. Dane podano jako wartość parametru GI_{50} [μM] dla badań przeciwtrypanosomowych oraz IC_{50} [μM] dla badań przeciwnowotworowych (średnia $\pm$ SD).

	Linia komórkowa lub pasożyt	IWR	artezunat	IV-16	mieszanina
		[μM]	[μM]	[μM]	[μM]
Badania przeciwtrypanosomowe	<i>T. b. brucei</i>	$2,9 \pm 0,1$	$3,6 \pm 0,2$	$0,39 \pm 0,2$	$9,0 \pm 3,5$
	HL-60	$30,0 \pm 1,1$	$30,8 \pm 9,0$	$18,0 \pm 6,0$	
Badania przeciwnowotworowe	PC3	$33,9 \pm 6,3$	$48,5 \pm 5,7$	$20,8 \pm 7,2$	$1,8 \pm 0,5$
	MDA-MB-231	$8,7 \pm 3,2$	$12,6 \pm 3,9$	$13,0 \pm 4,1$	$22,5 \pm 2,6$
	A549	$7,2 \pm 1,4$	$24,0 \pm 6,9$	$3,8 \pm 1,3$	$2,4 \pm 0,7$
	HCT-116	$9,9 \pm 1,1$	$37,8 \pm 4,2$	$4,5 \pm 0,6$	$1,7 \pm 0,2$
	HaCaT	$12,6 \pm 4,3$	$5,7 \pm 0,1$	>100	$16,1 \pm 2,9$

6.2. Modyfikacja chemiczna monenzyny



Schemat 3. Mapa syntez przeprowadzonych w celu chemicznej modyfikacji monenzyny. Warunki i reagenty:

(a) DBU, 1,4-dibromobutan lub 1,6-dibromoheksan, toluen, 90 °C; (b) fosfina, CH₃CN, refluks; (c) chlorek tosyłu, Na₂CO₃ (aq), toluen, tp; (d) NaN₃, DMSO, tp; (e) H₂, Pd/C, MeOH, tp; (f) DBU, bromek propargilu, toluen, 90 °C; (g) DCC, HOBT, propargiloamina, CH₂Cl₂, 0 °C do tp; (h) DIPEA, CuI, CH₃CN, N₂, tp; (i) HATU, DIPEA, DMF, tp; (j) dla amidów: chlorek kwasowy, TEA, CH₂Cl₂, 0 °C do tp lub kwas karboksylowy, chloromrówczan etylu, trietyloamina, DMF, -5 °C do tp; dla mocznika: izocyjanian, toluen, tp.

W ostatnich latach wiele zespołów badawczych podjęło się chemicznej modyfikacji monenzyny (**MON**) w celu otrzymania pochodnych o wyższej aktywności biologicznej niż związek wyjściowy. W cząsteczce tego jonoforu najbardziej interesujące wydają się modyfikacje grupy karboksylowej w pozycji C1 oraz grupy hydroksylowej w pozycji C26. Do tej pory otrzymano szereg pochodnych **MON** o interesujących właściwościach biologicznych (przeciwnowotworowych i/lub przeciwdrobnoustrojowych), między innymi estry, amidy, węglany, uretany oraz koniugaty ze związkami biologicznie aktywnymi (alkaloidami kory chinowej, sylibiną, innymi jonoforami) [91–98]. Potwierdza to, że racjonalna i selektywna modyfikacja wskazanych grup funkcyjnych może zwiększyć aktywność biologiczną związku wyjściowego. Warto jednak wspomnieć, że chemiczna modyfikacja **MON** nie należy do zadań prostych. Związek ten, tak jak wiele innych naturalnych jonoforów polieterowych, ulega nieodwracalnemu rozkładowi w obecności kwasów, zasad oraz w wyniku nadmiernego ogrzewania [99]. Konieczny jest więc dobór takich metod, które z jednej strony umożliwią selektywną modyfikację wybranej grupy funkcyjnej, a z drugiej strony nie doprowadzą do degradacji cząsteczki jonoforu.

Na [Schemacie 3](#) przedstawiłem mapę przeprowadzonych przekształceń chemicznych **MON**, które doprowadziły do otrzymania nowych pochodnych opisanych w publikacjach **V–VII**. W sumie otrzymałem 20 pochodnych **MON**, które zbadano pod kątem aktywności przeciwnowotworowej i/lub przeciwpasożytniczej. W grupie tej można wyróżnić 18 związków otrzymanych po raz pierwszy oraz 2, których syntezę ponowiono w oparciu o wcześniej opisane procedury syntetyczne. Do tej puli nie wliczam otrzymanych przeze mnie pochodnych **MON**, które stanowiły związki przejściowe dla końcowych produktów (np. azydowej lub aminowej pochodnej **MON**). Dane spektroskopowe potwierdzające strukturę i czystość otrzymanych analogów znajdują się w załączonych do niniejszej rozprawy publikacjach i/lub materiałach uzupełniających. Można w nich znaleźć również dane dotyczące aktywności biologicznej, a także właściwości strukturalnych i kompleksotwórczych wybranych związków.

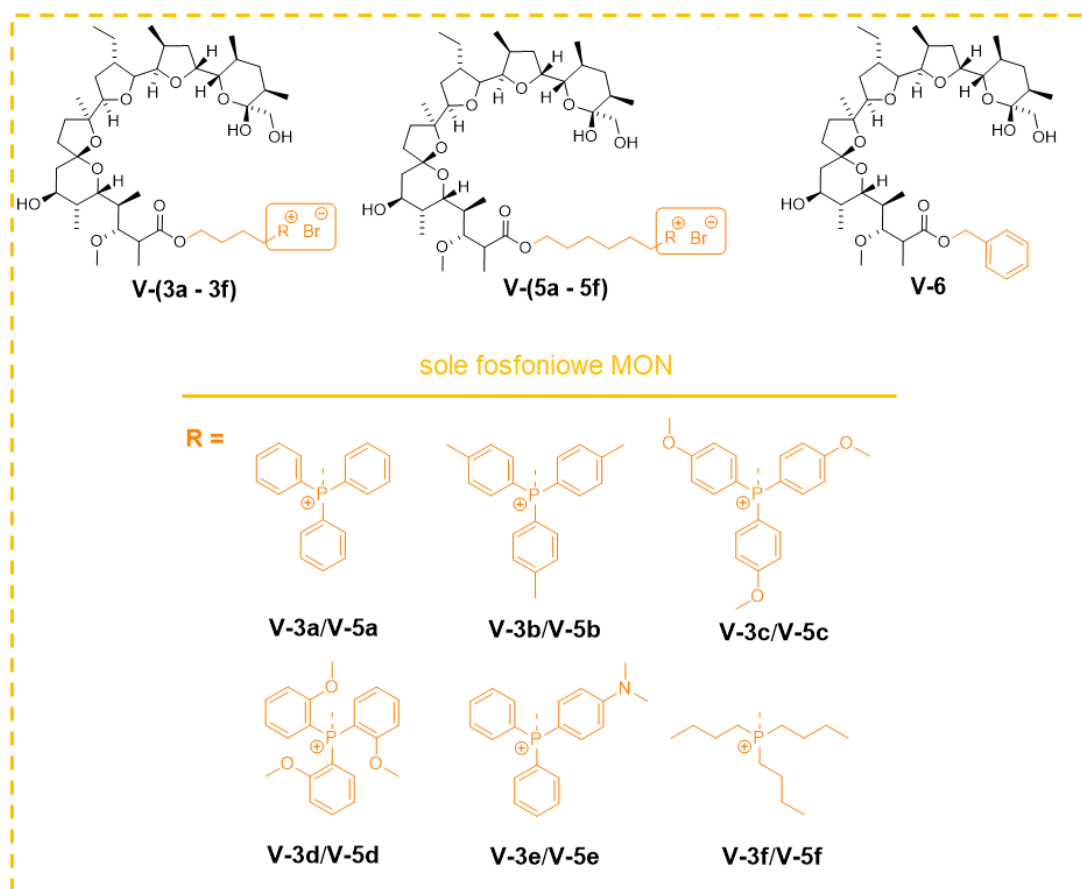
Zanim przejdę do omawiania wyników publikacji **V**, chciałbym w kilku zdaniach opisać strategię projektowania nowych, bioaktywnych związków, która zyskuje coraz większą popularność w chemii medycznej. Jak podkreślałem wcześniej, bardzo ważnym parametrem warunkującym skuteczność kandydatów na leki przeciwnowotworowe jest selektywność działania, czyli preferencja do atakowania komórek nowotworowych. Synteza związków o wysokiej selektywności jest aktualnie jednym z najbardziej

wymagających wyzwań chemii medycznej. W tym kontekście warto wspomnieć, że wiele chorób (nowotwory, cukrzyca, choroby neurodegeneracyjne) jest związanych z funkcjonowaniem mitochondriów [100–102]. Badania wskazują, że organelle te odgrywają ważną rolę w procesie śmierci komórki na drodze apoptozy, co czyni je ważnymi celami przy projektowaniu nowych środków przeciwnowotworowych. Permeabilizacja (zwiększenie przepuszczalności) błony mitochondrialnej jest zjawiskiem warunkującym skierowanie komórki nowotworowej na szlak apoptozy, prowadząc w konsekwencji do jej śmierci [100–102]. Znanych jest wiele związków i ugrupowań chemicznych, które wewnątrz komórki kierowane są do mitochondriów. Jednym z przykładów jest kation trifenylfosfoniowy (TPP⁺) [101,102].

Liczne badania udowodniły, że synteza biokoniugatów substancji biologicznie aktywnych z TPP⁺ prowadzi do zwiększenia ich aktywności przeciwnowotworowej i/lub przeciwdrobnoustrojowej [103–106]. Jak wspomniano wyżej, **MON** jest dobrym partnerem do biokoniugacji, a synteza jej hybryd z różnorodnymi związkami bioaktywnymi została opisana w kilku publikacjach naukowych [94,95,97]. W związku z powyższym, racjonalnym kierunkiem badań wydaje się być koniugacja **MON** z ugrupowaniami chemicznymi celującymi w mitochondria, takimi jak TPP⁺, jak również ocena wpływu tych modyfikacji na aktywność biologiczną otrzymanych pochodnych.

Badania opisane w publikacji V dotyczą syntezy, a także analizy właściwości biologicznych oraz biofizycznych 12 nowych koniugatów **MON** z TPP⁺ ([Schemat 3](#), [Rysunek 6](#)). Obie cząsteczki zostały połączone za pomocą wiązania estrowego przy zastosowaniu łączników alifatycznych o różnej długości. Poza trifenylfosfiną, do syntez użyto także jej analogi z różnorodnymi podstawnikami w pierścieniach aromatycznych oraz fosfinę alifatyczną – tributylfosfinę. W celach porównawczych przeprowadzono ponadto syntezę estru benzyloвого **MON**, aby wykluczyć wpływ samej estryfikacji w pozycji C1 na właściwości otrzymanych biokoniugatów ([Schemat 3](#), [Rysunek 6](#)). Wykonanie wszystkich założonych badań miało na celu określenie zależności pomiędzy strukturą a aktywnością biologiczną otrzymanych hybryd (SAR, ang. *structure-activity relationship*).

Publikacja V



Rysunek 6. Struktury pochodnych monenzyny opisane w publikacji V.

Synteza soli fosfoniowych **MON** przebiegała dwuetapowo i polegała na syntezie odpowiednich estrów **MON** z 1,4-dibromobutanem lub 1,6-dibromoheksanem, a następnie reakcji substytucji 1^o bromku z odpowiednią fosfiną (Schemat 3). Doprowadziło to finalnie do otrzymania koniugatów **V-(3a-3f)** oraz **V-(5b-5f)** z wydajnością reakcji substytucji w zakresie 14–36% w zależności od użytej fosfiny oraz długości łącznika alifatycznego (Rysunek 6). Najniższe wydajności zaobserwowałem w przypadku koniugatów **MON** z niemodyfikowaną tryfenylofosfiną. Nie udało się przez to otrzymać powyższą metodą hybrydy **V-5a** i konieczne było odwrócenie procedury syntetycznej – w pierwszym kroku przeprowadziłem reakcję substytucji atomu bromu w strukturze 1,6-dibromoheksanu za pomocą tryfenylofosfiny, produkt tej reakcji zaś poddałem estryfikacji z cząsteczką **MON**. Dzięki temu otrzymałem związek **V-5a** z wydajnością 43% (Rysunek 6).

W przypadku tego rodzaju koniugatów poza standardową analizą ¹H/¹³C NMR wykonano również pomiary widm ³¹P NMR. Najważniejsze analityczne sygnały w widmach ¹³C NMR pochodziły od nowo wprowadzonych grup estrowych w pozycji C1

jonoforu, które były obecne w wąskim zakresie 176,3–176,0 ppm. Z kolei zarejestrowanie sygnałów w widmach ^{31}P NMR w zakresie 34,9–22,8 ppm świadczyło o obecności atomów fosforu w strukturach zsyntetyzowanych pochodnych **MON**. Obecność reszt fosfoniowych pozwoliła natomiast na identyfikację oczekiwanych pików molekularnych $[\text{M}]^+$ w widmach HRMS.

Sole fosfoniowe **MON V-(3a-3f)** oraz **V-(5a-5f)**, ester benzyłowy **V-6**, a także niemodyfikowany chemicznie jonofor zbadano pod kątem aktywności przeciwproliferacyjnej we współpracy krajowej z zespołem prof. Marty Strugi z Katedry i Zakładu Biochemii Wydziału Lekarskiego Warszawskiego Uniwersytetu Medycznego wobec pięciu linii ludzkich komórek nowotworowych, tj. nowotworu jelita grubego (SW480, SW620), nowotworu prostaty (PC3), nowotworu piersi (MDA-MB-231) oraz nowotworu płuc (A549). Jako zdrowe linie referencyjne wybrano ludzkie keratynocyty (HaCaT) oraz fibroblasty płuc chomika chińskiego (V79). Wyniki aktywności przeciwproliferacyjnej wybranych związków na niektórych liniach komórkowych zostały zestawione w [Tabeli 3](#).

Tabela 3. Aktywność przeciwproliferacyjna wybranych pochodnych monenzyny. Dane podano jako wartość parametru IC_{50} [μM] (średnia $\pm$ SD).

Związek	Linie nowotworowe		Linia normalna
	PC3	MDA-MB-231	HaCaT
	IC_{50} [μM]	IC_{50} [μM]	IC_{50} [μM]
MON	$0,62 \pm 0,04$	$2,11 \pm 0,02$	$0,62 \pm 0,04$
Pochodne aromatyczne	V-3a	$0,52 \pm 0,12$	$0,97 \pm 0,20$
	V-3b	$0,59 \pm 0,19$	$0,72 \pm 0,08$
	V-3c	$0,50 \pm 0,11$	$0,93 \pm 0,29$
	V-3d	$0,48 \pm 0,09$	$0,66 \pm 0,17$
	V-3e	$0,97 \pm 0,22$	$0,69 \pm 0,10$
Pochodna alifatyczna	V-3f	$1,11 \pm 0,06$	$2,66 \pm 0,14$
Ester benzyłowy	V-6	$5,22 \pm 0,79$	$33,96 \pm 1,6$
Odnosićnik	Doksorubicyna	$0,59 \pm 0,02$	$0,83 \pm 0,03$

Wszystkie otrzymane sole fosfoniowe **MON** wykazały aktywność w stężeniach mikromolowych wobec testowanych linii komórek nowotworowych, w tym wiele z nich było bardziej aktywnych niż niemodyfikowany jonofor oraz doksorubicyna użyta jako referencyjny lek onkologiczny (np. IC_{50} , **v-3d** = $0,48 \mu\text{M}$, IC_{50} , **MON** = $0,62 \mu\text{M}$

i $IC_{50, \text{doksorubicyna}} = 0,59 \mu\text{M}$ wobec linii PC3). W większości przypadków koniugaty o łącznikach 4-węglowych **V-(3a-3f)** wykazały wyższą aktywność przeciwproliferacyjną niż ich 6-węglowe analogi **V-(5a-5f)** – stąd, jedynie aktywność tych pierwszych została zaprezentowana w Tabeli 3. Ester benzylowy **V-6**, jako próba negatywna, charakteryzował się drastycznym spadkiem aktywności przeciwproliferacyjnej w stosunku do niemodyfikowanego jonoforu (np. $IC_{50, \text{V-6}} = 33,96 \mu\text{M}$ i $IC_{50, \text{MON}} = 2,11 \mu\text{M}$ wobec linii MDA-MB-231, Tabela 3). Wszystkie otrzymane analogi wykazały ponadto mniejszą cytotoksyczność niż **MON** wobec linii referencyjnej HaCaT (Tabela 3). Dodatkowo, alifatyczna sól fosfoniowa **V-3f** wykazała niższą aktywność przeciwproliferacyjną niż koniugaty z trifenylfosfiną i jej analogami (np. $IC_{50, \text{V-3f}} = 2,66 \mu\text{M}$ i $IC_{50, \text{V-3a}} = 0,66 \mu\text{M}$ wobec linii MDA-MB-231, Tabela 3). Przeprowadzone testy *in vitro* potwierdziły, że koniugacja **MON** z aromatycznymi fosfinami może doprowadzić nie tylko do związków bardziej aktywnych przeciwnowotworowo, ale też wykazujących wyższą selektywność działania. W celu dokładniejszego potwierdzenia tej hipotezy, przeprowadzono szczegółowe badania biologiczne oraz biofizyczne wybranych analogów.

Badania mechanizmów aktywności biologicznej wykazały, że sole fosfoniowe **V-3a** oraz **V-3f** prowadzą między innymi do wytworzenia reaktywnych form tlenu (ROS), zwiększenia proporcji komórek nowotworowych w fazie wczesnej i późnej apoptozy, a także zahamowania cyklu komórkowego linii A549 i MDA-MB-231, powodując zwiększenie populacji komórek w fazie G0/G1. Głównym celem przeprowadzonych badań było jednak udowodnienie, że otrzymane hybrydy mogą być selektywnie kierowane do mitochondriów komórek nowotworowych. Badania przy użyciu barwnika JC-10 potwierdziły, że zastosowanie koniugatów **V-3a** oraz **V-3f** zwiększało intensywność zielonej fluorescencji (87,9% dla związku **V-3f** wobec linii MDA-MB-231), która była związana z utratą przez komórki nowotworowe mitochondrialnego potencjału błonowego. Wyniki te były w bardzo dobrej korelacji z przeprowadzonymi testami aktywności proapoptotycznej, co potwierdziło, że do śmierci badanych komórek nowotworowych dochodziło między innymi poprzez utratę ich mitochondrialnego potencjału błonowego.

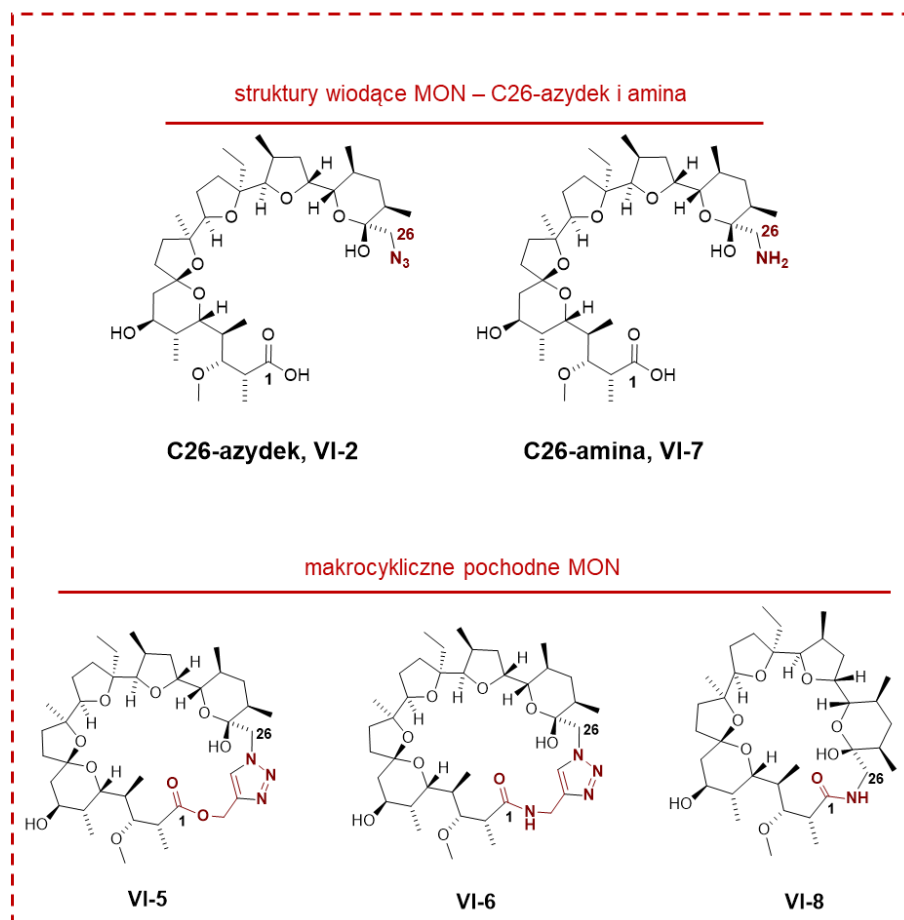
Postulowany mechanizm został również potwierdzony w badaniach biofizycznych za pomocą układu Oxygraph-2K wykonanych we współpracy krajowej z zespołem prof. Piotra Bednarczyka z Katedry Fizyki i Biofizyki, Instytutu Biologii, Szkoły Głównej Gospodarstwa Wiejskiego w Warszawie. Zauważono, że komórki linii nowotworowej A549 zużywały więcej tlenu po zastosowaniu soli **V-3a** oraz **V-3f** w stężeniach

odpowiednio 3 i 10 μM , co prowadziło do destabilizacji mitochondriów i ostatecznie zmniejszenia szybkości oddychania komórkowego. Efekt ten nie pojawiał się po zastosowaniu niemodyfikowanej **MON**. Badania biofizyczne pozwoliły również określić zdolność otrzymanych soli fosfoniowych do przenoszenia ładunku przez błony biologiczne. W eksperymencie BLM (ang. *black-lipid membrane*) zmierzono zależność pomiędzy generowanym natężeniem prądu a przyłożonym napięciem w obecności różnych stężeń badanych substancji. **MON** i jej ester benzylowy **V-6** nie powodowały zwiększenia natężenia prądu w żadnym badanym stężeniu, co oznaczało, że nie były w stanie efektywnie transportować ładunku przez modelową dwuwarstwę lipidową. Z kolei zastosowanie związków **V-3a** oraz **V-3e** umożliwiło wygenerowanie prądu o natężeniu tym wyższym, im wyższe było stężenie zastosowanej substancji. Wyniki te jednoznacznie potwierdziły, że otrzymane sole fosfoniowe były w stanie przenosić ładunek przez modelową dwuwarstwę lipidową oraz prowadzić do apoptozy komórek nowotworowych przez oddziaływanie na mitochondria.

Większość znanych chemicznych przekształceń cząsteczki **MON** zostało przeprowadzonych w obrębie grupy C1 karboksylowej lub grupy C26 hydroksylowej. Modyfikacje pozycji C26 były jednak ograniczone wyłącznie do estrów, *O*-uretanów oraz węglanów, a w literaturze naukowej nigdy nie opisano metody przekształcenia grupy C26 hydroksylowej w inną grupę funkcyjną, mogącą być dogodnym substratem wyjściowym do dalszych transformacji. Obszerne badania uwzględniające wpływ różnych reagentów, rozpuszczalników, warunków temperaturowych, zasad organicznych/nieorganicznych, itd., pozwoliły mi ostatecznie opracować pierwszą, efektywną, prostą do wykonania i możliwą do skalowania metodę syntezy azydku **MON** w pozycji C26 ([Schemat 3](#)). Metoda ta została szczegółowo opisana w publikacji **VI**. Polegała ona na tosylovaniu grupy C26 hydroksylowej **MON**, a następnie reakcji substytucji z NaN_3 ([Schemat 3](#)). Pozwoliło to na otrzymanie azydku **VI-2** z zadowalającą wydajnością w skali gramowej ([Rysunek 7](#)). Z jednej strony, azydek stanowił bardzo wygodny substrat wyjściowy do 1,3-dipolarnej cykloaddycji Huisgena, a z drugiej strony, mógł być interesującym prekursorem z punktu widzenia chemii medycznej do przekształcenia w grupę aminową, co otwiera nowe, atrakcyjne możliwości syntetyczne w kierunku oryginalnych pochodnych jonoforu, np. amidów lub moczników. **MON** nie posiada w swojej strukturze wiązań wielokrotnych, ani innych grup wrażliwych na redukcję wodorem, w związku z czym możliwe było

przekształcenie grupy C26 azydowej do aminowej w **VI-7** z zadowalającą wydajnością 63% (Schemat 3, Rysunek 7).

Publikacja VI



Rysunek 7. Struktury pochodnych monenzyny opisane w publikacji VI.

Dużą rolę w chemii medycznej odgrywają związki makrocykliczne [107,108]. Dzięki niskiej labilności konformacyjnej, wykazują one wiele pożądanych właściwości, w tym wysoką stabilność metaboliczną oraz selektywność działania, które umożliwiają ich zastosowanie między innymi jako inhibitory różnorodnych enzymów lub w katalizie organicznej [108–111]. Niezwykle ciekawym kierunkiem badań wydawała się więc być makrocyklizacja pseudocyklicznego jonoforu – **MON**. Historycznie pierwsze tego typu pochodne zostały otrzymane w wyniku reakcji makrolaktonizacji Corey-Nicolaou lub z użyciem chiralnych aminokwasów [112,113]. Mając dostęp do azydkowego oraz aminowego prekursora **MON**, postanowiłem zsyntetyzować nowe, makrocykliczne pochodne tego jonoforu. Wyniki badań zostały szczegółowo opisane w publikacji VI, a także były tematem pracy licencjackiej Roberta Granicznego – studenta, który realizował

pracę dyplomową w Zakładzie Chemii Medycznej Wydziału Chemii UAM i w której pełniłem rolę opiekuna pracy laboratoryjnej.

Nowe makrocykliczne pochodne **MON** zostały zsyntetyzowane za pomocą reakcji „klik” zgodnie z protokołem Meldala lub makrolaktamizacji. W tym celu w pierwszym kroku **VI-2** poddano reakcji estryfikacji z bromkiem propargilu w obecności DBU lub amidowania z propargiloaminą w obecności DCC i HOBt, co doprowadziło do otrzymania odpowiednio estru lub amidu propargilowego **MON** (Schemat 3). Dywersyfikacja charakteru wiązania chemicznego miała pozwolić na określenie jego wpływu na właściwości produktów makrocyklicznych. Następnie przeprowadzono reakcje „klik”, w wyniku czego otrzymano dwie makrocykliczne pochodne – z wiązaniem estrowym (**VI-5**) lub amidowym (**VI-6**), z wydajnościami odpowiednio 74% i 52% (Rysunek 7). Trzeci produkt makrocykliczny powstał na drodze makrolaktamizacji aminowej pochodnej **MON** (**VI-7**). Najefektywniejsza w tym przypadku okazała się reakcja z dodatkiem HATU, która pozwoliła na otrzymanie związku **VI-8** z wydajnością 72% (Schemat 3, Rysunek 7).

Najbardziej charakterystycznymi sygnałami dla makrocyklicznych pochodnych **MON** w widmach ^{13}C NMR były sygnały pochodzące od grup estrowych/amidowych w pozycji C1, które pojawiały się w zakresie 178,3–175,5 ppm. W widmach ^1H NMR laktamów **VI-6** i **VI-8** charakterystyczne były szerokie singlety pochodzące od grup amidowych, które obecne były w zakresie 7,41–6,50 ppm, a w przypadku pochodnych triazolowych (**VI-5** i **VI-6**) charakter analityczny miały singlety pochodzące od atomów wodoru pierścieni 1,2,3-triazolowych, które pojawiły się w przedziale 7,90–7,84 ppm. W widmach FT-IR najbardziej charakterystyczne pasma pochodziły od drgań rozciągających grup karbonylowych w estrach (1744–1741 cm^{-1}) oraz amidach, tzw. pasma amidowego (1657–1641 cm^{-1}). Związki **VI-2**, **VI-5** i **VI-8** zostały otrzymane w formie krystalicznej, co pozwoliło na przeprowadzenie analizy krystalograficznej we współpracy z prof. Janem Janczakiem z Instytutu Niskich Temperatur i Badań Strukturalnych Polskiej Akademii Nauk we Wrocławiu. Związek **VI-2** krystalizował w niecentrosymetrycznej grupie przestrzennej $P2_1$ układu jednoskośnego jako kompleks z cząsteczką wody. Co ciekawe, grupa azydowa była skierowana poza wewnętrzną „kieszę” jonoforu, a struktura **VI-2** była dodatkowo stabilizowana przez szereg wiązań wodorowych. Makrocykliczny lakton **VI-5**, będący produktem modyfikacji chemicznej azydku **VI-2**, również krystalizował w grupie przestrzennej $P2_1$ układu jednoskośnego, jednak w formie kompleksu z nadchloranem sodu. Atomy azotu pierścienia triazolowego były skierowane

na zewnątrz pierścienia makrocyklicznego i nie uczestniczyły w koordynowaniu kationu sodu, co może wynikać z przestrzennego ułożenia grupy azydowej w związku **VI-2**. Do dużych zmian strukturalnych doszło w przypadku związku **VI-8**, który krystalizował w niecentrosymetrycznej grupie przestrzennej $P2_12_12_1$ układu rombowego jako kompleks z nadchloranem sodu. Struktury obu makrocyklicznych pochodnych były dodatkowo stabilizowane przez szereg wiązań wodorowych. Porównanie podstawowych parametrów krystalograficznych dla wszystkich trzech związków zostało przedstawione w Tabeli 4, a bardziej szczegółowy opis jest częścią publikacji **VI** oraz materiałów uzupełniających.

Tabela 4. Dane krystalograficzne związków **VI-2**, **VI-5** i **VI-8**.

		VI-2	VI-5	VI-8
Wzór sumaryczny		$C_{36}H_{61}N_3O_{10} \cdot H_2O$	$C_{39}H_{63}ClN_3NaO_{14} \cdot C_2H_3N$	$C_{36}H_{61}ClNNaO_{13}$
Masa molowa		713,89 g/mol	897,41 g/mol	774,29 g/mol
Temperatura pomiaru		100 K	100 K	100 K
Układ krystalograficzny		Jednoskośny	Jednoskośny	Rombowy
Grupa przestrzenna		$P2_1$	$P2_1$	$P2_12_12_1$
Wymiary komórki elementarnej	a (Å)	8,2504(2)	8,2439(4)	12,0123(4)
	b (Å)	17,1298(5)	22,8701(10)	14,9800(5)
	c (Å)	13,7271(3)	11,9581(6)	22,0196(6)
	β (°)	97,865(2)	90,789(5)	—
V (Å ³)		1921,77(8)	2254,35(19)	3962,3(2)

Biorąc pod uwagę, że większość właściwości biologicznych jonoforów wynika z ich naturalnej zdolności do koordynowania kationów metali, postanowiłem sprawdzić, czy nowo otrzymane struktury makrocykliczne również wykazują właściwości kompleksotwórcze. W tym celu przygotowałem roztwory związków **VI-5**, **VI-6** i **VI-8** w acetonitrylu z dodatkiem soli nadchloranowej odpowiedniego metalu pierwszej ($LiClO_4$, $NaClO_4$, $KClO_4$, $RbClO_4$ lub $CsClO_4$) lub drugiej grupy ($Mg(ClO_4)_2$, $Ca(ClO_4)_2$, $Sr(ClO_4)_2$ lub $Ba(ClO_4)_2$). Tworzenie związków kompleksowych śledziłem za pomocą spektrometrii mas ESI MS, która jest powszechną metodą oceny oddziaływań w układach gość-gospodarz [114]. Badania z użyciem soli metali pierwszej grupy potwierdziły wytworzenie kompleksów typu $[VI-5 + M]^+$, $[VI-5 + M + H_2O]^+$, $[VI-6 + M]^+$ oraz $[VI-8 + M]^+$ ze wszystkimi testowanymi jonami metali, gdzie $M = Li, Na, K, Rb$ lub Cs , przy czym najwyższe powinowactwo stwierdziłem wobec kationów sodu. Testy kompleksowania z zastosowaniem soli metali drugiej grupy również potwierdziły wytworzenie związków

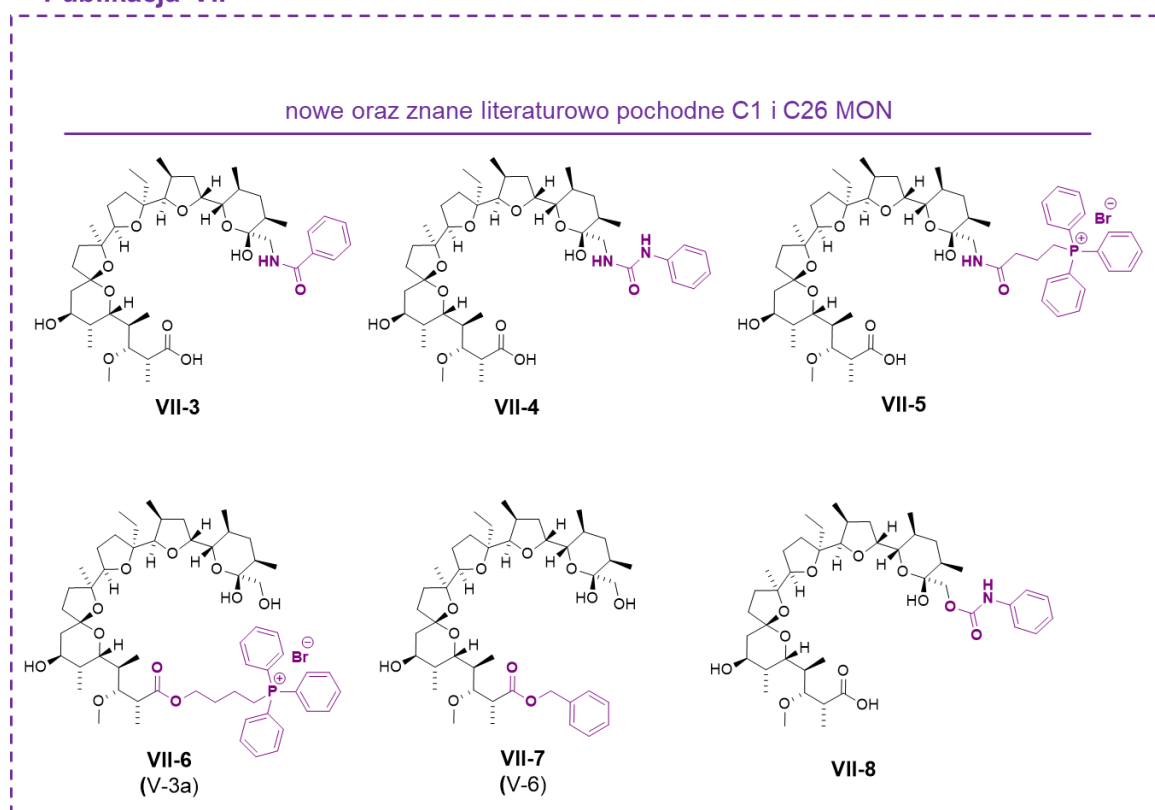
koordynacyjnych, jednak w tym przypadku jony nadchloranowe także pełniły rolę ligandów: $[\text{VI-5} + \text{MClO}_4]^+$, $[\text{VI-5} + \text{MClO}_4 + \text{H}_2\text{O}]^+$, $[\text{VI-6} + \text{MClO}_4]^+$, $[\text{VI-8} + \text{MClO}_4]^+$ oraz $[\text{VI-8} - \text{H} + \text{M}]^+$, gdzie $\text{M} = \text{Mg}, \text{Ca}, \text{Sr}$ lub Ba . W związku z tym, że jon nadchloranowy uzupełniał przestrzeń koordynacyjną kationu sodu w opisywanych powyżej strukturach krystalograficznych, należy stwierdzić, że makrocycliczne pochodne **MON** mogą pełnić rolę receptorów par jonowych [115].

Ostatnim krokiem badań nad makrocyclicznymi pochodnymi **MON** była ocena ich właściwości przeciwproliferacyjnych, która została przeprowadzona we współpracy z zespołem prof. Joanny Wietrzyk z Instytutu Immunologii i Terapii Doświadczalnej Polskiej Akademii Nauk im. Ludwika Hirszfelda we Wrocławiu. W testach uwzględniono cztery linie ludzkich komórek nowotworowych: A549 (nowotwór płuc), MCF-7 (nowotwór piersi), LoVo (nowotwór okrężnicy), LoVo/DX (nowotwór okrężnicy oporny na działanie doksorubicyny) oraz linię mysich fibroblastów BALB/3T3 jako odnośnik. Wszystkie pochodne makrocycliczne wykazały niższą aktywność przeciwproliferacyjną niż niemodyfikowana **MON**, co potwierdziło wyniki poprzednich badań [112]. Warto jednak zaznaczyć, że otrzymane analogi charakteryzowały się zdecydowanie niższą cytotoksycznością wobec linii referencyjnej BALB/3T3 niż związek wyjściowy. Najwyższą aktywność przeciwproliferacyjną spośród testowanych makrocycli wykazywał związek **VI-5** zawierający w swojej strukturze wiązanie estrowe. Był on ponad 2-krotnie bardziej aktywny niż amidowy odpowiednik ($\text{IC}_{50, \text{VI-5}} = 7,32 \mu\text{M}$ i $\text{IC}_{50, \text{VI-6}} = 15,5 \mu\text{M}$ wobec linii A549). Makrolaktam **VI-8** charakteryzował się najniższą cytotoksycznością wobec linii referencyjnej BALB/3T3 spośród testowanych związków, co sprawiło, że był on najbardziej selektywnym analogiem makrocyclicznym wobec linii A549 i LoVo (SI równe odpowiednio 2,1 oraz 3,3). Na wyróżnienie zasługuje również wysoka aktywność badanych związków wobec lekoopornej linii LoVo/DX. Związek **VI-5** był bardziej aktywny i selektywny wobec tej linii niż powszechnie stosowana w chemioterapii cisplatyna ($\text{IC}_{50, \text{VI-5}} = 2,19 \mu\text{M}$, $\text{SI} = 6,2$; $\text{IC}_{50, \text{cisplatyna}} = 2,4 \mu\text{M}$, $\text{SI} = 1,3$).

Znanych jest wiele przekształceń pozycji C26 **MON**, które prowadziły do pochodnych o wyższej aktywności przeciwproliferacyjnej i/lub przeciwtetrynosomowej [44,93,96,98], jednak niezwykle ubogi jest materiał dotyczący potencjalnych właściwości przeciwmalarycznych tego typu analogów. Pochodne **MON** otrzymane przez chemiczną modyfikację jej pozycji C26, takie jak pochodna tosylowana lub węglanowa, wykazały niezwykle silną skuteczność w zwalczaniu zarodźców *P. falciparum* (odpowiednio

$IC_{50} = 1,52$ nM oraz $1,08$ nM), przewyższając aktywność niemodyfikowanego jonoforu ($IC_{50} = 2,46$ nM) [49,116]. W świetle tych doniesień założyłem, że dalsza funkcjonalizacja otrzymanego przeze mnie prekursora C26 aminowego może prowadzić do nowych substancji o silnych właściwościach przeciwmalarycznych. W tym celu postanowiłem przeprowadzić syntezę kilku wybranych analogów i sprawdzić ich aktywność przeciwmalaryczną – wyniki badań zostały opisane w publikacji VII (Schemat 3, Rysunek 8).

Publikacja VII



Rysunek 8. Struktury pochodnych monenzyny opisane w publikacji VII.

Zdecydowałem się na syntezę pochodnych aromatycznych ze względu na wysoką aktywność przeciwpasożytniczą wykazywaną przez tego typu analogi w poprzednich badaniach [98]. W wyniku reakcji pomiędzy C26 aminą **MON** a chlorkiem benzoilu lub izocyjanianem fenylu otrzymałem odpowiednio amid **VII-3** oraz mocznik **VII-4** (Schemat 3, Rysunek 8). Biorąc pod uwagę, że mitochondria pasożytów *Plasmodium* mogą być ważnym celem terapeutycznym w projektowaniu nowych leków przeciwmalarycznych [117,118], przeprowadziłem ponadto syntezę C26 amidowego koniugatu **MON** z TPP⁺ (związek **VII-5**) (Schemat 3). Bibliotekę pochodnych **MON** rozszerzyłem dodatkowo o dwa związki opisane wcześniej w publikacji V – estrowy koniugat **MON** z TPP⁺ (**VII-6**)

oraz ester benzytowy **MON (VII-7)** ([Rysunek 8](#)). Serię uzupełniłem o związek **VII-8** będący *O*-uretanem fenylowym **MON** w pozycji C26. Pochodna ta wykazała wyjątkowo wysoką aktywność przeciwtrypanosomową w poprzednich badaniach [98], więc istniała duża szansa, że okaże się również skuteczna w walce z pasożytem malarii – *Plasmodium*.

Najbardziej charakterystyczne sygnały w widmach ^{13}C NMR pochodziły od nowo wprowadzonych ugrupowań, tj. grup estrowych w pozycji C1 dla związków **VII-6** i **VII-7** (176,0–175,2 ppm) oraz grup amidowych, mocznikowych lub uretanowych w pozycji C26 dla związków **VII-3**, **VII-4**, **VII-5** oraz **VII-8** (174,1–154,7 ppm). W widmach HRMS główne piki pochodziły od kompleksów otrzymanych produktów z kationem sodu $[\text{M}+\text{Na}]^+$, a dla koniugatów z TPP^+ były efektem wprowadzenia motywu fosfoniowego, tj. $[\text{M}]^+$. Ponadto, związki **VII-4** i **VII-5** zostały otrzymane w formie krystalicznej, co pozwoliło na przeprowadzenie analizy krystalograficznej we współpracy z prof. Janem Janczakiem z Instytutu Niskich Temperatur i Badań Strukturalnych Polskiej Akademii Nauk we Wrocławiu. Badania rentgenograficzne potwierdziły, że pochodne **VII-4** i **VII-5** efektywnie koordynują kation sodu, a ich podstawniki w pozycji C26 nie są zaangażowane w proces koordynacji. Pochodna mocznikowa **VII-4** krystalizowała w niecentrosymetrycznej grupie przestrzennej $R3:H$ układu trygonalnego jako kompleks z cząsteczkami wody, a pochodna amidowa **VII-5** krystalizowała w niecentrosymetrycznej grupie przestrzennej $P2_12_12_1$ układu rombowego jako kompleks z cząsteczką wody i jonami bromkowymi. Struktura pseudocykliczna w obu przypadkach była stabilizowana przez liczne wiązania wodorowe. Struktura krystalograficzna uretanu **VII-8** została już wcześniej opublikowana [119], dzięki czemu możliwe było przeprowadzenie analizy porównawczej pomiędzy uretanem **VII-8** a jego mocznikowym analogiem **VII-4**. Analiza rentgenograficzna potwierdziła podobieństwa strukturalne pomiędzy tymi związkami, w tym dotyczące rodzaju wytworzonych wiązań wodorowych. Co ciekawe, pomimo wielu podobieństw strukturalnych, oba związki krystalizowały w różnych grupach przestrzennych – mocznik **VII-4** w grupie przestrzennej $R3:H$ układu trygonalnego, a uretan **VII-8** w grupie przestrzennej $P1$ układu trójskośnego [119]. Porównanie podstawowych parametrów krystalograficznych dla wszystkich trzech związków zostało zaprezentowane w [Tabeli 5](#), a bardziej szczegółowy opis jest częścią publikacji **VII** oraz materiałów uzupełniających.

Tabela 5. Dane krystalograficzne związków VII-4, VII-5 i VII-8 [119].

		VII-4	VII-5	VII-8
Wzór sumaryczny		$C_{43}H_{69}N_2NaO_{12} \cdot 0,5H_2O$	$C_{58}H_{84}NNaO_{12}P \cdot 2Br$	$C_{43}H_{66}NO_{12}Na$
Masa molowa		838,00 g/mol	1201,04 g/mol	811,96 g/mol
Temperatura pomiaru		100 K	100 K	293 (2) K
Układ krystalograficzny		Trygonalny	Rombowy	Trójskośny
Grupa przestrzenna		$R\bar{3}:H$	$P2_12_12_1$	$P1$
Wymiary komórki elementarnej	a (Å)	26,4195 (8)	9,4554 (3)	9,5794 (12)
	b (Å)	—	12,5578 (5)	10,8654 (12)
	c (Å)	17,5811 (5)	50,0072 (19)	12,4681 (15)
	α (°)	—	—	107,478 (10)
	β (°)	—	—	111,536 (11)
	γ (°)	—	—	95,335 (9)
V (Å ³)		10627,4 (7)	5937,8 (4)	1120,8 (2)

Wszystkie otrzymane związki **VII-(3-8)**, niemodyfikowana **MON** oraz prymachina jako lek referencyjny zostały zbadane pod kątem aktywności przeciwmalarycznej wobec wątrobowego stadium pasożyta *P. berghei*. Badania przeprowadzono w ramach współpracy międzynarodowej z prof. Miguelem Prudêncio z Instituto de Medicina Molecular João Lobo Antunes, Faculdade de Medicina, Universidade de Lisboa & Gulbenkian Institute for Molecular Medicine, Portugalia. Analiza otrzymanych wyników udowodniła, że niemodyfikowany jonofor wykazywał bardzo wysoką aktywność przeciwmalaryczną na poziomie stężeń nanomolowych ($IC_{50, MON} = 5,5$ nM) osiągając ponad 1500-krotnie niższą wartość parametru IC_{50} niż stosowana w medycynie prymachina. Najważniejszym odkryciem było udowodnienie, że wszystkie otrzymane pochodne wykazały wielokrotnie wyższą aktywność przeciwmalaryczną niż lek referencyjny, a niektóre z nich były nawet bardziej aktywne niż **MON**. Pochodne otrzymane w wyniku chemicznej modyfikacji pozycji C26 były zdecydowanie skuteczniejsze w zwalczaniu pasożytów *P. berghei* niż pochodne w pozycji C1, z wyjątkiem związku **VII-5**, który okazał się najmniej aktywny spośród całej serii. Koniugacja **MON** z TPP^+ , niezależnie od tego, czy była przeprowadzona w pozycji C1 czy C26, nie wpłynęła pozytywnie na właściwości przeciwmalaryczne jonoforu. Amid **VII-3** oraz mocznik **VII-4** charakteryzowały się aktywnością przeciwmalaryczną również na poziomie stężeń nanomolowych ($IC_{50, VII-3} = 6,4$ nM oraz $IC_{50, VII-4} = 2,9$ nM). Najbardziej aktywnym związkiem okazał się uretan **VII-8**, który **wykazał aktywność poniżej 1 nM** ($IC_{50, VII-8} = 0,7$ nM). Co ważne,

wiele z otrzymanych związków poza swoją wysoką aktywnością przeciwmalaryczną, charakteryzowało się też znaczną selektywnością, preferencyjnie działając na komórki pasożyta. Wartość parametru SI dla uretanu **VII-8** przekroczyła 4000, co oznacza że jest on nie tylko najbardziej aktywnym, ale też najbardziej selektywnym związkiem w całej badanej serii. Przeprowadzone badania potwierdziły, że **MON** i jej pochodne w pozycji C26 wykazują silny potencjał przeciwmalaryczny, a kolejne generacje półsyntetycznych produktów wywodzących się ze struktury jonoforu mogą stać się w przyszłości potencjalnymi kandydatami na nowe leki skuteczne w walce z malarią.

7. Podsumowanie i wnioski

	Modyfikacja chemiczna iwermektyny (IWR)		Modyfikacja chemiczna monenzyny (MON)		
	Publikacje II i III	Publikacja IV	Publikacja V	Publikacja VI	Publikacja VII
Zsyntetyzowane pochodne	C13- <i>epi</i> -amidy IWR oraz ich C4-hydroksy analogi	Biokoniugaty IWR z bioaktywnymi partnerami	Estrowe koniugaty MON z TPP ⁺ w pozycji C1	Prekursor azydkowy i aminowy MON oraz ich makrocykliczne analogi	C26-acylowe pochodne MON
Badania spektroskopowe i spektrometryczne	¹³C NMR ¹H NMR - HRMS	¹³C NMR ¹H NMR - ESI-MS	¹³C NMR ¹H NMR ³¹P NMR - HRMS	¹³C NMR ¹H NMR - FT-IR - HRMS	¹³C NMR ¹H NMR ³¹P NMR - HRMS
Badania strukturalne	- Związek II-6a krystalizuje w grupie <i>P</i>₂₁ - Związek II-7a krystalizuje w grupie <i>P</i>₂₁,<i>2</i>,<i>2</i>			- Związek VI-2 krystalizuje w grupie <i>P</i>₂₁ - Związek VI-5 krystalizuje w grupie <i>P</i>₂₁ - Związek VI-8 krystalizuje w grupie <i>P</i>₂₁,<i>2</i>,<i>2</i>₁	- Związek VII-4 krystalizuje w grupie <i>R</i>3:<i>H</i> - Związek VII-5 krystalizuje w grupie <i>P</i>₂₁,<i>2</i>,<i>2</i>₁
Badania biologiczne <i>in vitro</i>	<i>T. b. brucei</i> - HL-60 <i>P. berghei</i> - PC3 - A549 - MDA-MB-231 - HCT-116 - HaCaT	<i>T. b. brucei</i> - HL-60 - PC3 - A549 - MDA-MB-231 - HCT-116 - HaCaT	- SW480 - SW620 - PC3 - MDA-MB-231 - A549 - HaCaT - V79	- A549 - MCF-7 - LoVo - LoVo/DX - BALB/3T3	<i>P. berghei</i>
Inne badania	Badania mechanizmu aktywności biologicznej: - wczesna i późna apoptoza - cykl komórkowy - poziom interleukiny-6 - stres oksydacyjny	Badania aktywności biologicznej <i>in vitro</i> mieszaniny iwermektyny i artezunatu	Badania mechanizmu aktywności biologicznej: - wczesna i późna apoptoza - cykl komórkowy - generowanie ROS - badania funkcji mitochondriów Badania biofizyczne: - Oxygraph-2K (zużywanie tlenu przez komórki) - Eksperyment BLM (zdolność do przenoszenia ładunku przez błony)	Badania zdolności do wytwarzania kompleksów: - z kationami metali pierwszej grupy (Li⁺, Na⁺, K⁺, Rb⁺, Cs⁺) - z kationami metali drugiej grupy (Mg²⁺, Ca²⁺, Sr²⁺, Ba²⁺)	

Rysunek 9. Podsumowanie badań przeprowadzonych w ramach niniejszej rozprawy doktorskiej.

Na [Rysunku 9](#) zaprezentowałem podsumowanie badań, które zostały wykonane w ramach niniejszej rozprawy doktorskiej i są częścią publikacji naukowych **II-VII**. Ich efektem jest bogaty materiał eksperymentalny, którego najważniejsze rezultaty, wnioski i odkrycia wymienilem poniżej.

- W wyniku serii chemo- oraz regioselektywnych przekształceń szkieletu **IWR** otrzymałem bibliotekę 11 nowych C13-*epi*-amidów, przy czym, w celu zwiększenia różnorodności strukturalnej, wprowadziłem podstawniki różniące się długością i charakterem chemicznym (alifatyczne, aromatyczne lub alifatyczno-aromatyczne).
- Przełomowym odkryciem była nieznana wcześniej reakcja przegrupowania układu heksahydrobenzofuranowego **IWR**, która umożliwiła otrzymanie serii 11 podwójnie modyfikowanych analogów **IWR** (przegrupowane C13-*epi*-amidy). Reakcji przegrupowania poddano ponadto **IWR** oraz jej aglikon.
- Większość z otrzymanych 24 analogów **IWR** wykazała wyższą aktywność biologiczną oraz selektywność działania wobec pasożyta śpiączki afrykańskiej – *Trypanosoma brucei brucei*, niż niemodyfikowany chemicznie lek. Dwa analogi – nieprzegrupowany (**II-6h**) oraz przegrupowany (**II-7h**) amid 2-chloroacetylowy – wykazały około 230-krotnie wyższą aktywność *in vitro* niż **IWR**. Jest to aktywność porównywalna do suraminy oraz bromku etydyny – leków powszechnie stosowanych w leczeniu śpiączki afrykańskiej (u ludzi) oraz nagany (u zwierząt).
- Wybrane związki zostały zbadane pod kątem ich aktywności przeciwmalarycznej wobec wątrobowego stadium *Plasmodium berghei*. Pojedynczo modyfikowane pochodne **IWR** wykazały niższą aktywność niż związek wyjściowy, ale amid acetylowy przegrupowanej **IWR** okazał się 2,5-krotnie bardziej aktywny niż **IWR** oraz 4-krotnie bardziej aktywny niż jej nieprzegrupowany analog.
- Wszystkie otrzymane pochodne **IWR** zostały po raz pierwszy zbadane pod kątem ich aktywności przeciwnowotworowej. Niektóre z analogów wykazały wyższą aktywność przeciwproliferacyjną i/lub wyższą selektywność działania niż związek wyjściowy.
- Badania nad mechanizmem aktywności przeciwnowotworowej wykazały, że aktywność pochodnych **IWR** była związana między innymi z wytwarzaniem reaktywnych form tlenu oraz peroksydacją lipidów. Ponadto, związki te hamowały wydzielanie interleukiny 6 (IL-6) w komórkach nowotworowych.

- W wyniku chemicznej modyfikacji grupy C13 hydroksylowej blokowanego aglikonu **IWR** otrzymałem serię 10 biokoniugatów z innymi bioaktywnymi partnerami (m.in. alkaloidami kory chinowej, analogami nukleozydów, artezunatem).
- Biokoniugat **IWR** z artezunatem (**IV-16**) wykazał najwyższą aktywność przeciwtrypanosomową ze wszystkich testowanych biokoniugatów, będąc 7-krotnie i 9-krotnie bardziej aktywnym wobec pasożytów *T. b. brucei* niż odpowiednio **IWR** i artezunat. Związek ten wykazał dużą skuteczność w badaniach przeciwproliferacyjnych, osiągając najniższe wartości współczynnika IC₅₀ spośród testowanych biokoniugatów oraz brak cytotoksyczności wobec linii referencyjnej.
- Badania biologiczne równomolowej mieszaniny **IWR** i artezunatu wykazały 23-krotnie mniejszą aktywność wobec pasożytów *T. b. brucei* niż skoniugowany związek **IV-16**, co potwierdziło korzyści płynące z biokoniugacji. Ponadto, związek ten wykazywał wielokrotnie niższą cytotoksyczność wobec referencyjnej linii HaCaT niż mieszanina obu leków.
- W wyniku dwuetapowej reakcji otrzymałem serię 12 biokoniugatów **MON** z solami fosfoniowymi.
- Otrzymane związki wykazały aktywność przeciwproliferacyjną na poziomie stężeń mikromolowych, a wiele z nich było bardziej aktywnych niż niemodyfikowana **MON** oraz doksorubicyna. Wszystkie sole fosfoniowe **MON** charakteryzowały się niższą cytotoksycznością niż związek wyjściowy wobec normalnej linii komórkowej HaCaT.
- Pogłębione badania biologiczne (eksperyment z użyciem barwnika JC-10) oraz biofizyczne (Oxygraph-2K oraz eksperyment BLM) wykazały, że badane pochodne były efektywnie transportowane do mitochondriów komórek nowotworowych, gdzie prowadziły do utraty mitochondrialnego potencjału błonowego, a w konsekwencji do apoptozy.
- Opracowałem prostą i efektywną metodę otrzymywania dogodnych substratów wyjściowych bazujących na strukturze **MON** – C26 azydek oraz C26 aminę. Substraty te posłużyły do otrzymania trzech makrocyklicznych pochodnych **MON**.
- Przeprowadzono badania strukturalne makrocyklicznych pochodnych **MON** oraz jej C26 azydku. Sprawdziłem zdolność makrocykli do koordynowania kationów metali pierwszej i drugiej grupy. Wykazałem formowanie kompleksów z badanymi kationami metali z najwyższym powinowactwem do kationów sodu. Ponadto udowodniłem, że makrocykliczne pochodne **MON** mogą pełnić rolę receptorów par jonowych.

- Prekursor C26 aminowy **MON** posłużył mi do otrzymania serii nowych analogów – amidu, mocznika oraz amidowego koniugatu z kationem trifenylofosfoniowym.
- Badania wykazały, że wiele z otrzymanych pochodnych wykazuje aktywność na poziomie stężeń nanomolowych wobec pasożytów *P. berghei* odpowiedzialnych za wywoływanie malarii, będąc przy tym bardziej skutecznymi niż stosowana w medycynie prymachina. Jeden z badanych analogów (*O*-uretan fenyłowy) charakteryzował się wartością parametru $IC_{50} = 0,7$ nM, będąc prawie 8-krotnie bardziej aktywny niż niemodyfikowany jonofor, oraz współczynnikiem SI większym niż 4000, będąc przy tym najbardziej selektywnym związkiem z całej serii.

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9. Spis osiągnięć naukowych

9.1. Publikacje naukowe i zgłoszenia patentowe

- Publikacje wchodzące w skład rozprawy doktorskiej:
 - **M. Sulik**, M. Antoszczak, A. Huczyński D. Steverding, Antiparasitic activity of ivermectin: Four decades of research into a “wonder drug”, *Eur. J. Med. Chem.* 261 (2023), 115838.
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 - M. Antoszczak, J. Krzywik, G. Klejborowska, **M. Sulik**, S. Sobczak, D. Czerwonka, E. Maj, M. Ullrich, T. Sobierajski, J. Sukiennik, J. Wietrzyk, W. Mozga, P. Pilaszek, A. Huczyński, Effect of stereochemistry at position

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 - A. Urbaniak, M.R. Reed, D. Fil, A. Moorjani, S. Heflin, M. Antoszczak, **M. Sulik**, A. Huczyński, M. Kupsik, R.L. Eoff, M.C. MacNicol, T.C. Chambers, A.M. MacNicol, Single and double modified salinomycin analogs target stem-like cells in 2D and 3D breast cancer models, *Biomed. Pharmacother.* 141 (2021), 111815.
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- Recenzowane materiały pokonferencyjne:
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- Rozdział w monografii:
 - **M. Sulik**, M. Antoszczak, A. Huczyński, Zastosowanie antybiotyków jonoforowych oraz ich pochodnych w walce ze śpiączką afrykańską, w: H. Koroniak, J. Barciszewski, *Na pograniczu chemii i biologii*, t. XXXIX, 2019, Poznań, Wydawnictwo Naukowe Uniwersytetu im. Adama Mickiewicza, 222 p., ISBN 9788323235934.
- Zgłoszenia patentowe:
 - Zgłoszenie do Urzędu Patentowego RP nr P.444419 pt. „Nowe pochodne iwermektyny, sposób ich otrzymywania oraz zastosowanie do leczenia onkologicznego”. Twórcy: A. Huczyński, **M. Sulik**, M. Struga, D. Otto-Ślusarczyk.

9.2. Projekty badawcze

- 10.2023 – 10.2024: **Kierownik projektu** pt. „*Biokoniugaty iwermektyny ze związkami cytostatycznymi jako nowe środki przeciwnowotworowe*” finansowanego w ramach programu „Inicjatywa Doskonałości – Uczelnia Badawcza” przez Uniwersytet im. Adama Mickiewicza w Poznaniu (nr projektu: 102/13/SNŚ/0002).
- 01.2022 – 01.2025: **Wykonawca projektu** OPUS 21 nr 2021/41/B/ST4/00088, pt. „*Koniugaty celujące w mitochondria jako nowe środki przeciwnowotworowe*” finansowanego przez Narodowe Centrum Nauki. Kierownik projektu: prof. dr hab. Adam Huczyński.
- 11.2020 – 11.2023: **Kierownik projektu** Diamentowy Grant nr 0159/DIA/2020/49, pt. „*Synteza oraz badania właściwości przeciwnowotworowych*”

i przeciwpasożytniczych nowych pochodnych iwermektyny” finansowanego przez Ministerstwo Nauki i Szkolnictwa Wyższego.

- 01.2018 – 07.2020: **Wykonawca projektu SONATA 12 nr 2016/23/D/ST5/00242**, pt. *„Drugie życie salinomycyny. Chemo- i regioselektywne modyfikacje salinomycyny oraz ich wpływ na aktywność przeciwnowotworową i przeciwdrobnoustrojową”* finansowanego przez Narodowe Centrum Nauki. Kierownik projektu: prof. UAM dr hab. Michał Antoszczak.

9.3. Konferencje naukowe

W okresie kształcenia w Szkole Doktorskiej UAM:

- **M. Sulik**, M. Jędrzejczyk, M. Mielczarek-Puta, J. Hoser, P. Bednarczyk, M. Struga, A. Huczyński, *„Sole trifenylofosfoniowe antybiotyków jonoforowych, jako koniugaty celujące w mitochondria komórek nowotworowych”* – wystąpienie ustne na 66. Zjeździe Polskiego Towarzystwa Chemicznego w Poznaniu w dniach 15–20.09.2024 r.
- **M. Sulik**, M. Jędrzejczyk, M. Mielczarek-Puta, J. Hoser, P. Bednarczyk, M. Struga, A. Huczyński, *“Mitochondria targeting triphenylphosphonium salts of monensin – synthesis and antiproliferative activity”* – prezentacja posteru podczas 4th MMCS: Harnessing the Power of New Drug Modalities w Barcelonie (Hiszpania) w dniach 24–26.04.2024 r.
- **M. Sulik**, D. Otto-Ślusarczyk, M. Struga, A. Huczyński, *“Synthesis and anticancer activity of new ivermectin derivatives”* – ustna prezentacja posteru podczas XII Meeting of the Paul Ehrlich Euro-PhD Network w Salonikach (Grecja) w dniach 16–18.07.2023 r.
- **M. Sulik**, D. Otto-Ślusarczyk, M. Struga, A. Huczyński, *„Synthesis and anticancer activity of new ivermectin derivatives”* – prezentacja posteru podczas 30th SCT Young Research Fellows Meeting w Paryżu (Francja) w dniach 1–3.02.2023 r.
- **M. Sulik**, D. Fontinha, M. Prudêncio, D. Steverding, D. Otto-Ślusarczyk, M. Struga, S. Sobczak, A. Huczyński, *“Synteza i analiza właściwości biologicznych nowych pochodnych iwermektyny”* – wystąpienie ustne na IV Ogólnopolskim Sympozjum Chemii Bioorganicznej, Organicznej i Biomateriałów w Poznaniu w dniu 3.12.2022 r.

- **M. Sulik**, D. Fontinha, M. Prudêncio, S. Sobczak, A. Huczyński, „*Synteza i analiza właściwości przeciwmalarycznych nowych pochodnych ivermektyny*” – wystąpienie ustne na 64. Zjeździe Polskiego Towarzystwa Chemicznego w Lublinie w dniach 11–16 września 2022 r.
- **M. Sulik**, D. Fontinha, M. Prudêncio, S. Sobczak, A. Huczyński, „*Synthesis and antimalarial activity of new ivermectin derivatives*” – prezentacja posteru na 9th EFMC Young Medicinal Chemists' Symposium w Nicei (Francja) w dniach 8–9.09.2022 r.

Przed rozpoczęciem kształcenia w Szkole Doktorskiej UAM:

- **M. Sulik**, M. Antoszczak, D. Steverding, J. Janczak, A. Huczyński, „*Synteza oraz aktywność przeciwpasożytnicza podwójnie modyfikowanych pochodnych salinomycyny*” – wystąpienie ustne na 62. Zjeździe Polskiego Towarzystwa Chemicznego w Warszawie w dniach 2–6.09.2019 r.
- **M. Sulik**, M. Antoszczak, D. Steverding, J. Janczak, A. Huczyński, „*Synteza i analiza właściwości przeciwpasożytniczych podwójnie modyfikowanych pochodnych salinomycyny*” – wystąpienie ustne na 17. Ogólnopolskim Seminarium Doktorantów i Studentów "Na pograniczu chemii i biologii" w Jastrzębiej Górze w dniach 12–15.05.2019 r.
- **M. Sulik**, M. Antoszczak, A. Urbaniak, M. Delgado, T.C. Chambers, A. Huczyński, „*Synthesis and anticancer activity of doubly-modified salinomycin derivatives*” – prezentacja posteru na 61. Zjeździe Polskiego Towarzystwa Chemicznego w Krakowie w dniach 17–21.09.2018 r.
- **M. Sulik**, M. Antoszczak, A. Urbaniak, E. Maj, J. Wietrzyk, T.C. Chambers, A. Huczyński, „*Influence of double-modification on the anticancer activity of salinomycin derivatives*” – prezentacja posteru na IX Konwersatorium Chemii Medycznej w Lublinie w dniach 13–15.09.2018 r.
- **M. Sulik**, „*Synteza oraz aktywność przeciwnowotworowa i przeciwdrobnoustrojowa podwójnie modyfikowanych pochodnych salinomycyny*” – wystąpienie ustne na konferencji Wolontariat studencki organizowanej na Wydziale Chemii UAM w Poznaniu.

9.4. Szkolenia, staże, rozwój kwalifikacji

W okresie kształcenia w Szkole Doktorskiej UAM:

- 03.2025: 2-tygodniowy staż naukowo-badawczy w grupie Prof. Miguela Prudêncio w Gulbenkian Institute for Molecular Medicine w Lizbonie. W czasie stażu zajmowałem się oznaczaniem aktywności przeciwmalarycznej pochodnych produktów naturalnych wobec pasożytów *Plasmodium*. Staż sfinansowano w ramach projektu ID-UB Uniwersytetu im. Adama Mickiewicza w Poznaniu (nr projektu: 158/13/UAM/0030).
- 09.2023: miesięczny staż naukowo-badawczy w grupie Prof. Hiroki Oguriego w Uniwersytecie Tokijskim, gdzie zajmowałem się syntezą alkaloidów bis-tetrahydroizochinolinowych wspomaganą enzymami. Staż sfinansowano w ramach projektu „Uniwersytet Jutra” Uniwersytetu im. Adama Mickiewicza w Poznaniu.
- 10.2022 – 06.2023: studia podyplomowe Badania kliniczne i biomedyczne badania naukowe realizowane w Uniwersytecie Medycznym im. Karola Marcinkowskiego w Poznaniu.
- 08.2022 – 09.2022: 2-tygodniowy staż naukowo-badawczy w grupie Prof. Jacka Tuszyńskiego w Politechnice Turyńskiej, gdzie zajmowałem się dokowaniem molekularnym pochodnych iwermektyny do konkretnych celów molekularnych. Staż sfinansowano w ramach projektu ID-UB Uniwersytetu im. Adama Mickiewicza w Poznaniu (nr projektu: 048/13/UAM/0005).

Przed rozpoczęciem kształcenia w Szkole Doktorskiej UAM:

- 07.2020 – 09.2020: 2-miesięczny staż na stanowisku Chemik Organik w firmie TriMen Chemicals Sp. z o.o. w Łodzi.
- 25.04.2019: jednodniowe szkolenie pt. „Szkola 2D NMR” organizowane przez Instytut Chemii Bioorganicznej Polskiej Akademii Nauk w Poznaniu.

9.5. Stypendia, nagrody i wyróżnienia

W okresie kształcenia w Szkole Doktorskiej UAM:

- Laureat Ogólnopolskich Eliminacji Polskiego Towarzystwa Chemii Medycznej do ogłoszenia komunikatu na 12th EFMC Young Medicinal Chemists' Symposium.
- **Stypendium Miasta Poznania dla młodych badaczy.**

- Drugie miejsce w konkursie European 360RG-CHEM Challenge w Katowicach, którego celem była popularyzacja chemii organicznej.
- **Stypendium Fundacji UAM dla najlepszych doktorantów na rok akademicki 2024/25.**
- Nagroda zespołowa Rektora za osiągnięcia naukowe w roku akademickim 2023/24.
- **Nagroda Marszałka Województwa Wielkopolskiego w kategorii: nauka w konkursie „Wielkopolska dla Planety 2030”.**
- **Nagroda Polskiego Towarzystwa Chemicznego za najlepszą pracę magisterską obronioną w roku akademickim 2020/21.**

Przed rozpoczęciem kształcenia w Szkole Doktorskiej UAM:

- **Medal Uniwersytetu im. Adama Mickiewicza w Poznaniu dla absolwentów za wybitne osiągnięcia naukowe.**
- Nagroda Dziekana za najlepszą pracę magisterską wykonaną na Wydziale Chemii UAM w roku akademickim 2020/21.
- **Dwukrotny laureat stypendium Ministra Nauki i Szkolnictwa Wyższego dla wybitnych studentów (rok akademicki 2019/20 i 2020/21).**
- Finalista konkursu „Złoty Medal Chemii” organizowanego przez Instytut Chemii Fizycznej Polskiej Akademii Nauk w Warszawie, za najlepszą chemiczną pracę licencjacką/inżynierską.
- Nagroda Polskiego Towarzystwa Chemicznego za najlepsze wystąpienie konferencyjne podczas 17. Ogólnopolskiego Seminarium Doktorantów i Studentów "Na pograniczu chemii i biologii" w Jastrzębiej Górze.
- Nagroda za najlepsze wystąpienie ustne podczas konferencji „Wolontariat studencki” organizowanej przez Wydział Chemii UAM.
- Stypendia Rektora UAM dla najlepszych studentów w latach akademickich: 2020/21, 2019/20, 2018/19 oraz 2017/18.

10. Spis rysunków, schematów i tabel

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11. Artykuły naukowe stanowiące rozprawę doktorską

11.1. Publikacja I

Michał Sulik, Michał Antoszczak, Adam Huczyński, Dietmar Steverding

Antiparasitic activity of ivermectin: Four decades of research into a “wonder drug”

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Review article

Antiparasitic activity of ivermectin: Four decades of research into a “wonder drug”

Michał Sulik^a, Michał Antoszczak^a, Adam Huczyński^{a,*}, Dietmar Steverding^b^a Department of Medical Chemistry, Faculty of Chemistry, Adam Mickiewicz University, Uniwersytetu Poznańskiego 8, 61-614, Poznań, Poland^b Bob Champion Research & Education Building, Norwich Medical School, University of East Angles, Norwich, UK

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ABSTRACT

Parasitic diseases still pose a serious threat to human and animal health, particularly for millions of people and their livelihoods in low-income countries. Therefore, research into the development of effective antiparasitic drugs remains a priority. Ivermectin, a sixteen-membered macrocyclic lactone, exhibits a broad spectrum of antiparasitic activities, which, combined with its low toxicity, has allowed the drug to be widely used in the treatment of parasitic diseases affecting humans and animals. In addition to its licensed use against river blindness and strongyloidiasis in humans, and against roundworm and arthropod infestations in animals, ivermectin is also used “off-label” to treat many other worm-related parasitic diseases, particularly in domestic animals. In addition, several experimental studies indicate that ivermectin displays also potent activity against viruses, bacteria, protozoans, trematodes, and insects. This review article summarizes the last 40 years of research on the antiparasitic effects of ivermectin, and the use of the drug in the treatment of parasitic diseases in humans and animals.

1. Introduction

Ivermectin is a mixture of two semi-synthetic macrocyclic lactones and is widely used as an antiparasitic drug for the treatment of intestinal roundworm infestation in veterinary medicine. It has also a few applications in human medicine, in particular for the treatment and control of onchocerciasis (river blindness), and therefore, is on the World Health Organization's (WHO) model list of essential medicines [1]. There are only a few drugs that can claim the title of “Wonder Drug”. Alongside penicillin and aspirin, two drugs that probably had the greatest medicative impact on human health and well-being, ivermectin is also a worthy contender for this title as its effect on global health to date has been extraordinary. Firstly, ivermectin played an important role in global food production by fighting roundworm infestation in livestock, and secondly, the drug was paramount in relieving the burden of filarial diseases from millions of people living in the poorest countries, which can be regarded as the most successful public-private partnerships in global health [2]. Although ivermectin has been studied for over 40 years, the mechanism of its action and resistance are not fully established, mainly because the drug exhibits a wide range of diverse effects in many different organisms. The versatility of ivermectin makes this

drug an interesting compound to be investigated as a medication for other diseases and a potential source for derivatization to improve its activity and efficacy. The focus of this review article is a comprehensive literature appraisal on the licensed, “off-label” and experimental uses of ivermectin in humans and domestic animals.

2. Discovery and mode of action of ivermectin

Ivermectin is a sixteen-membered macrocyclic lactone obtained as a 22,23-dihydro derivative of avermectin by selective hydrogenation using rhodium-based Wilkinson's catalyst (Fig. 1) [3]. Avermectins were first isolated from a *Streptomyces* sp. strain in Japan in 1967 [4]. Ivermectin is a mixture of two forms – B1a with an ethyl group at the C-26 position, and B1b with a methyl group at this position (Fig. 1). This mixture contains at least 80% ivermectin B1a and a maximum of 20% ivermectin B1b [5]. Ivermectin exhibits a wide spectrum of anti-helminthic activities and is therefore used in the treatment of various diseases caused by parasitic nematodes [6]. The approval of ivermectin for use in humans in 1987 significantly improved the quality of life of many people in sub-Saharan Africa, India, and elsewhere, enabling the treatment of diseases that had previously been devastating

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[2]. The success of ivermectin is also due to the fact that its use is generally safe with LD₅₀ values ranging between 24 mg kg⁻¹ (monkeys [7]) and 80 mg kg⁻¹ (beagle [7]), while the maximum treatment dose in animals and humans is around 200 µg kg⁻¹.

The first person to discover a microorganism capable of producing avermectin was Satoshi Omura, a microbiologist working at the Kitasato Institute in Japan. The researcher focused on *Streptomyces* bacteria, which are commonly found in soil and are known for producing various biologically active substances [8]. He isolated and characterized a new strain of *Streptomyces* from soil samples and successfully cultured these bacteria [8]. Starting from a few thousand samples, he was able to narrow down the sample size to about 50 strains that looked most promising in terms of producing novel bioactive metabolites [9]. One of these strains (NRRL 8165-a) would later be called *Streptomyces avermectilis* [9]. The second scientist who contributed to the discovery of the unusual biological activity of avermectin was William Campbell, who worked as a biologist and parasitologist at the Merck Sharp and Dohme Research Laboratories (MDRL) in New Jersey, USA. He investigated Omura's bacterial broths for their biological properties and activities [6, 10, 11]. One of Campbell's collaborators, Thomas Miller, was the first to chemically characterize avermectin, the active component of Omura's sample NRRL 8165-a [11]. Campbell conducted a number of studies that confirmed the high antiparasitic activity of avermectin against many worms in domestic animals [12, 13]. Then, together with other MDRL scientists, they chemically modified avermectin to obtain ivermectin, which turned out to exhibit even stronger antiparasitic activity [14]. According to Campbell, this molecule showed extraordinary biological activity against many parasites, was active even against benzimidazole-resistant nematodes, and was well-tolerated by the host species [15]. This discovery was the cornerstone that changed the world in the fight against helminthic diseases.

For the discovery of ivermectin, Satoshi Omura and William Campbell were awarded the Nobel Prize in Physiology/Medicine in 2015, specifically, "(...) for their discoveries concerning a novel therapy against infections caused by roundworm parasites" [16]. Ivermectin, thanks to its high efficacy, versatility, and safety of use, has so significantly influenced medical treatments, that many scientists call it a "wonder drug", comparing ivermectin to aspirin or penicillin [17].

Mechanistically, ivermectin acts as a positive allosteric modulator that selectively opens inhibitory glutamate-gated chloride ion channels (GluCl) of muscle and nerve cells of microfilariæ, female reproductive tracts, and the excretory/secretory pores of nematodes, as well as of muscles and nerves of insects (Fig. 2) [7, 18, 19]. GluCl channels consist of five subunits, each of which has four transmembrane α -helices: M₁, M₂, M₃, and M₄ [20, 21]. Their mode of action relies on glutamate binding to the orthosteric agonist site of the channels, which leads to the opening of the channel and subsequent flow of ions [20, 21]. Ivermectin, in turn, binds to the allosteric site of the channels (Fig. 2). The drug, due to its lipophilicity, can insert deeply into the subunits of the channels, which stabilizes the open-pore conformation and thus extends its opening time [7, 22]. The binding between the drug and the channel is characterized by high affinity and leads to an increased influx of chloride ions, and subsequently to hyperpolarization, paralysis, and death of

the parasite [7, 18, 19].

Moreover, ivermectin has also the ability to inhibit the conductance of γ -aminobutyric acid (GABA) channels in *Ascaris suum* at concentrations of <0.2 µM [22]. This additional action may, together with its binding to GluCl channels, synergistically enhance the antiparasitic activity of the drug [7]. What is important, ivermectin does not cross the blood-brain barrier, and therefore, does not affect mammals, including humans, in which GABA receptors are located mainly in the central nervous system [17]. Nevertheless, accumulation of ivermectin in the human brain has been observed after administration of doses about 100 times higher than the recommended one [23]. Such an overdose of the drug may lead to coma and subsequent death of the patient [23]. Oral administration of doses several times higher than recommended was found to be lethal to mice and rats as well [24]. The symptoms of an overdose of ivermectin manifest as significant ataxia, bradypnea, decreased activity, and mydriasis [24]. In addition to overdosing, ivermectin can also enter the brain when mutated multi-drug-resistance transporters present in the blood-brain barrier are unable to effectively eliminate drugs from the plasma [7]. It has been noted that ivermectin, in addition to binding to GABA receptors in the mammalian brain, also targets glycine receptors (GlyRs) and nicotinic acetylcholine receptors (nAChRs) [25–27].

Ivermectin has also been found to be effective against *Plasmodium falciparum*, the parasite species responsible for the most severe form of malaria (see section 5.1.3 for details). The drug inhibits the signal recognition particle (SRP) nuclear import in malaria cells by blocking the motility of the heterodimer carrier IMP α / β , which leads to the subsequent death of the parasite [28]. SRPs are eukaryote ribonucleoprotein complexes that are present in *P. falciparum* cells and are responsible for targeting proteins to the endoplasmic reticulum [28].

3. Licensed use of ivermectin

Ivermectin was introduced for use in humans about 20 years after its discovery [29]. The extremely high microfilaricidal activity of ivermectin and its extraordinary safety in human use, which have been confirmed in many studies, enabled the implementation of this compound in healthcare systems. However, the turning point came in 1987, when Dr. Roy Vagelos (CEO of Merck & Co., Inc.) decided to donate ivermectin "as much as needed, for as long as needed, to anyone who needed it" [29]. This decision greatly improved the quality of life of people living in sub-Saharan regions of Africa, India, and elsewhere, where people could not afford the drug. The introduction of ivermectin made it possible to treat diseases that had affected the poorest people in the world. In particular, ivermectin has been pivotal in the fight against river blindness (*Onchocerca volvulus*) (Table 1). The drug is also used to treat strongyloidiasis (*Strongyloides stercoralis*) and scabies (*Sarcoptes scabiei* var. *hominis*) infections in humans (Table 1) [30].

In addition to being greatly important for human medicine, ivermectin's widespread use in veterinary medicine should be emphasized. Ivermectin and other macrocyclic lactones are commonly used in the treatment and prevention of parasitic diseases in livestock and pets. They are one of the most frequently used anthelmintics in the UK sheep

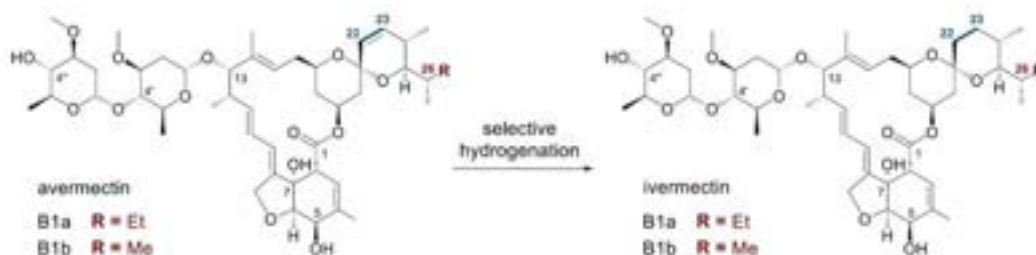


Fig. 1. Chemical structure of avermectin and ivermectin.

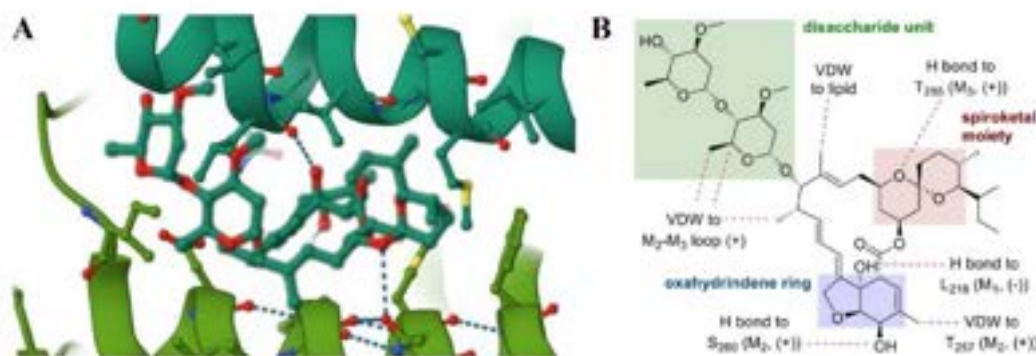


Fig. 2. (A) Ivermectin-binding site of a glutamate-gated chloride ion channel (GluCl) subunit. Ribbons are part of the GluCl while the stick molecule is ivermectin; (B) Interactions between the ivermectin molecule and GluCl. VDW, van der Waals interactions [20].

industry and the US cattle industry [2,31,32]. There are many animal parasitic diseases for which ivermectin is the drug of first choice. Parasitic diseases treated with ivermectin include heartworm disease, intestinal roundworm infestations, eye worm infestation, lungworm infestation, equine onchocerciasis, mange, lice infestation, myiasis, and gastric myiasis (Table 1).

3.1. In humans

3.1.1. River blindness

River blindness (onchocerciasis) is a helminthic disease caused by the nematode *Onchocerca volvulus*. This filarial worm is transmitted by infected blackflies (*Simulium* spp.). In the human body, the adult worms produce embryonic larvae (microfilariae) which migrate to the eyes, skin, and other organs, consequently causing the clinical symptoms of the disease, which include severe itching, various skin changes, and eye lesions that can lead to permanent blindness [33]. According to the WHO, more than 99% of people affected by river blindness live in 31 sub-Saharan African countries [33]. An estimation by the Global Burden of Disease Study indicated that at least 220 million people required preventive chemotherapy against river blindness in 2017, with 14.6 million individuals who already had skin disease and 1.15 million vision loss [33].

Mass drug administration (MDA) with ivermectin remains the main strategy in combating river blindness. In 2006, Busáñez et al. [34] performed a systematic review of individual and population-based ivermectin trials to examine the temporal dynamics of the microfilaricidal and embryostatic efficacy after single-dose administration at $150 \mu\text{g kg}^{-1}$ of the drug. Analysis of the data collected from 26 microfilarial and 15 macrofilarial studies was supported by a mathematical model, describing the dynamics of potentially fertile female worms to skin microfilariae [34]. According to the authors, 2 days after treatment with ivermectin, the dermal microfilarial load was reduced by 78% [34]. After 3 days, this value increased to 90%, after 7–8 days to 92–95%, and after 14–60 days to ~98% [34]. The combination of meta-analysis and mathematical modeling suggested that the placebo-corrected microfilaricidal efficacy of ivermectin was 92–99% [34].

Combination therapies of ivermectin with other drugs have been also tested to establish whether these may increase the effectiveness of the treatment. Such studies indicated that 30 years of monotherapy with ivermectin resulted in the emergence of drug-resistant variants of *O. volvulus* [35]. It has been confirmed that in some communities, adult female worms did not respond or were resistant to the anti-fecundity effect of ivermectin-based treatment [36]. Unfortunately, the efficacy of doxycycline-ivermectin co-therapy to target also adult worms remained unclear [37]. It should be noted that the survival of adult worms of *O. volvulus* depends on symbiotic *Wolbachia* bacteria that can

be killed with the antibiotic doxycycline. Although in a 6-month follow-up, more patients in the ivermectin plus doxycycline group showed improvement in iridocyclitis and punctate keratitis than those in the ivermectin alone group, the authors concluded that the results are of insufficient quality, and thus cannot be treated as decisive [37]. In another randomized, open-label clinical trial the effectiveness of ivermectin plus albendazole co-therapy was evaluated [38]. The results demonstrated that the combination of ivermectin and albendazole was not better than ivermectin alone in sterilizing and killing adult worms, or achieving sustained microfilariae clearance [38]. Nevertheless, it was found that after 36 months, a 6-monthly treatment regimen was superior compared to a yearly treatment regimen in achieving sustained clearance of *O. volvulus* microfilariae from the skin [38].

3.1.2. Strongyloidiasis

Strongyloidiasis is a chronic parasitic disease caused by nematodes from the genus *Strongyloides*, with *S. stercoralis* being mainly responsible for the human variant of the disease. This parasite belongs to the group of soil-transmitted helminths, i.e., microfilariae released into the soil directly penetrate the skin of the human host [39]. The main symptoms of the disease are intermittent or persistent diarrhea, pruritus, coughing, and wheezing. It is estimated that around 30–100 million people are suffering from strongyloidiasis worldwide [39].

Currently, the most effective treatment option for strongyloidiasis is the use of ivermectin, thiabendazole, or albendazole [39]. However, ivermectin is better tolerated than thiabendazole and more effective in achieving larval clearance than albendazole [40–42]. The standard treatment regimen for uncomplicated *S. stercoralis* infections in the US is oral ivermectin at a dose of $200 \mu\text{g kg}^{-1}$ per day for two consecutive days, but some specialists suggest repeating the cycle after 2 weeks to prevent possible autoinfections [42,43]. In severe cases of strongyloidiasis, the parasite can appear as two different clinical entities, which is known as hyperinfection syndrome [44]. In this situation, the classic life cycle of the parasite is exaggerated, resulting in an increase in the parasitic burden as a result of autoinfections [44]. In such cases, it is suggested that ivermectin should be administered daily at $200 \mu\text{g kg}^{-1}$ per day for at least 2 weeks [42]. If needed, treatment should be extended until no more larvae are found in stool specimens [42].

In 2004, Igual-Adell et al. [45] compared the effectiveness of ivermectin and thiabendazole in the treatment of strongyloidiasis. A total of 88 patients were treated using the following protocols: 31 patients – thiabendazole 25 mg kg^{-1} every 12 h for 3 consecutive days; 22 patients – ivermectin $200 \mu\text{g kg}^{-1}$ as a single dose; and 35 patients – ivermectin $200 \mu\text{g kg}^{-1}$ for 2 consecutive days [45]. The criterion for cure was the absence of parasites in the feces of three samples collected every other day [45]. Among the 31 patients treated with thiabendazole, 25 (78%) met the criteria for a cure, and 5 (16%) experienced side effects, such as

Table 1
Licensed use of ivermectin in humans and animals.

Species to be treated	Disease	Parasitic species (type)
Humans	River blindness	<i>Onchocerca volvulus</i> (nematode)
	Strongyloidiasis	<i>Strongyloides stercoralis</i> (nematode)
	Scabies	<i>Sarcoptes scabiei</i> var. <i>hominis</i> (mite)
Dogs, cats	Heartworm disease	<i>Dirofilaria immitis</i> (nematode)
Cattle, sheep, goats (ruminants)	Intestinal roundworm infestation	<i>Haemonchus placei</i> , <i>Chabertia ovina</i> , <i>Cyberus curtipis</i> , <i>C. ocrephorus</i> , <i>C. pectinatus</i> , <i>C. punctatus</i> , <i>Haemonchus contortus</i> , <i>H. placei</i> , <i>Nematodirus battus</i> , <i>N. helveticus</i> , <i>N. spathiger</i> , <i>Oesophagostomum columbianum</i> , <i>O. radiatum</i> , <i>O. venosum</i> , <i>Ostertagia circumcincta</i> , <i>O. ostertagi</i> , <i>O. lyoni</i> , <i>Strongyloides papillatus</i> , <i>Trichostrongylus axei</i> , <i>T. colubriformis</i> , <i>Trichostrongylus axei</i> (nematodes)
	Eye worm infestation	<i>Thelazia</i> sp. (nematodes)
	Lungworm infestation	<i>Dictyocaulus filaria</i> , <i>D. viviparus</i> (nematodes)
	Mange	<i>Psoroptes ovis</i> , <i>Sarcoptes scabiei</i> var. <i>bovis</i> , <i>S. a. var. ovis</i> (mites)
	Lice infestation	<i>Bovicapripes erythrogastrus</i> , <i>Linguatula africana</i> , <i>L. ovalis</i> , <i>L. pedalis</i> , <i>L. stragalis</i> , <i>L. vituli</i> , <i>Solenopotes capillatus</i> (lice)
	Myiasis	<i>Hypoderma bovis</i> , <i>H. lineatum</i> , <i>Oestrus ovis</i> (flies)
Horses	Intestinal roundworm infestation	<i>Oxyuris equi</i> , <i>Cyathostomum</i> sp., <i>Cylicocyclus</i> sp., <i>Cylicodontophorus</i> sp., <i>Cylicostephanus</i> sp., <i>Cylicocapulus</i> sp., <i>Habronema muscivorum</i> , <i>H. microstomum</i> , <i>H. muscae</i> , <i>Oesophagostomum</i> sp., <i>Oxyuris equi</i> , <i>Parascaris equorum</i> , <i>Potamostrongylus</i> sp., <i>Strongyloides westeri</i> , <i>Strongylus edentatus</i> , <i>S. equinus</i> , <i>S. vulgaris</i> , <i>Trichostrongylus</i> sp. (nematodes)
	Lungworm infestation	<i>Dictyocaulus arvensis</i> (nematode)
	Equine onchocerciasis	<i>Onchocerca cervicalis</i> , <i>Camellia reticulata</i> (nematodes)
	Eye worm infestation	<i>Thelazia</i> sp. (nematodes)
	Gastric myiasis	<i>Gasterophilus haemorrhoidalis</i> , <i>G. intestinalis</i> , <i>G. nasalis</i> (flies)
Swine	Intestinal roundworm infestation	<i>Acaris suum</i> , <i>Hyostrongylus rubidus</i> , <i>Oesophagostomum breviscaputum</i> , <i>O. dentatus</i> , <i>O. quadrispinatus</i> , <i>Strongyloides ransomi</i> (nematodes)
	Lungworm infestation	<i>Metastrongylus apri</i> , <i>M. pulmonis</i> , <i>M. salm</i> (nematodes)
	Mange	<i>Sarcoptes scabiei</i> var. <i>swi</i> (mite)
	Lice infestation	<i>Bovicapripes suis</i> (louse)

asthenia, epigastralgia, and disorientation [45]. Of the 22 patients treated with a single dose of ivermectin, 17 (77%) met the criteria for a cure, and only 2 (9%) reported side effects, like dizziness and dyspepsia [45]. Among the 35 patients treated with ivermectin on 2 consecutive days, all patients were cured and no one experienced side effects [45]. This outcome showed that the treatment regimen with ivermectin was the most effective and safe. Moreover, in a study that compared the effectiveness of ivermectin versus albendazole, it was found that parasitological cure was higher in the ivermectin group and that ivermectin was as well tolerated as albendazole [46].

Ivermectin was also shown to be effective in the treatment of strongyloidiasis in primary school children. With a single dose of 200 µg kg⁻¹, a cure rate of ~95% was achieved in the pupils, with no side effects [47]. Moreover, ivermectin has also been tried in the treatment of strongyloidiasis in human immunodeficiency virus (HIV)-positive men [48]. After 3 weeks of oral administration of the drug, no changes were observed [48]. However, a high plasma concentration of ivermectin was

achieved after parenteral administration resulting in a rapid disappearance of the parasite from biological samples and a significant improvement in the clinical condition of the men without any adverse reaction [48].

3.1.3. Scabies

Scabies is a parasitic skin condition that is caused by the *Sarcoptes scabiei* var. *hominis* mite in humans. The main symptoms of the disease are severe itching, linear burrows, and vesicles around the finger webs, wrists, upper and lower limbs, and belt area [49]. Scabies is one of the most common dermatological diseases in the world, and it is estimated that more than 200 million people suffer from it at any time [49]. Current treatment methods are based on the use of 5% permethrin, 0.5% malathion lotion, 10–25% benzyl benzoate emulsion, or 5–10% sulfur ointment [49]. In addition, oral administration of ivermectin at a dose of 200 µg kg⁻¹ is also very effective in treating scabies [50]. However, as ivermectin seems not to be immediately effective against all forms of the parasite, it was found that taking a second dose 7–10 days after the first dose can increase the cure rate [50].

In 2018, Rosumek et al. [51] analyzed the results of 13 studies comprising 1456 patients to evaluate the effectiveness of systemic ivermectin versus permethrin. Oral administration of ivermectin at a dose of 200 µg kg⁻¹ led to slightly lower rates of complete clearance of the mites after one week than topical treatment with 5% permethrin cream (extrapolated cure rates: permethrin 65%, ivermectin 43%) [51]. However, after two weeks, no significant difference in the rates of complete clearance was observed (extrapolated cure rates: permethrin 74%, ivermectin 68%) [51]. Considering the life cycle of the scabies mite (10–17 days), the authors suggested repeating the treatment to increase the effectiveness of the therapy, because ivermectin, unlike permethrin, only affects the adult mites, but not the eggs [51].

According to the WHO, the safety of ivermectin in children under 15 kg body weight has not been established [49]. An observational study performed by Levy et al. [52] in 2020 suggested that ivermectin is safe and effective for the treatment of scabies in infants and young children [52]. Data were collected from 170 infants and children aged 1–64 months, with a body weight of 4–14.5 kg, who were treated with oral ivermectin [52]. The administered dose was 223 µg kg⁻¹, and a second dose was given to 89% of the young patients [52]. Mild adverse events were reported in 7 children (4%) [52]. At the follow-up visit, 139 children (85%) were completely cured [52]. In addition, ivermectin may also be an effective drug in the treatment of scabies in patients with HIV infection [53]. In an open-label study, 11 otherwise healthy patients and 11 HIV patients with scabies were treated with a single oral dose of 200 µg kg⁻¹ ivermectin [53]. After 4 weeks, all 11 healthy patients had fully recovered with no evidence of the parasite [53]. Ten of the 11 HIV patients (91%) also showed no evidence of scabies 4 weeks after having been treated with ivermectin [53]. Moreover, ivermectin seems to be effective in treating patients with therapy-resistant scabies too, as some studies report successful interventions with ivermectin in patients with previous treatment failures [54].

3.2. In animals

3.2.1. Heartworm disease

Heartworm disease is caused by the nematode *Dirofilaria immitis*. It is a very serious parasitic disease in dogs and cats that can lead to severe lung damage, heart failure, other organ damage, and death [55]. The infectious larvae of *D. immitis* are transmitted by infected mosquitoes. The average worm burden in dogs is about 15 worms, but the number can vary from 1 up to 250 [55].

Ivermectin administered subcutaneously at a dose of 200 µg kg⁻¹ was shown to be effective against *D. immitis* [56]. For example, 90% of dogs treated with ivermectin (pre-treated with sodium thiocetarsamide at a dose of 2.2 mg kg⁻¹ twice daily for 2 days) showed no signs of microfilariae in their blood 21 days after starting the therapy [56].

Interestingly, other studies have shown that ivermectin is even effective against *D. immitis* larvae at much lower doses [57]. Larvicidal activity of ivermectin was fully achieved at a dose of $3.0 \mu\text{g kg}^{-1}$, while lower doses of $0.5 \mu\text{g kg}^{-1}$, $1.0 \mu\text{g kg}^{-1}$, and $2.0 \mu\text{g kg}^{-1}$ showed incomplete anti-larval activities [57]. These results have been confirmed by other authors. For example, a group of 42 dogs was injected with 50 infective larvae of *D. immitis* [58]. The animals were then divided into six groups: group 1 was a vehicle-treated control, groups 2–5 were treated with oral ivermectin at doses of $0.3 \mu\text{g kg}^{-1}$, $1.0 \mu\text{g kg}^{-1}$, $2.0 \mu\text{g kg}^{-1}$, and $3.3 \mu\text{g kg}^{-1}$, respectively, 30 days after infection while group 6 was given ivermectin at a dose of $2.0 \mu\text{g kg}^{-1}$ 45 days after being inoculated with the parasite [58]. The efficacies for preventing heartworm maturation were 0% (group 2), 53% (group 3), 97% (group 4), 98% (group 5), and 64% (group 6), demonstrating that therapies with orally administered doses of $2.0 \mu\text{g kg}^{-1}$ or $3.3 \mu\text{g kg}^{-1}$ ivermectin 30 days after *D. immitis* infection were the most effective treatment regimens [58].

Ivermectin was also successfully used in the form of subcutaneous implants, providing long-term prevention of *D. immitis* infection in dogs [59]. A study by Genchi et al. [59] evaluated whether implants consisting of an ethylcellulose matrix containing ivermectin had prophylactic properties. At necropsy, all control dogs were found to be infected, however, implanted dogs were negative for the presence of microfilaria and *D. immitis* antigens [59]. The implants protected dogs against the parasite for at least one year without side effects [59]. It should be noted that ivermectin and other macrocyclic lactones are highly potent against *D. immitis*, but in some cases, the efficacy of ivermectin can be reduced due to emerging drug resistance in this parasite [60].

3.2.2. Intestinal roundworm infestations

There are numerous nematodes responsible for causing intestinal roundworm infestations in livestock (Table 2). The most common roundworms in northern Europe are *Ostertagia ostertagi* and *Cooperia oncophora*, but all intestinal nematodes can be a serious threat to animal breeding, as they can reduce growth rates by up to 30% [61]. Ivermectin has been successfully used in the treatment and prevention of intestinal roundworm infestations in livestock (cattle, sheep, goats, horses, and swine) in various formulations [62].

With respect to ruminants, ivermectin administered in the form of a paste at a dose of $200 \mu\text{g kg}^{-1}$ to calves experimentally infected with *Bunostomum phlebotomum* 18 and 60 days after infection proved to be very effective, with 100% and 99.8% cure rates, respectively [62]. In another study with calves, ivermectin at the dose of $200 \mu\text{g kg}^{-1}$ was 100% effective against a resistant variant of *C. oncophora* [63]. At the same dose administered subcutaneously to cattle infected with different nematode species (*B. phlebotomum*, *Cooperia pectinata*, *C. punctata*, *Haemonchus placei*, *Oesophagostomum radiatum*, *Ostertagia ostertagi*), ivermectin showed an efficacy of 80% against all the roundworms in 80% of the treated animals after 7 or 9 days of treatment [64]. Sustained-release ivermectin delivering approximately 8 mg of the drug per day for the duration of 120 days protected 100% of cattle when challenged with infective larvae of *B. phlebotomum*, *H. placei*, *O. radiatum*, *O. ostertagi*, and *Trichostrongylus axei* 4–6 weeks after the bolus administration [65].

Ivermectin has also been given to infected sheep in the form of a controlled-release capsule which delivers the drug at the rate of 1.6 mg per day for 100 days [66]. This technique worked successfully against numerous parasites with an effectiveness of $\geq 99\%$ [66]. The results suggested that the controlled-release formulation of the drug for sheep may be an effective method in fighting nematode infestations [66]. Oral administration of ivermectin to sheep at a dose of $200 \mu\text{g kg}^{-1}$ to clear away larvae and adult worms of other nematode species, including benzimidazole-resistant strains achieved 80% efficacy in more than 80% of the treated animals within 9–43 days [67]. Worrying, however, is the emergence of ivermectin-resistant variants in some nematode species. For instance, it has been found that ivermectin treatment of lambs reduced the level of *Ostertagia circumcincta* and *Cooperia curticei* by only

Table 2
Licensed use of ivermectin in the treatment of intestinal roundworm infestations.

Animals	Dose and formulation of ivermectin	Parasitic species	Efficacy	Ref.
Calves	paste, $200 \mu\text{g kg}^{-1}$	<i>Bunostomum phlebotomum</i>	100% cure rate after 18 days and 99.8% cure rate after 60 days	[62]
Calves	subcutaneous administration, $200 \mu\text{g kg}^{-1}$	<i>Cooperia oncophora</i>	100% cure rate after 7 and 14 days	[63]
Cattle	subcutaneous administration, $200 \mu\text{g kg}^{-1}$	<i>Bunostomum phlebotomum</i> , <i>Cooperia pectinata</i> , <i>C. punctata</i> , <i>Haemonchus placei</i> , <i>Oesophagostomum radiatum</i> , <i>Ostertagia ostertagi</i>	80% effective in 80% of the treated animals after 7 or 9 days	[64]
Cattle	sustained release, 8 mg per day for 120 days	<i>Bunostomum phlebotomum</i> , <i>Haemonchus placei</i> , <i>Oesophagostomum radiatum</i> , <i>Ostertagia ostertagi</i> , <i>Trichostrongylus axei</i>	100% efficacy	[65]
Sheep	controlled-release capsule, 1.6 mg per day for 100 days	<i>Chabertia ovina</i> , <i>Cooperia curticei</i> , <i>Dictyocaulus filaria</i> , <i>Haemonchus contortus</i> , <i>Nematodirus battus</i> , <i>N. filicollis</i> , <i>Oesophagostomum circumcincta</i> , <i>Ostertagia circumcincta</i> , <i>O. pinnata</i> , <i>O. trifurcata</i> , <i>Trichostrongylus colubriformis</i> , <i>Strongylus edentatus</i> , <i>Strongylus papillorum</i> , <i>Trichostrongylus axei</i> , <i>T. colubriformis</i> , <i>T. circumcincta</i> , <i>T. vitellus</i> , <i>Trichostrongylus axei</i> , <i>T. axei</i>	$\geq 99\%$ cure rate after 21, 28, 35 and 56 days	[66]
Sheep	oral administration, $200 \mu\text{g kg}^{-1}$	<i>Chabertia ovina</i> , <i>Dictyocaulus filaria</i> , <i>Cooperia pectinata</i> , <i>Haemonchus contortus</i> , <i>Nematodirus battus</i> , <i>Oesophagostomum circumcincta</i> , <i>O. columbianus</i> , <i>Trichostrongylus colubriformis</i> , <i>Trichostrongylus axei</i>	80% effective in more than 80% of the treated animals after 9–43 days	[67]
Horses	oral 2% ivermectin formulation, $200 \mu\text{g kg}^{-1}$	<i>Coronocylus silabofortis</i> , <i>Cyathostomum aculeolatum</i> , <i>Cyathostomum catenatum</i> , <i>C. parvum</i> , <i>Cylicocyclus levisqualatus</i> , <i>C. vulgus</i> , <i>C. leptostomus</i> , <i>C. nasutus</i> , <i>C. silabofortis</i> , <i>Cylicocyclus</i> spp., <i>Cylicostephanus colicatus</i> , <i>C. longicollis</i> , <i>C. pectinatus</i> , <i>Halobutyrus mucosus</i> , <i>Halobutyrus</i> spp., <i>Oxyuris equi</i> , <i>Parascaris equorum</i> , <i>Parascaris equorum</i> , <i>Trichostrongylus axei</i> , <i>Trichostrongylus axei</i>	100% efficacy after 5 days, 99% after 14 days and 100% after 19 days	[68]
Ponies	paste, $200 \mu\text{g kg}^{-1}$	<i>Coronocylus silabofortis</i> , <i>C. silabofortis</i> , <i>C. silabofortis</i> , <i>C. silabofortis</i>	reduction in the number of parasites from 94% to $>99\%$	[69]

(continued on next page)

Table 2 (continued)

Animals	Dose and formulation of ivermectin	Parasitic species	Efficacy	Ref.
		<i>Cyathostomum catellatum</i> , <i>C. punctatum</i> , <i>Cylicocyclus subseriis</i> , <i>C. ringatus</i> , <i>C. insigne</i> , <i>C. leptostomum</i> , <i>C. insensu</i> , <i>C. radiatus</i> , <i>Cylicodactylomorphus bicornutus</i> , <i>Cylicodactylomorphus asymmetricus</i> , <i>C. bidentatus</i> , <i>C. calicatus</i> , <i>C. goldi</i> , <i>C. longibaratus</i> , <i>C. sinensis</i> , <i>Gasterophilus intestinalis</i> , <i>Gastrophilus capitatus</i> , <i>Habronema</i> spp., <i>Oxyuris equi</i> , <i>Parascaris equorum</i> , <i>Potamostrongylus equorum</i> , <i>Potamostrongylus pectinatus</i> , <i>Potamostrongylus impendens</i> , <i>P. rotuli</i> , <i>Strongylus edentatus</i> , <i>S. vulgaris</i> , <i>Trichostrongylus flexuatus</i> , <i>T. serratus</i> , <i>Strongylus westeri</i>	after 14 or 15 days	
Foals	intramuscular administration, 200 µg kg ⁻¹		≥99% reduction in the number of parasites after 21 days	[70]
Pigs	feed formulation, 100 µg kg ⁻¹ or 200 µg kg ⁻¹ per day	<i>Ascaris suum</i> , <i>A. scrofae</i> , <i>Trichostrongylus axei</i> , <i>Trichostrongylus colubriformis</i> , <i>Macronchylus</i> spp., <i>M. colubriformis</i> , <i>M. axei</i> , <i>M. colubriformis</i> spp., <i>Oesophagostomum</i> spp.	efficacy from 86% to 100%, depending on the type of the parasite	[71]
Pigs	100 µg kg ⁻¹ per day	<i>Ascaris suum</i> , <i>Trichostrongylus axei</i> , <i>Trichostrongylus colubriformis</i> , <i>Macronchylus</i> spp., <i>M. colubriformis</i> , <i>M. axei</i> , <i>M. colubriformis</i> spp., <i>Oesophagostomum</i> spp.	92% cure rate after 14 days	[72]

37% and 19%, respectively [73].

Ivermectin was also shown to be effective in treating intestinal roundworm infestations in horses. For example, 20 horses naturally infected with 19 different nematode species were divided into an ivermectin-treated group (single oral dose of 200 µg kg⁻¹) and a control group [68]. The efficacy of the treatment on days 5, 14, and 19 post-infection was 100%, 99%, and 100%, respectively [68]. Similar results were obtained when ivermectin was administered as a paste at a dose of 200 µg kg⁻¹ on ponies naturally infected with small and large strongyle nematodes [69]. A reduction in the number of parasites between 94% and 99% was observed [69]. At a dose of 200 µg kg⁻¹, ivermectin was also effective against the *Strongyloides westeri* in naturally infected foals [70]. Intramuscular injection of the drug reduced the number of parasite egg output by more than 99% [70].

The effectiveness of ivermectin has also been confirmed in the treatment of pigs infected with roundworms when the drug was administered to the animals in feed for 7 days [71]. At a concentration that provided a dose of $100 \mu\text{g kg}^{-1}$ per day, the efficacy against naturally acquired and induced (fourth-stage larvae) roundworm infections ranged between 90% and 100%, depending on nematode species [71]. At a quantity that afforded a dose of $200 \mu\text{g kg}^{-1}$ per day, ivermectin was found to be particularly potent in eliminating *Ascaris suum*, *Ascaris strongylina*, and *Metastrongylus* spp [71]. The effectiveness of ivermectin in the treatment of swine was also confirmed in another study in which animals were given either ivermectin or abamectin (avermectin B1) at a dose of $100 \mu\text{g kg}^{-1}$ per day for 7 days [72]. Both drugs were more than

93% effective against *A. suum*, *Hyostromylos rubidus*, *Metastrongylus salmi*, and *Strongyloides ransomi* [72].

3.2.3. Eye worm infestation

Eye worm infestation (thelaziasis) is a disease caused by nematodes of the genus *Thelazia*, which affects cattle and horses worldwide. These roundworms are transmitted by face flies [74]. Thelaziasis causes inflammation of the eye with symptoms ranging from lacrimation, epiphora, conjunctivitis, and ulcers, to blindness. The drugs used to treat eye worm infestation are ivermectin, doramectin (an ivermectin derivative), and levamisole [74].

Kennedy et al. [75] have performed a study to test the effectiveness of ivermectin in a pour-on formulation on cattle naturally infected with *Thelazia* sp. The drug (IVOMEK®, 0.5% w/v ivermectin) was applied topically at 1.0 ml per 10 kg body weight [75]. After 14 days, the total elimination of *T. gulosa* worms and a 97% reduction in the number of *T. skrjabini* worms were observed, compared to the control group [75]. Total elimination of *T. skrjabini* nematodes was also observed 2 weeks after subcutaneous administration of ivermectin at a dose of 200 µg kg⁻¹ to experimentally infected calves [76]. Similar results have been reported after subcutaneous administration of the drug at the same dose to cattle naturally infected with *T. rhodesii* [77]. The parasite reduction exceeded 99%, reaching 100% in two-thirds of the cases [77].

3.2.4. Lungworm infestation

Lungworm infestation (verminous bronchitis, verminous pneumonia) is a disease of the lower respiratory tract in livestock caused by various nematodes. The disease in cattle, sheep, goats, and horses is induced by *Dictyocaulus* spp, while in swine it is caused by *Metastrongylus* spp [78]. The most common symptoms are coughing and dyspnea, however, the condition of the animal can get worse due to simultaneous bacterial or viral infections. A commonly used treatment involves the use of anthelmintics [78].

In a study by Pouplard et al. [79], the effectiveness of ivermectin was compared to that of levamisole in the treatment of calves infected with *D. viviparus* larvae. One group of calves was given levamisole at a dose of 10 mg kg⁻¹, while the other group was administered ivermectin at a dose of 200 µg kg⁻¹ [79]. Ivermectin turned out to be more effective than levamisole [79]. These results were confirmed in another study with calves experimentally infected with *D. viviparus* larvae [80]. One group was given an injectable formulation of ivermectin at a dose of 200 µg kg⁻¹, while the second and third groups were treated with a pour-on formulation of levamisole at 10 mg kg⁻¹ or ivermectin at a dose of 500 µg kg⁻¹ [80]. The fourth group was left untreated (control group) [80]. After 28 days, the presence of *D. viviparus* worms was checked in 2 calves from each group [80]. On day 35, the remaining calves were re-infected [80]. Levamisole treatment was ~95% effective, while both ivermectin treatment regimens eradicated 100% of the parasites [80]. In addition, the calves treated with ivermectin were protected from reinfection, which subsequently became an issue in the control calves and those treated with levamisole [80]. Ivermectin has also been shown to be effective in treating calves infected with inhibited larvae of *D. viviparus* [81]. After subcutaneous treatment at a dose of 200 µg kg⁻¹, no traces of adult worms or inhibited L5 stages of *D. viviparus* were found in the lungs of the treated animals [81].

Ivermectin was also successfully used in the treatment of lungworm infections in ponies caused by *D. ænfieldi* [82]. Oral administration of the drug in the form of a paste at a single dose of $200 \mu\text{g kg}^{-1}$ resulted in a complete cure of the infected ponies [83]. The possibility of using ivermectin in the treatment of *Metastrongylus* spp. responsible for lungworm infestation of swine was also studied. A dose of $100 \mu\text{g kg}^{-1}$ allowed the elimination of the parasite with an efficacy of 95% or better, while a double dose was 100% effective [71,72].

3.2.5. Equine onchocercosis

Equine onchocercosis is a skin disease in horses caused by

microfilariae produced by *Onchocerca* sp. adult worms. The parasites are transmitted by different species of biting flies. Unfortunately, there is no treatment that is effective against the adult worms of the parasite [83]. However, ivermectin is commonly used in the fight against *O. cervicifilis* and *O. reticulatus* microfilariae.

In a study, a group of 40 horses who were infected with microfilariae of *O. cervicifilis* and suffering from dermatitis, alopecia, and pruritus, were given a single injection of 200 µg kg⁻¹ of ivermectin [84]. Skin snips were taken from all horses after 4 and 33 days post-treatment [84]. All samples were found to be negative for the presence of *O. cervicifilis* microfilariae [84]. Significant clinical improvement in health was observed within 2–3 weeks after treatment when the lesions were replaced by healthy skin and new hair [84]. Ivermectin at a single dose of 200 µg kg⁻¹ in injectable paste formulation was also effective against microfilariae of *O. cervicifilis* in naturally infected horses [85]. The number of microfilariae was reduced to zero 21 days after treatment in 19 out of 20 horses [85]. Active lesions improved or completely disappeared within 63 days after treatment [85]. Another study compared the efficacy of moxidectin (a macrocyclic lactone related to ivermectin) 2% oral gel (dose 400 µg kg⁻¹) with the efficacy of ivermectin 2% oral paste (dose 200 µg kg⁻¹) in horses infected with *O. cervicifilis* [86]. After analyzing skin snips taken 14 days after treatment, it was concluded that both drugs were 100% effective when used as a single oral dose [86].

3.2.6. Mange

Mange is a disease caused by parasitic mites. Species of the genera *Chorioptes*, *Demodex*, *Psorobia* (formerly *Psorergates*), *Psoroptes*, and *Sarcoptes*, are the main pathogens responsible for mange in domestic animals. However, the widespread use of macrocyclic lactones over the last 30 years has reduced the risk of mite infestation [87,88]. Ivermectin is commonly used in the treatment of mange in ruminants (cattle, sheep, and goats), especially against *Psoroptes ovis*, *Sarcoptes scabiei* var. *bovis*, and *S. scabiei* var. *ovis*. The drug is also used in the treatment of mange in swine.

The efficacy of a controlled-release formulation of ivermectin in the treatment and prevention of *P. ovis* in sheep has been described by Forbes et al. [89]. The results clearly indicated that an intraruminal controlled-delivery system (bolus) to release ivermectin at a rate of 20–40 µg kg⁻¹ per day for 100 days completely eliminated the parasite [89]. The prophylactic effect was maintained throughout the active life of the bolus [89]. Another study investigated how effectively a long-acting injectable formulation of the drug protects cattle against *P. ovis* [90]. The animals were divided into 5 groups, including one control group that was not treated with any drug [90]. The treatment groups were administered with ivermectin at a dose of 630 µg kg⁻¹, 56, 42, or 35 days prior to infection, or with doramectin (a macrocyclic lactone closely related to ivermectin) at a dose of 200 µg kg⁻¹, 35 days prior to infection [90]. Then, all animals were challenged with *P. ovis* [90]. Viable mites were present in 33%, 67%, and 83% of the controls after 14, 21, and 28 days of infection, respectively [90]. However, no parasites were present in animals treated with ivermectin, indicating that a long-acting injectable formulation of this drug may protect cattle against the disease for at least 56 days [90]. On the other hand, doramectin showed no prophylactic effect 35 days after treatment [90]. Ivermectin was also successfully applied topically at 500 µg kg⁻¹ against *S. scabiei* var. *bovis* in cattle [91]. Out of 12 cattle naturally infected with *S. scabiei* var. *bovis*, only in one case three mites were recovered on day 28 [91]. In the remaining cases, no mites were found in scrapings from ivermectin-treated animals after 2 weeks [91].

Ivermectin can also be successfully used to treat *S. scabiei* var. *sis* in pigs. When administered by esophageal intubation at doses of 300 µg kg⁻¹, 400 µg kg⁻¹, or 500 µg kg⁻¹, a significant reduction in the number of mites was observed in pigs [92]. Of the 18 pigs treated with ivermectin, 10 were found to be mite-free after a week [92]. This number increased to 16 pigs after 14 days and to 18 pigs after 21 days,

respectively, and then decreased to 16 pigs after 28 days [92]. In another study, sows infected with *S. scabiei* var. *sis* were treated with a single subcutaneous injection of ivermectin at doses of 75 µg kg⁻¹, 150 µg kg⁻¹, or 300 µg kg⁻¹ [93]. There was a significant decline in the number of mites, especially in the animals treated with the highest dose [93]. In the latter case, almost all mites and eggs were eradicated after one week of ivermectin treatment [93]. The drug was also tried in the form of a single subcutaneous injection at a dose of 300 µg kg⁻¹ on a commercial herd of 146 pigs, 80% of which were infected with the mite [94]. Ivermectin was administered to all animals, except 6 which served as controls [94]. At 28 and 42 days post-treatment, no parasites were found in the scrapings of ivermectin-treated pigs [94]. In contrast, live mites were present in the scrapings of all control animals [94].

3.2.7. Lice infestation

Lice infestation is a big problem in livestock farming, especially in winter, when animals are crammed together. It affects, among others, cattle and swine, leading to skin and hair damage, weight loss, and decreased milk production [95,96]. The disease in ruminants is mainly caused by the following lice species: *Haematopinus eurysternus*, *Linognathus africanus*, *L. ovalis*, *L. pedalis*, *L. stenopsis*, *L. vituli*, and *Solenopotes capillatus*. In swine, the disease is caused by the *H. suis* louse (Table 1).

Ivermectin has been successfully used to combat the lice species listed above. Both subcutaneous and oral drug administration at a dose of 200 µg kg⁻¹ allowed complete elimination of sucking lice (*L. vituli*) from infected cattle as early as 3 or 4 days post-treatment [97]. The high efficacy of ivermectin was also confirmed in other studies. The drug completely eliminated *L. vituli* and *S. capillatus* from heavily infested cattle [98]. The efficacy of a combination of ivermectin with the anti-parasitic drug triclofenazole was also evaluated in cattle [99]. Two combined formulations providing ivermectin at a dose of 200 µg kg⁻¹ and triclofenazole at a similar dose of 10 mg kg⁻¹ or 12 mg kg⁻¹, respectively, were tested orally or subcutaneously [99]. Both formulations proved to be effective against three different lice species [99]. The efficacy against *L. vituli* was greater than 99% after 7 days of treatment [99]. For *S. capillatus* and *H. eurysternus*, the efficacy was 100% and 98% and thus equally high after one week of treatment [99]. Subcutaneous administration of ivermectin was also highly effective against *H. suis* in swine [100]. The drug was given at a dose of 20 µg kg⁻¹, 100 µg kg⁻¹, or 200 µg kg⁻¹, and the efficacy range between 99% and 100% [100].

3.2.8. Myiasis

Myiasis is a disease of cattle and other livestock caused by cattle grubs (*Hypoderma bovis* and *H. lineatum*) and the sheep bot fly (*Oestrus ovis*). The larvae of these flies enter the host's body, causing increased susceptibility to other diseases, poor growth rate, decreased meat and milk production, and thus significant economic losses [101]. Macrocyclic lactones, including ivermectin, are being used in the treatment of myiasis.

IVOMEC® is a type of ivermectin formulation that can be used in the fight against myiasis in cattle [102]. The formulation (0.5% ivermectin) is used at the dose of 1 mL per 10 kg body weight [102]. Studies indicated that it is 100% effective against migrating first-instar larvae of *H. lineatum* for 3 weeks after administration [102]. After 4 weeks, its efficacy was still high (96%) [102]. Another study conducted on cattle selected from herds with a history of cattle grubs has shown that long-acting formulation of ivermectin administered subcutaneously at a dose of 630 µg kg⁻¹ was 100% effective in treating the animals naturally infected with *H. bovis* and *H. lineatum* [103]. A study on yaks was conducted to establish the lowest effective dose of ivermectin against *Hypoderma* spp [104]. The drug was administered to the animals subcutaneously at doses of 1 µg kg⁻¹, 5 µg kg⁻¹, and 10 µg kg⁻¹, respectively [104]. After 4 and 6 months, no warbles were found on treated animals, although they were present in the control group [104]. This study showed that ivermectin at a relatively low dose of 1 µg kg⁻¹ was sufficiently effective to protect animals against *Hypoderma* spp. larvae

[104].

Ivermectin can also be used to treat effectively sheep infected with *O. ovis*. Oral administration of the drug at a dose of 200 µg kg⁻¹ showed 100% efficacy in combating *O. ovis* larval infestations [67,105]. As mentioned above, ivermectin in the form of a controlled-release capsule that delivered the drug at the rate of 1.6 mg per day for 100 days showed ≥99% efficacy against *O. ovis* larvae [66]. In a study by Bello et al. [106], the prophylactic effect of ivermectin and closantel to control *O. ovis* infestation in sheep was compared. Ten sheep each were assigned to the following groups: control, treated with ivermectin given subcutaneously at a dose of 200 µg kg⁻¹, and treated with closantel given orally at a dose of 10 mg kg⁻¹ [106]. All animals were kept together, and after examination, it was found that 7 of the control lambs were found to be infected with *O. ovis* larvae [106]. The botfly larvae were intact and active [106]. Three of the lambs treated with ivermectin had *O. ovis* larvae, but they were dead, while no botfly larvae were found in animals treated with closantel [106]. According to the authors, both drugs showed high efficacy against *O. ovis* [106].

3.2.9. Gastric myiasis

Gastric myiasis is a disease of horses caused by *Gasterophilus* spp. maggots. Adult females lay eggs on single hairs of the horse's front legs, abdomen, flanks, and shoulders. After the larvae have hatched, they are ingested by the horse and bury themselves in the tongue, gums, or lining of the mouth. The second stage larvae move into the stomach and can cause inflammatory reactions and in large numbers blockages and colics [107]. Ivermectin is the treatment of choice as it shows efficacy against both oral and gastric stages [107].

In one study, the efficacy of ivermectin in treating ponies naturally or experimentally infected with *G. intestinalis* was investigated [108]. The drug was administered at a dose of 200 µg kg⁻¹ by intravenous injection, by intramuscular injection, or as oral paste [108]. After 21 days of treatment, no maggots were found in the ponies treated with ivermectin [108]. The drug showed 100% efficacy against oral and gastric stages of *Gasterophilus* spp [108]. However, one pony that was given the drug intravenously had an anaphylactic reaction, resulting in its death [108]. This effect was also seen in a control pony given the vehicle intravenously, while no adverse reactions occurred in ponies treated orally or intramuscularly [108]. The high potential of ivermectin in treating ponies was also confirmed in two other studies. In one study, the drug was administered at a dose of 200 µg kg⁻¹ in the form of an injectable micelle solution administered intramuscularly or in the form of an oral paste formulation [109]. In both cases, the efficacy of ivermectin was higher than 90% against *G. intestinalis* and *G. nasalis* larvae [109]. In the second study, it was shown that ivermectin was ≥97% effective against *G. intestinalis* larvae when administered intramuscularly to ponies [110]. The study tested ivermectin at doses of 200 µg kg⁻¹, 300 µg kg⁻¹, and 500 µg kg⁻¹, and the effect was similar for each dosage [110].

4. "Off-label" use of ivermectin

In addition to the licensed use of ivermectin for the treatment of onchocerciasis, strongyloidiasis, and scabies in humans, as well as nematodiasis in veterinary medicine, this semi-synthetic macrocyclic lactone was also found to be effective against a number of other parasitic nematodes and arthropods for which the drug has not been officially approved. This practice is known as "off-label" use, which refers to the use of a pharmaceutical drug for an unlicensed indication or in an unlicensed dosage or route of administration [111]. Generally, healthcare authorities may prescribe a drug for unlicensed use when they judge that it is medically appropriate for their patients [112]. The "off-label" use is more common when the patients are less likely to be included in clinical trials (for example, those suffering from neglected tropical diseases), as well as in veterinary medicine, as the available pharmacopeia for veterinarians is smaller than for human practitioners. Unlicensed use of ivermectin involves certain intestinal roundworm infestations,

manoselliosis, cutaneous larva migrans, gnathostomiasis, demodicosis, and lice infestations in humans, and mange, ear mites, and certain intestinal roundworm and lungworm infestations in animals (Table 3).

4.1. In humans

4.1.1. Intestinal roundworm infestations

Numerous nematodes are responsible for causing intestinal roundworm infestations in humans. Ascariasis, caused by *Ascaris lumbricoidea*, is one of the most commonly neglected soil-transmitted helminth infections of major public health importance [113]. This human parasite is mainly distributed in tropical and subtropical regions, with more than a billion individuals affected worldwide [114]. According to the data published by the Global Burden of Diseases Study, 446 million cases of *A. lumbricoidea* infections were reported worldwide in 2019 [115]. *Trichuris trichiura*, also known as the human whipworm is another example of a soil-transmitted nematode species. Both ascariasis and trichuriasis are classified as neglected tropical diseases.

Ascariasis may cause shortness of breath and fever at the beginning of the disease, while abdominal swelling and pain combined with diarrhea are the symptoms during the advanced stage of the parasitosis. Trichuriasis is typically asymptomatic [116], but individuals with heavy infections may have gastrointestinal problems, growth retardation, or other mild pathologies [116]. The current treatment for ascariasis and trichuriasis in humans is based on the administration of benzimidazoles (Fig. 3). Unfortunately, the effectiveness of benzimidazoles in the treatment and prophylaxis of soil-transmitted helminthiasis is limited, and thus the search for new treatment options is a priority. In this context, ivermectin has been investigated as an alternative treatment and prevention option for these human parasitic nematodes.

In a randomized, double-blind, multicenter clinical trial to evaluate the efficacy and side effects of ivermectin and albendazole against *A. lumbricoidea*, the cure rates of the drugs at a dose of 0.1 mg kg⁻¹ and 6.7 mg kg⁻¹, were found to be 100% (102/102 individuals) and 99% (101/102 individuals), respectively [117]. In addition to a lower effective dose of ivermectin compared with albendazole, the use of the macrocyclic lactone resulted only in rare, mild, and transient side effects, with no special treatment required [117]. Importantly, the elimination of the worms was faster in the ivermectin-treated group as well [117], which was suggested to be a consequence of the different anti-parasitic mechanisms of action of the two drugs. The better efficacy of ivermectin was also reported by other authors. For example, single doses

Table 3
"Off-label" use of ivermectin in humans and animals.

Species to be treated	Disease	Parasitic species (type)
Humans	Intestinal roundworm infestation	<i>Ascaris lumbricoidea</i> , <i>Trichuris trichiura</i> , <i>T. vulpis</i> (nematodes)
	Manoselliosis	<i>Manosella acardi</i> , <i>M. periana</i> , <i>M. streptocera</i> (nematodes)
	Cutaneous larva migrans	<i>Ancylostoma braziliense</i> , <i>A. caninum</i> , <i>A. ruberformis</i> , <i>Gnathostoma hispidum</i> , <i>G. spinigerum</i> (nematodes)
	Gnathostomiasis	<i>Gnathostoma spinigerum</i> (nematode)
	Demodicosis	<i>Demodex folliculorum</i> (mite)
	Lice infestation	<i>Pediculus humanus corporis</i> , <i>P. A. corporis</i> , <i>Phthirus pubis</i> (lice)
Dogs, cats	Ear mite infestation	<i>Notoedres cati</i> , <i>Otodectes cynotis</i> (mites)
	Mange	<i>Chyletiella blattai</i> , <i>C. yagouzi</i> , <i>Demodex canis</i> , <i>D. cati</i> , <i>D. gatoi</i> , <i>Sarcoptes scabiei var. canis</i> (mites)
Dogs	Intestinal roundworm infestation	<i>Ancylostoma caninum</i> , <i>Tamiasciurus leonine</i> , <i>Tamiasciurus canis</i> (nematodes)
	Lungworm infestation	<i>Capillaria aerophilus</i> (nematode)
Rabbits, guinea pigs	Ear mite infestation	<i>Notoedres cati</i> , <i>N. muris</i> (mites)
	Mange	<i>Chyletiella parasitivorax</i> , <i>Chirodactylus corvici</i> , <i>Psoroptes cuniculi</i> , <i>Trichostrongylus axei</i> (mites)

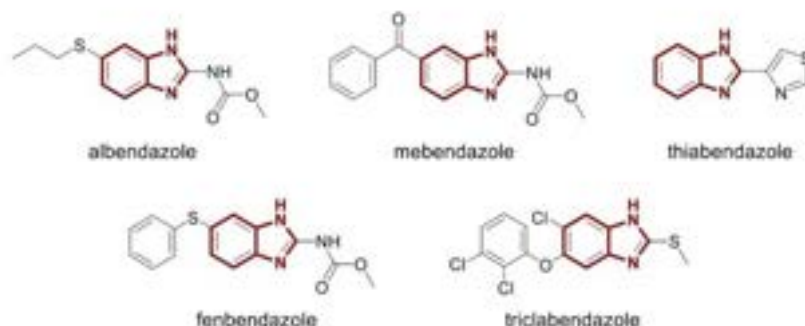


Fig. 3. Chemical structures of the antiparasitic drugs from the benzimidazole group. The benzimidazole scaffold present in the molecules is highlighted in red.

of albendazole (400 mg once a day), mebendazole (500 mg once a day), and ivermectin ($0.1\text{--}0.4\text{ mg kg}^{-1}$) were found to produce comparable effects against infection with *A. lumbricoides*, resulting in a high parasitological cure, i.e., the elimination of the parasite from stool samples, without serious side effects [118]. Of note, treatment failure after single-dose administration was the lowest with ivermectin [118]. A 100% overall cure rate in ascariasis-positive patients was also reported after ivermectin administration by Naquira et al. [119] and others [120].

A promising strategy to fight intestinal roundworm infestation seems also to be a combination therapy of ivermectin with albendazole [121–126]. Although an MDA trial with ivermectin/albendazole ($200\text{ }\mu\text{g kg}^{-1}$ of ivermectin plus 400 mg of albendazole) revealed that the combination therapy had little effect on the prevalence of *A. lumbricoides*, it showed that the intensities of infections were reduced [121]. On the other hand, combinational treatment with ivermectin was shown to be not superior to albendazole alone against *A. lumbricoides*, but increased the effectiveness of the benzimidazole drug against *T. trichiura* [122,127]. A similar effect of ivermectin in enhancing the efficacy of albendazole was also confirmed by other authors [125,126]. This finding is of importance, as benzimidazoles are known to be less effective against trichuriasis than against ascariasis or hookworm infestations, and as there are concerns that benzimidazole monotherapies could lead to the emergence of resistant intestinal roundworms in patients.

There is also an urgent need for up-to-date knowledge on the prevalence and intensity of soil-transmitted helminth infections in children in general and, in particular, in children under the age of five. If soil-transmitted helminthiasis are not controlled in children, youngsters can constitute a human reservoir for the geoparasites and thus may contribute to the spread of the diseases to the general population. For example, in a study by Nana-Djeunga et al. [128] including 421 children aged 2–4 years, it was found that the overall prevalence of infections with *A. lumbricoides* and *T. trichiura* was ~10% (95% CI 6.5–13.9), with a maximum infection rate of ~30% in a specific district. Although Moncayo et al. [129] have shown that the administration of ivermectin to school-age children had no significant impact on *A. lumbricoides* or hookworm infections, the drug was effective against infections caused by *T. trichiura*. Moreover, combination therapy of this drug with albendazole seems to be a highly effective and well-tolerated treatment strategy against *T. trichiura* infections in children and adolescents [130–132]. Interestingly, Knopp et al. [133] have found that the addition of ivermectin improves the therapeutic outcome of both albendazole and mebendazole in the fight against trichuriasis, with the ivermectin-mebendazole combination producing a higher reduction in egg output (97%, 95% CI 95%–98%) than the ivermectin-albendazole combination (91%, 95% CI 87%–94%). It has also been shown that ivermectin can be safely administered to preschool-aged children infected with trichuriasis [134]. The pharmacokinetic properties of ascending doses of ivermectin ($100\text{--}600\text{ }\mu\text{g kg}^{-1}$) studied in

T. trichiura-infected children aged 2–12 years showed a lower exposure profile of the drug in children compared with adults [135]. However, in an ivermectin/albendazole combination MDA study with 228 pupils in Tanzania, it was found that the prevalence of *A. lumbricoides* and *T. trichiura* had only insignificantly decreased from 0.9 to 0.7% ($p = 0.84$) [136].

Analysis of the long-term benefits of treating ascariasis with ivermectin suggests that the efficacy of the drug to control this intestinal nematode may also depend on the geographical region (i.e., different geographical strains) [117,137] and on the genetic disposition of the individuals living in local communities [138]. Similar conclusions regarding the effectiveness of anthelmintic drugs used alone or in combination were also reached for other roundworm infestations [139–142].

4.1.2. Mansonellosis

Mansonellosis is a filarial disease caused by three species, *Mansonella ozzardi*, *M. perstans*, and *M. streptocera*. The three species vary in their geographic distribution and location within their human host [143]. The parasites are transmitted by the bite of infected midges. While the adult worms of *M. perstans* reside in body cavities (pleural and peritoneal cavities, pericardium), adult worms of *M. ozzardi* and *M. streptocera* live in subcutaneous tissue and dermis, respectively. The microfilariae of the three *Mansonella* species enter the peripheral circulation, and in the case of *M. streptocera* also the skin [143]. The main common symptoms of mansonellosis are pruritus, arthralgias, headaches, and fever. There is no standard treatment for mansonellosis but the most commonly used drug to treat this filarial disease is diethylcarbamazine [144].

Although the ineffectiveness of single dose of ivermectin treatment has been independently confirmed for *M. ozzardi* and *M. perstans* filariasis [145,146], in a double-blind, randomized, placebo-controlled study, a single oral dose of the drug ($150\text{ }\mu\text{g kg}^{-1}$) led to an almost complete reduction (99.9%) of the *M. ozzardi* microfilaraemia in infected people [147], suggesting that the drug has some effect on female worm survival or fertility. Filaricidal activity of ivermectin against *M. ozzardi* microfilariae, which could last for up to one year, has also been reported by other authors [148–150]. When using ivermectin, the possibility of early adverse reactions, including the Mazzotti reaction (severe, potentially life-threatening allergic response), should always be taken into account, but in most studies evaluating the effectiveness of the drug in the treatment of mansonellosis, nearly all patients recovered rapidly without any additional antiallergic therapy required [147,148,151].

Human mansonellosis caused by *M. perstans* is usually regarded as the most difficult to treat among the three forms of the disease [144]. Ivermectin has been shown to reduce the motility of microfilariae *in vitro* to 50–65%, irrespective of the concentration of the drug [152]. Specifically, both $5\text{ }\mu\text{g mL}^{-1}$ and $10\text{ }\mu\text{g mL}^{-1}$ of ivermectin had almost the same effect on microfilariae survival (~50%) by day 5, but its activity was lower than those of mefloquine and artesunate [152]. Ivermectin

generally produced no significant changes in microfilaraemia in vivo, suggesting it is inefficient against *M. perstans* infections when used alone [153,154]. However, the efficacy of ivermectin treatment against this parasite may be strictly related to cumulative doses of the drug. A 3-year trial showed that the microfilariae loads were only reduced by two-thirds in patients treated with the standard dose ($150 \mu\text{g kg}^{-1}$ annually), while in another group treated with high doses (two doses of $400 \mu\text{g kg}^{-1}$, followed by ten doses of $800 \mu\text{g kg}^{-1}$ 3-monthly) the reduction was 97%, and in two other groups treated with medium doses (one dose of $400 \mu\text{g kg}^{-1}$, followed by two doses of $800 \mu\text{g kg}^{-1}$ or $150 \mu\text{g kg}^{-1}$ every three months) the reduction was 85% [155]. Ivermectin has also been successfully used in the treatment of infections caused by *M. streptocerca* [156,157]. Moreover, the same authors showed that a single dose of ivermectin ($150 \mu\text{g kg}^{-1}$) can lead to long-lasting suppression of *M. streptocerca* microfilariae, with almost half of the patients displaying no detectable microfilariae in skin biopsies one year after the therapy [157].

Worth mentioning is that a combination treatment with ivermectin and albendazole was found to slightly decrease the microfilariae load in patients infected with *M. perstans* [158,159]. A randomized, double-blind study performed by Asio et al. [139] showed that the combination of ivermectin ($150\text{--}200 \mu\text{g kg}^{-1}$) with albendazole (once 400 mg) appears more effective than ivermectin alone, but the differences were small and not statistically significant. The combination treatment regimens including ivermectin, albendazole, and doxycycline were found to be effective in two cases of *M. perstans* infections imported to Europe [160].

4.1.3. Cutaneous larva migrans

Cutaneous larva migrans (creeping disease) is a neglected parasitic skin disease that is widespread worldwide, particularly in low-income countries with tropical or subtropical climates [161]. This syndrome may be caused by larval forms of nematodes, including *Ancylostoma* and *Gnathostoma* spp. In humans, the larvae cannot penetrate the basement membrane of the epidermis, and therefore the larvae keep migrating within the skin, causing local inflammation and intense itching [162]. For multiple lesions or severe infestation, ivermectin is a first-line systemic treatment option.

Ivermectin was found to be $\sim 40\text{--}50$ times and 300 times more effective against *Ancylostoma ceylanicum* larvae than against *Necator americanus* larvae in vitro [163] and in vivo (hamster model) [164], respectively. While complete clearance of *A. ceylanicum* was achieved with a single dose of $100 \mu\text{g kg}^{-1}$ of the drug [164], the clinical cure of an 18-year-old patient with extensive cutaneous larva migrans was accomplished with a single dose of $200 \mu\text{g kg}^{-1}$ of ivermectin [165]. Numerous cases of successful ivermectin-based therapy for creeping disease have also been documented by other authors [166–169]. In a prospective study performed by Bouchaud et al. [166], 64 patients with cutaneous larva migrans were enrolled and treated with a single dose of $200 \mu\text{g kg}^{-1}$ ivermectin, which resulted in a 77% cure rate. The overall cure rate increased to 97% after one or two supplementary doses [166]. Caumes [167] reported that a similar single dose of ivermectin (once 12 mg) was effective in achieving a cure of 98% in French tourists suffering from cutaneous larva migrans.

A prospective open-labeled study showed that ivermectin when given orally at dosages of up to $200 \mu\text{g kg}^{-1}$ was safe in patients diagnosed with cutaneous gnathostomiasis, with no serious adverse events [170]. In a placebo-controlled study on 17 patients with a serologically confirmed diagnosis of cutaneous gnathostomiasis, 41% of individuals responded to the therapy when treated with $200 \mu\text{g kg}^{-1}$ of ivermectin [171]. A higher cure rate (76%) was observed by Karavichian et al. [168], when a group of 17 patients suffering from cutaneous gnathostomiasis was treated with the same dose of the antiparasitic drug. Ivermectin proved also to be successful in a returning traveler with cutaneous gnathostomiasis when the initial albendazole therapy failed [172].

4.1.4. Gnathostomiasis

Human gnathostomiasis is a food-borne parasitic disease that is caused by several species of the genus *Gnathostoma*. Definitive hosts are dogs, cats, and wild mammals, but humans can also be accidental hosts after ingesting third-stage larvae [173]. People become infected primarily by eating raw or undercooked fish, frogs, lobsters, crabs, snakes, and poultry or drinking contaminated water [174]. Moreover, gnathostomiasis is increasingly reported among travelers returning from endemic areas. The *Gnathostoma* larvae typically cause migratory swelling, but the parasites can also enter other tissues, resulting in vision loss or blindness, nerve pain, paralysis, coma, and death. If the nematodes can be easily removed, surgical intervention is recommended. Otherwise, ivermectin can be used as a non-invasive drug against gnathostomiasis.

After two rounds of ivermectin therapy ($200 \mu\text{g kg}^{-1}$), followed by a 3-week course of albendazole (400 mg, twice per day), a patient diagnosed with invasive gnathostomiasis was lesion-free at 40 weeks post-initial administration [175]. In a comparative treatment study, ivermectin showed slightly greater activity than albendazole, with a cure rate of $\sim 95\%$ and $\sim 94\%$, respectively [176]. In the ivermectin-treated group, the side effects were hypotension, dizziness, weakness, and diarrhea [176]. Similarly, higher values of cure rate after ivermectin administration (0.2 mg kg^{-1} for 2 days) compared with that after albendazole therapy (400 mg twice daily for 21 days) have been further confirmed by the same research team [177], but the difference between the two drugs was statistically insignificant.

4.1.5. Demodicosis

Demodicosis is a skin disease of the phlebotomous units, involving predominantly the face and head [178]. In humans, the disease is caused by *Demodex* mites (*D. folliculorum* and *D. brevis*) [179]. Demodicosis may have a primary or secondary form, with a rosacea-like presentation, and itching, hair loss, and inflammation as the most common symptoms. In immunocompromised patients, the disease may be more frequent and severe, and therefore, systemic therapy is usually needed to achieve a clinical cure. Worth mentioning is that ivermectin has potent acaricidal activity and therefore may be useful in the treatment of human demodicosis [180], particularly in HIV-positive patients [181,182].

Oral administration of ivermectin, followed by topical application of 5% permethrin cream, demonstrated good therapeutic effects in the fight against demodicosis [183,184]. In a single clinical case, three months after the initiation of the co-therapy, the facial eruption had completely resolved in a 6-year-old child with acute leukemia during chemotherapy [185]. Interestingly, there is growing evidence that the pathogenesis of rosacea may involve *Demodex* mites [186]. According to Brown et al. [187], the causative role of *D. folliculorum* should be considered in immunocompetent children with rosacea or rosacea-like refractory eruptions. In such cases, ivermectin-based treatment should be safe and beneficial [187]. Other formulations of ivermectin or combinations with other antiparasitic drugs for the treatment of demodicosis have been reported by some authors. For example, single or double application of ivermectin in the form of 1% cream was not only well tolerated but also highly effective in reducing or eliminating the characteristic sleeves – the primary clinical sign of *D. folliculorum* infestation of the eyelids [188]. In a randomized, single-blind, controlled clinical trial it was found that a combination therapy of ivermectin with metronidazole was superior in decreasing the *D. folliculorum* count compared to ivermectin monotherapy [189]. After 4 weeks, 72% of individuals in the combined therapy group showed complete remission of the disease, while only 45% of patients from the monotherapy group fully recovered [189].

4.1.6. Lice infestation

Human pediculosis can be caused by two species of obligate blood-sucking lice, *Pediculus humanus*, and *Phthirus pubis*. The infestation affects people in both developing and developed countries. Pediculosis

humans occurs in two ecotypes, the head louse (*P. h. capitis*) and the body louse (*P. h. corporis*) [190]. *Phthirus pubis*, the crab or pubic louse resides mainly in the pubic hair. The parasites spread most frequently from person to person by close contact, and symptoms include itching and scratching, which can lead to secondary bacterial infections [191]. In recent years, a growing problem seems to be the development of drug-resistant lice, including double and cross-resistance to insecticides [192–195]. Treatment of lice infestations generally involves shampoos and creams usually containing insecticides (pyrethrins, permethrin, and malathion). Also, both topical and oral ivermectin have been found promising for the treatment of all forms of pediculosis [196–201].

Ivermectin in the form of 0.5% lotion was approved by the Food and Drug Administration (FDA) in 2012 for topical use against head lice infestation (pediculosis capitis) in patients aged ≥ 6 months [197]. The efficacy of this treatment is generally about 75% [202,203], but this value varied between studies. For instance, in a study by Hamedanian et al. [204], about 91% of individuals who were treated with ivermectin lotion had no head lice one month after the intervention. Additionally, ivermectin given orally may be an alternative way of drug administration against lice infestation [205]. In a multicenter, cluster-randomized, double-blind controlled trial, eradication of head lice was achieved by day 15 in more than 95% of patients when treated orally on days 1 and 8 with ivermectin at a dose of $400 \mu\text{g kg}^{-1}$ [206]. Similarly, oral ivermectin used at the standard dose of $200 \mu\text{g kg}^{-1}$, administered on days 1 and 8, reduced the burden of active pediculosis capitis [207]. A reduction of $\sim 89\%$ and $\sim 71\%$ was achieved at 2-week and 3-month follow-ups, respectively [207]. Ahmad et al. [208] have shown that both topical and oral ivermectin are characterized by high efficacy and tolerability in the treatment of head lice infestation. It should, however, be noted that the authors of this study compared the effects of a high 1% topical ivermectin formulation with the standard oral dose of $200 \mu\text{g kg}^{-1}$ ivermectin. The co-therapy of ivermectin with diethylcarbamazine or albendazole was found to successfully reduce head lice infestation in endemic rural communities [209].

In a laboratory study, it was shown that 81%–100% of nymphs and females of the human body louse (*P. h. humanus*) died after artificial feeding on blood containing ivermectin at concentrations ranging from 2.5 to 10 ng mL^{-1} [210]. Using toxicity bioassays, Lamassiane et al. [211] further confirmed the activity of ivermectin against *P. h. humanus*. A combination of ivermectin with various antibiotics (doxycycline, erythromycin, rifampicin, and azithromycin) demonstrated synergistic effects in the killing of body lice, which should be of great value when considering drug-resistant lice populations [212]. Moreover, oral ivermectin at a dose of $250 \mu\text{g kg}^{-1}$ was successfully used in the treatment of *P. pubis* infestations, with no side effects or recurrences [213,214].

4.2. In animals

4.2.1. Intestinal roundworm infestations

Bhanjades et al. [215] have documented a 100% efficiency of ivermectin against the canine hookworm *Ancylostoma caninum* when used at oral doses of $200 \mu\text{g kg}^{-1}$. The compound was found to remove all adult worms after the initial dose, bringing down the fecal egg count from 1725 ± 331 to zero on days 15 and 30 [215]. Even lower single doses of 10 – $100 \mu\text{g kg}^{-1}$ ivermectin were reported to successfully eliminate *A. caninum* in dogs [216]. The ivermectin therapy was actually superior compared to treatments with the anti-hookworm agent albendazole [216]. An efficacy of $\geq 96\%$ was observed for ivermectin at a dosage of $24 \mu\text{g kg}^{-1}$ in the treatment of *A. caninum* and *Uncinaria stenocephala* infections in pups, regardless of the administration route (subcutaneous or oral) [217]. Closer inspection of the results revealed that the estimated oral doses of ivermectin required to achieve maximal efficacy ranged from $14 \mu\text{g kg}^{-1}$ for adult worms of *A. caninum* to $44 \mu\text{g kg}^{-1}$ for fourth-stage larvae of *U. stenocephala* [217]. Anderson and Roberson [218] have evaluated the activity of ivermectin not only against hookworms (*A. caninum*, *A. braziliense*) but also against dog roundworms

(*Toxocara canis*, *T. leonina*) and whipworms (*Trichuris vulpis*), as well as experimentally induced infections caused by fourth-stage larvae of *A. caninum* and *T. canis*. Single subcutaneous injections of $50 \mu\text{g kg}^{-1}$, $100 \mu\text{g kg}^{-1}$, $200 \mu\text{g kg}^{-1}$, or $400 \mu\text{g kg}^{-1}$ of ivermectin expelled $>99\%$ of the adult forms of both species of *Ancylostoma* hookworms as well as intestinal larval forms of *A. caninum* [218]. At a dose of $200 \mu\text{g kg}^{-1}$, ivermectin was found to be effective against larval stages of *T. canis* (97%), but only marginally efficacious (up to $\sim 69\%$) against *T. leonina* [218]. The effect of ivermectin on preventing transplacental transmission of reactivated encysted *T. canis* larvae to pups during pregnancy in female greyhounds was studied by Payne and Ridley [219]. The strategic use of the drug at a dose of $300 \mu\text{g kg}^{-1}$ in the female dogs on days 0, 20, and 60 of gestation reduced the worm burden in puppies by 90% [219].

In a study by Heredia Cardenas et al. [220], the efficacy of a combined therapy of ivermectin ($200 \mu\text{g kg}^{-1}$) and praziquantel (5 mg kg^{-1}) was evaluated in 100 dogs with confirmed diagnosis of *Toxocara* spp. infestation. After a single dose of the drug combination, the number of parasite eggs decreased by 71% and 88% on day 14 and 28 post-treatment, respectively [220]. In another study, the combination of ivermectin ($6 \mu\text{g kg}^{-1}$) and pyrantel (as pamoate salt, 5 mg of active drug kg^{-1}) in a chewable formulation, was shown to be 100% effective in preventing the development of *Dirofilaria immitis* larvae in dogs, while the efficacy of the combination therapy against *A. caninum*, *T. canis*, *T. leonina*, and *U. stenocephala* was 98.5%, 90.1%, 99.2%, and 98.7%, respectively [221]. The formulation was also effective against *A. caninum* and *U. stenocephala* in experimentally infected dogs and reduced the worm burden in the animals by 99.6% without any adverse side effects [222]. The ivermectin/pyrantel combination therapy was tested for its efficacy against *A. braziliense* in Beagles as well and 100% efficacy was observed against the adult form of this hookworm species [223].

4.2.2. Lungworm infestation

Capillaria aerophila is among the most frequently diagnosed parasites of the respiratory system in carnivorous mammals. The nematode settles in the mucous membrane mainly of the trachea and bronchi. Most infections caused by *C. aerophila* are asymptomatic and only periodically can lead to mild catarrhal inflammation. Severe infestations cause irritation that can obstruct the lumen of the airways, which can be followed by a chronic cough, by occasional shortness of breath, and by secondary bacterial bronchitis.

A single dose of ivermectin administered orally was found to efficaciously eliminate *C. aerophila* infecting the nasal passages in a dog [224]. No side effects of the treatment were observed [224]. Ivermectin ($300 \mu\text{g}$ per 100 g body weight) was also shown to be an effective and well-tolerated treatment against *Capillaria* spp. of hedgehogs, providing 100% efficacy against the nematodes [225]. In adult gray foxes naturally infected with *C. aerophila*, ivermectin together with febantel and fenbendazole, effectively eliminated the lungworm, and the activity of the drug combination was higher than that of mebendazole [226].

4.2.3. Ear mite infestation

Ear mite infestations, caused by *Notedres cati*, *N. muris*, and *Otodectes cynotis*, are frequently diagnosed in cats, dogs, rabbits, and other pet animals. The parasites are usually found in the ear canal, but they can also live on the skin surface, causing intense itching, which triggers scratching at the affected ear(s) and can finally lead to serious bacterial infections. Most older ear mite treatment products contain insecticides and are applied topically. However, these products must be used for at least 3 weeks as they do not kill the eggs. Modern topical ear mite medications include isoxazolines (fluralaner and sarolaner) or macrocyclic lactones (selamectin, moxidectin, and ivermectin). Only ivermectin-based preparations can be administered orally or subcutaneously.

Administration of 0.5 mL of 0.01% ivermectin otic suspension

removed ear mites in kittens more effectively than the related macrocyclic lactone selamectin [227]. Importantly, no evidence of toxicity was observed with the preparation [227]. Noteworthy is that the formulation even prevented the hatching of *O. cynotis* larvae from eggs in *in vitro* tests [228]. A complete clinical recovery was also found in cats after four doses of ivermectin administered orally ($200 \mu\text{g kg}^{-1}$), along with supportive daily therapy, i.e., multi-vitamin and mineral syrup [229].

On the other hand, ivermectin given subcutaneously at a dosage of $200 \mu\text{g kg}^{-1}$ twice at 3-weekly intervals was shown to be effective against *O. cynotis* infestation in the American red foxes (*Vulpes fulva*), with an efficacy of about 97% [230]. Toxic side effects associated with the drug administration were not observed, even after increasing the dose to 1.0 mg kg^{-1} [230]. In addition, the activity of ivermectin to eliminate *N. muris* has been confirmed in marsh rats [231]. In a therapeutic trial study it was found that 6 days after treatment with ivermectin ($400 \mu\text{g kg}^{-1}$, single subcutaneous injection), a complete visual shedding of lesions was observed in a group of 15 rabbits infected with *N. cori* var. *cuniculi* [232]. In another study, it was shown that ivermectin injected subcutaneously at weekly intervals for 4 weeks resulted in remission of clinical signs and improvement of the health condition in rabbits with mixed infestations of *N. cori*, *Sarcoptes cuniculi*, and *Psoroptes cuniculi* [233].

4.2.4. Mange

In addition to the licensed use of ivermectin to treat mange in ruminants and swine (see section 3.2.6 and Table 1 for details), the drug can also be used "off-label" against a series of other parasitic mites. Ivermectin-based therapy was found to be effective in the fight against feline and canine cheyletiellosis, especially when large numbers of animals are involved [234,235]. For example, the subcutaneous injection of the drug at the dose of $300 \mu\text{g kg}^{-1}$ twice, at a 3-weekly interval, resulted in a complete cure of adult dogs infected with *Cheyletiella yasguri* with no adverse reactions [234]. In a retrospective treatment study of cheyletiellosis in rabbits, 2–3 subcutaneous injections of ivermectin at doses ranging between 200 and $476 \mu\text{g kg}^{-1}$ in intervals of 11 days, resulted in remission of almost 82% of the animals [236]. The effective elimination of these mites is important, as infected pet animals can become a source of *Cheyletiella dermatitis* in humans [237,238].

On the basis of the literature evidence, oral daily administration of ivermectin ($300 \mu\text{g kg}^{-1}$) can be recommended for the treatment of generalized canine demodicosis, but the administration of the drug should be initiated at lower doses and animals need to be monitored for possible adverse reactions [239]. A slightly lower dose of ivermectin ($250 \mu\text{g kg}^{-1}$) every other day was used in a study on two cats suffering from feline demodicosis [240]. This treatment regimen was found to be effective in both cases in eradicating *Demodex gato* from the skin, but after 4 months of therapy, neurological symptoms were observed in one animal, confirming the need for close veterinary monitoring during the treatment [240]. Demodicosis caused by *D. cori* and *D. corni* was also successfully treated with ivermectin in dogs by daily oral administration of $500 \mu\text{g kg}^{-1}$ of the drug together with external application of amitraz (non-systemic acaricide) as well as a supportive therapy [241]. Complete recovery from the disease was achieved in 45 days without any side reactions [241]. The successful use of ivermectin to treat canine demodicosis has also been confirmed by other authors [242]. In a blinded, randomized three-phase clinical trial with 58 dogs, ivermectin was found to be more effective than 2.5% moxidectin plus 10% imidacloprid [243]. Interestingly, Saridomichelakis et al. [244] have shown that systemic treatment using a combination of ivermectin and cephalixin led to a complete resolution of the skin lesions and the disappearance of demodectic mites in two dogs after two months. On the other hand, topical use of the drug (1.5 mg kg^{-1} of 0.5% pour-on ivermectin) was found to have a rather limited efficacy in the treatment of chronic generalized demodicosis in dogs [245].

Effective treatment of the mange mite *Sarcoptes scabiei* var. *canis* with

ivermectin was also confirmed in animals [246]. A progressive clinical improvement of mange lesions was observed in foxes, whereby the best results were obtained when ivermectin was given subcutaneously at the initial dose of $400 \mu\text{g kg}^{-1}$, followed by a subsequent dose of $200 \mu\text{g kg}^{-1}$ of the drug after 2 or 3 weeks of the initial treatment [246]. Nevertheless, it should be noted that canine scabies resistant to the action of ivermectin-based therapy have been reported [247].

Ivermectin has also been shown to be effective against *Psoroptes* mites. At a single dose of $400 \mu\text{g kg}^{-1}$ and regardless of the injection route (intramuscularly or subcutaneously), the drug eliminated completely *Psoroptes canis* and *P. ovis* mites in rabbits [248]. In a study by McKellar et al. [249] it was found that $400 \mu\text{g kg}^{-1}$ of ivermectin given subcutaneously, significantly reduced the clinical score in rabbits infected with *P. canis*, while a slightly higher dose ($500 \mu\text{g kg}^{-1}$) resulted in a clinical cure in guinea pigs suffering from mange due to *Trichostrongylus axei*. Other studies confirmed that rabbits naturally infested with *P. canis* can be effectively treated with ivermectin [250–252]. For example, Pandey [251] reported that the administration of a single dose of the drug of $200 \mu\text{g kg}^{-1}$ and $400 \mu\text{g kg}^{-1}$ injected subcutaneously, led to the elimination of *P. canis* in rabbits within 6 days and that the animals remained negative for the presence of the mite until the end of the trial. Nevertheless, the regression of lesions was faster in rabbits who received twice the dose of the drug [251]. Although ivermectin seems to have no direct effect on the immune response of mite-infested rabbits [253], it can enhance the production of specific antibodies, particularly in weakly-infested animals [254]. A clinical trial with guinea pigs revealed that subcutaneous injection of ivermectin at a dose of $400 \mu\text{g kg}^{-1}$ led to the elimination of *T. axei* mites within 40 days [255]. The promising use of ivermectin to treat and control *T. axei* infestations in guinea pigs has also been confirmed in another study [256].

5. Experimental use of ivermectin

As described above, ivermectin is widely used in the treatment of many parasitic diseases, both in licensed and "off-label" use. In many cases, the drug is the first choice, due to its high efficiency at low dosage (usually $200 \mu\text{g kg}^{-1}$). Taking into consideration that the use of ivermectin in humans and animals is very safe, this drug has also been tested as experimental therapy against other parasitoses and diseases, such as viral and bacterial infections, and cancer [4,257]. Although drug repurposing can be a useful strategy to find new therapeutic applications for existing medications, it should only complement the process of drug discovery but not be its alternative [257].

Taking into account the constant need for new antiparasitic drugs due to the growing problem of the emergence of parasites that are resistant to commonly available therapeutics, ivermectin should be considered as an alternative drug in the treatment of many parasitic diseases, including leishmaniasis, trypanosomiasis, malaria, schistosomiasis, and trichinosis. Ivermectin may also be useful in the fight against bedbugs, which feed on human blood [4,258].

5.1. In protozoans

5.1.1. Leishmaniasis

Leishmaniasis is caused by *Leishmania* parasites, which are transmitted by the bites of infected female phlebotomine sandflies [259]. There are over twenty *Leishmania* species and three main forms of the disease – visceral (the most serious, fatal without treatment), cutaneous (causing skin ulcers), and mucocutaneous (affecting mouth, nose, and throat) [259]. There are an estimated 700,000 to one million new cases of the disease annually [259]. Several drugs are available for the treatment of the different forms of leishmaniasis (including amphotericin B and paromomycin) [260], but ivermectin has also been shown to exhibit antileishmanial activity.

The *in vitro* and *in vivo* activity of ivermectin was evaluated against

L. infantum [261]. The *in vitro* testing of the drug against promastigotes of *L. infantum* and macrophages gave IC_{50} (50% Leishmania inhibitory concentration) and CC_{50} (50% macrophage inhibitory concentration) values of $3.64 \pm 0.48 \mu M$ and $427.50 \pm 17.60 \mu M$, respectively, and thus a selectivity index (SI) of around 117 [261]. For comparison, the corresponding values for amphotericin B were: $IC_{50} = 0.12 \pm 0.05 \mu M$, $CC_{50} = 1.06 \pm 0.23 \mu M$, and SI ~ 9 [261]. Although the *in vitro* activity of ivermectin was lower than that of amphotericin B, the macrocyclic lactone showed a more promising selectivity [261]. In addition, this drug showed prophylactic activity by inhibiting macrophage infection with pre-treated parasites [261]. Importantly, ivermectin in free format or incorporated in polymeric micelles (5 mg kg^{-1} every two days for 10 days) could significantly reduce the parasite load in mice infected with *L. infantum* [261]. In a study by Rifaat et al. [262], the authors evaluated the antileishmanial activity of ivermectin against *L. donovani* infections in hamsters and mice. The activity of the drug was compared with that of other medicaments, including pentostam, levamisole, and thymic extract [262]. Ivermectin was administered at a dose of $300 \mu g$ per 100 g body weight every day for 10 days [262]. At the end of the study, it was found that the reduction in parasite burden was $\sim 89\%$ and $\sim 76\%$ in hamsters and mice, respectively [262]. Compared with pentostam, levamisole, and pentostam/thymic extract treatment regimens, ivermectin administration gave the best results [262].

Freitas et al. [263] tested the leishmanicidal activity of ivermectin against *L. amazonensis* and *L. donovani* in infected macrophages, using different drug concentrations ($1 \mu g \text{ mL}^{-1}$, $5 \mu g \text{ mL}^{-1}$, and $10 \mu g \text{ mL}^{-1}$). Amphotericin B was used as a reference at the concentrations of $1 \mu g \text{ mL}^{-1}$, $2 \mu g \text{ mL}^{-1}$, and $5 \mu g \text{ mL}^{-1}$ [263]. The results indicated a similar decrease in the percentage of infected macrophages after treatment with increasing concentrations of both drugs (Fig. 4) [263]. In addition, the authors showed that ivermectin incorporated into polymeric micelles and administered to chronically infected mice may cause a better cellular and humoral response than in animals treated with free ivermectin [263].

In the prevention of leishmaniasis, it is also important to control the phlebotomine sandfly vectors. To determine the insecticidal activity of ivermectin against sandflies, the drug was administered subcutaneously to hamsters at a dose of $200 \mu g \text{ kg}^{-1}$ or $400 \mu g \text{ kg}^{-1}$ [264]. Then, *Phlebotomus papatasi* infected with *L. major* were allowed to blood-feed on ivermectin-treated hamsters at various times post-treatment (4 h, days 1, 2, 6, and 10) [264]. The highest mortality rate was recorded in the sandflies that fed closest to the time of drug administration [264].

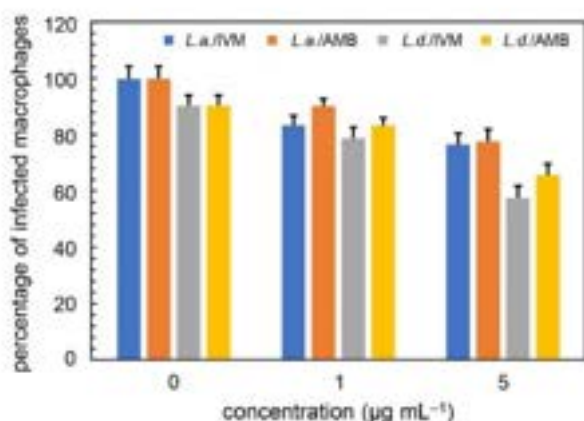


Fig. 4. Effect of ivermectin (IVM) and amphotericin B (AMB) on the infection of macrophages with *L. amazonensis* (L.a.) and *L. donovani* (L.d.). The percentage of infected macrophages after 48 h incubation with the drugs at $0 \mu g \text{ mL}^{-1}$, $1 \mu g \text{ mL}^{-1}$, and $5 \mu g \text{ mL}^{-1}$ is shown. Figure created using data published by Freitas et al. [263].

While the average survival rate of control insects that fed on non-treated hamsters was 11.5 days, the survival of sandflies that fed on hamsters treated with a dose of $400 \mu g \text{ kg}^{-1}$ ivermectin after 4 h, 1 day, or 2 days was 1.6, 2.1 and 2.7 days, respectively [264]. Sandflies that fed on low-dose ($200 \mu g \text{ kg}^{-1}$) treated hamsters still showed higher mortality rates than the controls [264]. However, it was also found that *L. major* promastigotes in sandflies that survived feeding on ivermectin-treated hamsters were unaffected by the drug [264]. Therefore, the findings of this study indicate that ivermectin can be considered as an agent for vector control to prevent leishmaniasis.

5.1.2. Trypanosomiasis

Trypanosomiasis comprises diseases caused by parasites from the genus *Trypanosoma*, including human African trypanosomiasis (sleeping sickness) caused by *T. brucei gambiense* and *T. b. rhodesiense*, human American trypanosomiasis (Chagas disease) caused by *T. cruzi*, and animal trypanosomiasis (nagana disease) caused by *T. b. brucei*, *T. evansi*, *T. congolense*, and *T. vivax* [265,266]. African trypanosomes are transmitted by tsetse flies (*Glossina* sp.), and mainly affect humans and animals in sub-Saharan Africa [265]. The parasites that cause Chagas disease are transmitted by triatomine bugs, but humans can get also infected by consuming contaminated food and beverages, by transfusion of contaminated blood products, during pregnancy from mother to child, and by laboratory accidents [266]. Chagas disease mainly affects people living in rural regions of Central and South America [266]. If left untreated, trypanosomiasis can lead to death in humans and animals [265,266]. Treatment of trypanosomiasis depends on a few drugs that have limited efficacy and can cause serious side effects [265,266]. Vector control plays also an important role in the prevention of trypanosomiasis [265,266]. Due to the emergence of drug-resistant trypanosome strains, it is necessary to search for new trypanocidal drugs. In this context, the potential antitrypanosomal activity of ivermectin may be of interest.

Fraccaroli et al. [267] have evaluated the activity of ivermectin against various strains and life cycle stages of *T. cruzi*. The EC_{50} values for epimastigotes were determined after 72 h of culture, while those for trypomastigotes and amastigotes after 24 h [267]. It was found that the EC_{50} values for epimastigotes ranged between $5.3 \mu M$ and $12.5 \mu M$, whereas those for amastigotes and trypomastigotes were $0.3 \mu M$ and $10.4 \mu M$, respectively [267]. Noteworthy is that the activity of ivermectin against amastigote and trypomastigote stages was better than that of the commonly used drugs benznidazole and nifurtimox [267]. Moreover, the SI of ivermectin calculated for *T. cruzi* amastigotes was 12, which meets the criteria for candidates for infectious disease drugs (SI > 10) [267,268]. The study also determined whether the effects of ivermectin on *T. cruzi* were trypanostatic or trypanocidal [267]. A drug is considered trypanostatic when the proliferation of the parasite recovers after transfer into a drug-free medium, while it is trypanocidal when the drug irreversibly affects the proliferation of the parasites [267,269]. It has been shown that ivermectin can exhibit both trypanostatic and trypanocidal activity depending on the dose used [267]. At a dose of $50 \mu M$ ($4 \times EC_{50}$), epimastigote proliferation was recovered by removing the drug after incubation for up to an hour, while at twice the dose ($8 \times EC_{50}$), the drug irreversibly inhibited the proliferation of the epimastigotes [267].

The *in vitro* activity of ivermectin against the *T. evansi* bloodstream forms was also investigated [270]. An IC_{50} value of $13.82 \mu M$ was determined for the drug and an SI value ranging from ~ 1.3 to ~ 1.6 was established with mammalian cells [270]. The antitrypanosomal activity of ivermectin was also confirmed in a study on mice infected with *T. brucei* [271]. A dose of $300 \mu g \text{ mL}^{-1}$ per kg body weight was most effective for both treatment and prophylaxis, increasing the average survival time of infected mice from 5 to 12 days [271]. Further research is, however, needed to determine whether the appropriate form of treatment should be a single dose (as in the study), multiple doses, or a combination with other drugs [271].

In addition to the antitrypanosomal activity of ivermectin, another important aspect of the drug is whether it can also be used in the control of the vectors that transmit trypanosomes. In a study by Pooda et al. [272], cows were injected with a therapeutic dose of ivermectin ($200 \mu\text{g kg}^{-1}$) or a 10 times higher dose (2 mg kg^{-1}). Then, tsetse flies (*Glossina palpalis gambiense*) were allowed to feed on treated and control cattle [272]. A significant decrease in the survival rate of the insects that fed on the ivermectin-treated cows was observed [272]. After 8 days, the reduction in survival ranged from $\sim 21\%$ to $\sim 84\%$ for tsetse flies that fed on cattle treated with $200 \mu\text{g kg}^{-1}$, and after 14 days from $\sim 78\%$ to $\sim 94\%$ for flies that fed on cattle treated with 2 mg kg^{-1} [272]. Ivermectin also led to 100% mortality in adult teneral males, mature males, and fertile females of *G. morsitans* when feeding on a single meal of defibrinated pig blood containing the drug at concentrations of $0.1 \mu\text{g mL}^{-1}$, $1.6 \mu\text{g mL}^{-1}$ or $>1.6 \mu\text{g mL}^{-1}$, respectively [273]. The lethal dose was $<0.04 \mu\text{g mL}^{-1}$ for teneral males when fed repeatedly on treated blood [273]. Moreover, after oral administration of ivermectin to a horse at a dose of $400 \mu\text{g kg}^{-1}$, the drug concentration in the blood reached $0.14 \mu\text{g mL}^{-1}$ within 24 h, which reduced the fecundity of tsetse flies to zero after a single meal [273].

5.1.3. Malaria

Malaria is still one of the most life-threatening parasitic diseases to humans. According to WHO data, nearly half of the world's population was at risk of malaria in 2021 [274]. In humans, the disease is mainly caused by 5 malaria species, *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi*, with the first two being the most dangerous [274]. Malaria parasites are transmitted by infected female *Anopheles* mosquitoes, but humans can get also infected via blood transfusion and contaminated needles [274]. The initial symptoms of the disease include fever and headache, and thus, the infection is often mistaken for flu. However, if left untreated, the disease can lead to extreme tiredness and fatigue, abnormal bleeding, and subsequent death [274]. The drugs commonly used to treat malaria are chloroquine, primaquine, and artemisinin-based combination therapies [274]. Importantly, the first malaria vaccine was approved for use in humans in 2021 [274].

Ivermectin has been shown to exhibit antimalarial activity and has been used in experimental therapies for malaria. In a study performed by Kobylinski et al. [275], the effect of ivermectin on the survival and re-feeding of *A. darlingi* as well as the development of *P. vivax* in the mosquito were evaluated. Different concentrations of the drug were mixed with human blood and fed to *A. darlingi* whose viability was monitored for the next 7 days [275]. After this time, the LC_{50} , LC_{25} , and LC_5 values were determined, which were 43.2 ng mL^{-1} , 27.8 ng mL^{-1} , and 14.62 ng mL^{-1} , respectively [275]. In the next step, the sporontocidal activity of ivermectin against *P. vivax* in *A. darlingi* was determined [275]. For this purpose, blood samples were taken from malaria patients and administered to mosquitoes with or without ivermectin at doses corresponding to the LC_{50} , LC_{25} , or LC_5 values [275]. The drug was found to be sporontocidal to *P. vivax* in *A. darlingi* at the LC_{50} and LC_{25} concentrations, reducing the prevalence by $\sim 23\%$ and $\sim 17\%$, respectively, but it showed no activity at the LC_5 concentration [275]. To investigate whether ivermectin inhibits re-feeding, estimated concentrations of the drug occurring after a single oral dose of $200 \mu\text{g kg}^{-1}$ were given to *A. darlingi* [275]. For the next 12 days, the mosquitoes had the opportunity to re-feed on a volunteer [275]. It was found that the drug significantly delayed the time of re-feeding at predicted 4-h (48.7 ng mL^{-1}) and 12-h (26.9 ng mL^{-1}) concentrations [275]. Other studies have also confirmed that ivermectin is sporontocidal to *P. falciparum* in mosquitoes at sub-lethal concentrations to *A. gambiae* [276]. Sporogony was inhibited in *A. gambiae* when the mosquitoes ingested ivermectin at a concentration of 10.7 ng mL^{-1} together or after the uptake of the parasites [276]. Mendes et al. [277] have demonstrated the effectiveness of ivermectin against the liver stage of the parasite in both *in vitro* and *in vivo* experiments. The drug was found to reduce *P. berghei* infection in human hepatoma cells *in vitro*, with similar

efficacy as primaquine, the only licensed liver-stage antiparasitic drug ($\text{IC}_{50} = 2.1 \mu\text{M}$ and $2.4 \mu\text{M}$, respectively) [277,278]. Worth mentioning is that ivermectin given to mice at a dose of 10 mg kg^{-1} reduced liver infections by 80% after 44–46 h post parasite infection [277]. In a study by Batiha et al. [279] it was shown that ivermectin has also the potential to be an alternative remedy in inhibiting the growth of the malaria-related parasite *Babesia* sp. and *Theileria* sp. both *in vitro* and *in vivo*.

Various studies have shown that ivermectin also exhibits mosquitoicidal and larvicidal activities, and therefore, may be used to control the spread of malaria vectors. In a study by Pampiglione et al. [280], the effectiveness of ivermectin against four mosquito species was determined. The drug showed larvicidal properties against *Culex pipiens* ($\text{LC}_{50} = 3.94 \text{ ppb}$), *A. stephensi* ($\text{LC}_{50} = 5.85 \text{ ppb}$), and *A. aegypti* ($\text{LC}_{50} = 23.41 \text{ ppb}$) [280]. When given to adult female mosquitoes (*A. stephensi*, *A. aegypti*, and *C. quinquefasciatus*) at a concentration of 2.6 mg L^{-1} (in sucrose), ivermectin killed the insects within 60 h [280]. *In vivo* studies were also conducted using mice injected subcutaneously with ivermectin at a dose of 82 mg of active ingredient per kg body weight [280]. After 12 h, female mosquitoes were allowed to blood-feed on the treated mice [280]. After 36 h, 100% mortality was observed for *A. stephensi*, 60% mortality for *A. aegypti*, and 50% mortality for *C. quinquefasciatus* [280]. The mosquitoicidal properties of ivermectin administered orally to dogs at various doses ($10 \mu\text{g kg}^{-1}$, $500 \mu\text{g kg}^{-1}$, $1000 \mu\text{g kg}^{-1}$, and $2500 \mu\text{g kg}^{-1}$) were also studied [281]. *A. quadrimaculatus* mosquitoes were allowed to blood-feed on treated dogs or were given blood previously collected from one of the dogs [281]. Mosquito mortality was observed 24 h and 48 h after feeding [281]. More than 90% of mosquitoes from the groups that fed on dogs died, while the mortality of mosquitoes that fed on the blood collected from a dog treated with $10 \mu\text{g kg}^{-1}$ was $\sim 65\%$ [281]. In another study, a volunteer took ivermectin at a single dose of $250 \mu\text{g kg}^{-1}$ and then allowed *A. farauti* mosquitoes to blood-feed on him [282]. High mosquito mortality was observed, with at least 50% of insects dying within 3 days [282].

A major problem in the use of ivermectin as a prophylactic agent is the limited retention time of therapeutic concentrations in the blood [283]. This problem can be overcome by using a sustained-release formulation of the drug. In a study by Chaccour et al. [284], rabbits were given silicone implants with ivermectin, deoxycholate, and sucrose, which made it possible to maintain a concentration of ivermectin in the blood of the animals at a level corresponding to the LC_{50} value against *A. gambiae* for at least 12 weeks [284]. Mathematical modeling based on the experimental data predicted a 96% reduction in infectious vector density by using a long-lasting ivermectin formulation for 12 weeks [284]. An oral long-lasting drug formulation was also developed by encapsulation of ivermectin in poly(ϵ -caprolactone) [285]. In a swine model, it was shown that the long-lasting formulation maintained therapeutic concentrations of ivermectin for up to 2 weeks, which was supposed to have a significant impact on malaria transmission [285]. The antimalarial activity of ivermectin and its derivatives has been recently comprehensively reviewed [286].

5.2. In helminths

5.2.1. Schistosomiasis

Schistosomiasis is a parasitic disease caused by blood flukes of the genus *Schistosoma* [287]. According to WHO data, in 2021, at least 251.4 million people required preventive treatment against schistosomiasis [287]. The intermediate hosts of the disease are different species of freshwater snails that release free-swimming larvae (cercariae) into the water. Infection occurs when cercariae from infested water penetrate the human skin [287]. The symptoms of the disease are diarrhea and blood in the stool or urine (depending on the schistosome species), among others, and they are mainly related to the body's reaction to the eggs of the parasite [287]. Praziquantel is mainly used to treat the disease, but albendazole and ivermectin should be considered as therapeutic agents

as well [287,288].

A study by Taman et al. [289] was conducted to test the effectiveness of ivermectin against *S. mansoni* in experimentally infected mice. The drug was administered 42 days after infection in two regimens, a single dose of 25 mg kg⁻¹ given orally and the same dose for two consecutive days [289]. In both cases, a significant decrease in the number of female worms, hepatic tissue eggs, and early immature eggs was observed [289]. The use of the same dose for 2 days significantly reduced the number of male worms and intestinal tissue egg load [289]. However, another study questioned the effectiveness of ivermectin in treating *S. mansoni*-infected mice [290]. Ivermectin was administered orally at a lower dose of 1 mg kg⁻¹ alone, or in combination with other drugs (cobiscistat or elacridar), 3 days before infection [290]. It was found that ivermectin alone or in combination with cobiscistat or elacridar showed no prophylactic activity against infection by *S. mansoni* cercariae [290]. In addition, mice treated with ivermectin and elacridar displayed severe neurotoxic adverse effects [290]. Thus, it seems to be necessary to perform further studies to validate the effectiveness and safety of ivermectin in the treatment of schistosomiasis.

One strategy to prevent infections with schistosomes is the control of the intermediate host snails with molluscicides. However, the treatment of freshwater bodies with pesticides may be harmful to other organisms living in the environment. Katz et al. [291] evaluated the effects of ivermectin on three Biomphalaria species (*B. glabrata*, *B. tenagophila*, *B. straminea*), on *B. glabrata* infected with *S. mansoni*, and on snail egg masses, miracidia, and cercariae, as well as on guppies (*Poecilia reticulata*). According to the authors, the calculated LD₅₀ and LD₉₀ doses for the three species of snails ranged between 0.03 and 0.13 µg mL⁻¹ and 0.3–1.0 µg mL⁻¹, respectively [291]. *B. glabrata* snails that were shedding cercariae died already when exposed to the drug at a concentration of only 0.01 µg mL⁻¹ [291]. Interestingly, only ivermectin 81b, which constitutes a maximum of 20% of the commercially available drug, was responsible for the death of snails [291]. Regarding the life-cycle stages of the parasite, ivermectin at a dose of 0.2 µg mL⁻¹ was sufficient to kill 50% and 90% of the cercariae and miracidia within 5 min and 30 min, respectively [291]. Of concern is the observation that guppies were also very sensitive to the action of ivermectin. At the concentrations of 0.5 µg mL⁻¹ and 0.01 µg mL⁻¹, the mortality rates after 24 h of exposure were 100% and 30%, respectively [291]. Thus, at the low concentration of 0.01 µg mL⁻¹ that was sufficient to kill 100% of schistosome-infected snails, ivermectin still killed 30% of the guppies [291]. Furthermore, ivermectin was ineffective against snail egg masses up to 100 µg mL⁻¹, the highest concentration tested [291].

5.2.2. Trichinosis

Trichinosis (trichinellosis) is a disease caused by roundworms of the genus *Trichinella*. Infection in humans occurs most often as a result of eating undercooked meat (mainly pork) infected with larvae of the parasite [292]. There are about 10,000 infections annually worldwide, and initial symptoms are gastrointestinal problems, such as diarrhea or vomiting, when the ingested larvae develop into adult worms in the small intestine [292]. After several weeks, the adult worms produce larvae that migrate through the body to reach striated muscle tissue. Once larvae have entered the muscle tissues, patients can experience muscle aches and pain, muscle stiffness, and muscle weakness [292]. The disease may also affect any mammals and even birds can be experimentally infected with trichinae. For the treatment of the disease in humans, albendazole and mebendazole are used, which are effective in eliminating adult worms, but unfortunately, are ineffective in combating encysted larvae residing in the muscles [292]. Thus, it is necessary to investigate the activity of other drugs against trichinae.

A study by Soliman et al. [293] compared the efficacy of ivermectin and doramectin (a derivative of ivermectin) at the dose of 200 µg kg⁻¹, along with levamisole at the dose of 7.5 mg kg⁻¹ against *T. spiralis* in experimentally infected rats [293]. The drugs were tested against adult worms on day 4 post-infection, migrating larvae on day 10

post-infection, and encysted larvae on day 35 post-infection [293]. Both macrocyclic lactones were shown to be highly effective in eliminating mature worms and migrating larvae with efficacies of ~98% and ~86% for doramectin and 95% and ~84% for ivermectin, respectively [293]. However, both drugs were ineffective in killing encysted larvae residing in the diaphragm [293]. In another study, the effectiveness of ivermectin and verapamil in eliminating intestinal adult worms and encysted muscle larvae was examined [294]. Ivermectin was orally administered to mice at a dose of 4 µg per mouse per day on days 1, 5, 15, and 35 post-infection, while verapamil was given to the animals from 1 to 35 days post-infection at a dose of 30 µg per mouse per day in the form of intraperitoneal injections [294]. Verapamil was found to be ineffective against adult worms but it was highly effective in reducing the number of encysted muscle larvae by ~94% [294]. In contrast, ivermectin was active against both life-cycle forms and reduced adult worms count by ~85% and muscle larval count by ~90% [294]. The combined use of ivermectin and verapamil resulted in a reduction in adult worms by ~69% and in muscle larvae by 99% [294]. Elmehy et al. [295] compared the effectiveness of ivermectin administered in two different formulations, i.e., 200 µg kg⁻¹ in a single oral dose of nanocrystalline ivermectin and 200 µg kg⁻¹ in a single oral dose of microsomal ivermectin [295]. The study was conducted on mice infected with different life-cycle stages of *T. spiralis* (adult worms, migrating larvae, and encysted larvae) [295]. In each case, the microsomal administration of ivermectin was more effective than the nanocrystalline formulation [295]. The percentages of reduction were ~92% and ~73% for adult worms, ~70% and ~35% for migrating larvae, and ~63% and ~51% for encysted larvae, for microsomal and nanocrystalline ivermectin, respectively [295]. Moreover, ivermectin was also effective in treating monkeys and baboons infected with *T. simbabwensis* [296].

5.3. In bedbugs

Bedbugs are parasitic wingless insects that feed on the blood of humans and animals during sleep [297,298]. Although the insects do not spread diseases, they can cause itching and severe allergic reactions in some people that may lead to subsequent bacterial skin infections [297, 298]. There are two species that mainly feed on humans, *Cimex lectularius* and *C. hemipterus* [297]. Preventive measures include controlling the spread of the insects, for which ivermectin seems to be an effective tool.

To determine the mortality and morbidity of *C. lectularius* after ivermectin intervention, an artificial feeding membrane, and pre-treated mice and humans were used in the tests [299]. The results indicated that after 13 days of feeding on mouse blood containing ivermectin at a concentration of 260 ng mL⁻¹ through an artificial membrane, the bedbugs mortality rate was 98%, while the mortality rate in the control group (just mouse blood) was 0% [299]. Similar results (86% mortality) were observed with bedbugs that fed on mice treated with 200 µg kg⁻¹ ivermectin [299]. In addition, bedbugs that fed on a human who had been given 200 µg kg⁻¹ of oral ivermectin 3 h earlier, showed a mortality rate of 63% after 20 days (mortality rate of the control was 8%) [299]. Thus, it seems that ivermectin may be useful in controlling bedbugs. Similar results were obtained in a study by Ridge et al. [300], in which a rabbit was treated with subcutaneous ivermectin at a dose of 300 µg kg⁻¹, and *C. lectularius* bedbugs were allowed to feed on the animal (before and after drug administration) [300]. At blood concentrations of ~2 ng mL⁻¹, a reduction in bedbug fecundity was observed, while blood concentrations of ~8 ng mL⁻¹ led to the death of the insects or long-term morbidity, including a reduction in refeeding, mobility, and molting [300]. A relationship between the concentration of the drug in the blood and the reaction of bedbugs has also been demonstrated by other authors [301]. The mortality rate of *C. lectularius* bedbugs that fed on blood containing ivermectin or moxidectin (an ivermectin-like macrocyclic lactone) at a concentration as little as 25 ng mL⁻¹ was 100% after 13 days, compared to 0%–6% mortality of the control [301].

Bedbugs that survived blood meals containing ivermectin at a concentration of 2.5 ng mL^{-1} showed reduced fecundity, feeding difficulty, and incomplete ecdysis [301].

González-Morales et al. [302] have determined the LC_{50} and LC_{90} values of ivermectin against bedbugs of the Harlan strain. Ivermectin was dissolved in DMSO and added to human blood, and the insects were fed using an artificial feeding system. LC_{50} and LC_{90} values were determined to be 61.0 ng mL^{-1} and 114.9 ng mL^{-1} , respectively [302]. However, in *in vivo* studies, ivermectin did not show high efficacy [302]. Chickens were injected with ivermectin at the dose of $200 \mu\text{g kg}^{-1}$, but it did not result in the killing of the bedbugs that fed on the treated animals [302]. When administered orally at the same dose, mortality was achieved only for a few insects (5–11 out of 15 bedbugs per replicate), and a relatively low bioavailability of ivermectin in chicken blood may explain the low efficacy of the drug in this animal model [302]. It was also found that drug levels in bedbugs decreased rapidly during the first week of treatment but remained relatively constant between weeks 1 and 4 [302]. This observation was confirmed in another study in which it was discovered that ivermectin can persist in the blood of bedbugs for up to one month after a blood meal [303]. The long retention time of ivermectin in bedbugs may explain why those insects that survived the treatment exhibit long-term morbidity [302].

5.4. In other diseases

In addition to the broad antiparasitic potency, ivermectin also exhibits biological activities against bacteria, viruses, and cancer cells [4]. Previously it was believed that ivermectin did not have antibacterial properties, but reports from the last decade have shown that the drug is effective against *Chlamydia trachomatis*, *Mycobacterium tuberculosis*, and *M. ulcerans* [304–306], although some studies could not confirm the antimycobacterial activity of the drug [307,308]. Thus, additional studies are needed to validate the real antibacterial potential of this compound. Regarding the antiviral activity of ivermectin, the drug effectively inhibited the replication of the yellow fever virus and other flaviviruses [309]. Ivermectin exhibits also activity against RNA viruses, such as HIV-1 and dengue viruses [310]. In recent years, the drug has gained popularity as a potential treatment option against COVID-19. For example, ivermectin at a concentration of $5 \mu\text{M}$ allowed for an approximately 5000-fold reduction of the SARS-CoV-2 virus in cell culture after 48 h [311]. However, despite many clinical trials, ivermectin has never been introduced as a treatment for COVID-19.

Several recently published reports described the high anticancer activity of ivermectin against various types of tumors, including colorectal, breast, glioblastoma, head and neck, leukemia, melanoma, pancreatic, and prostate cancers [257,312]. The mechanism of anticancer activity of ivermectin is extremely diverse and affects many biochemical processes. Briefly, the drug can inhibit the synthesis of proteins responsible for multi-drug resistance (MDR) and the AKT/mTOR pathways, which are the main regulators of ovarian cancer progression, but it can also block the Wnt/TCF pathway responsible for the proliferation process of cancer cells [5,312]. Moreover, the mode of action of ivermectin is associated with the degradation of PAK-1, the main kinase responsible for the process of carcinogenesis, as well as with an increase in the level of reactive oxygen species (ROS) in tumor cells, leading to oxidative stress and subsequent DNA damage [5,312]. Ivermectin has also been shown to significantly reduce the number of cancer stem cells, a small subpopulation of cancer cells (5%–10% of the tumor mass), whose presence is associated with the progression, metastasis, and recurrence of cancer [5,312]. Importantly, ivermectin can reach clinically relevant concentrations to inhibit tumor growth in humans [312]. Further studies are necessary to demonstrate whether ivermectin can be used in anticancer therapy.

6. Opportunities and challenges of ivermectin

Ivermectin is one of the most extensively used antiparasitic drugs worldwide against various nematode, insect, and acarine parasites. The drug may be administered at relatively low doses via different routes (orally, topically, or subcutaneously). However, the limited retention time of therapeutic concentrations of ivermectin in the blood is a major problem in its use as a prophylactic agent [283]. For example, it has been shown that the mosquitocidal concentration of ivermectin against *A. gambiae* lasts for 2–3 days after a single standard dose of the drug ($200 \mu\text{g mL}^{-1}$) in the blood meal [283,313,314], which clearly illustrates the need for multiple doses of the drug to achieve appropriate concentration levels for longer times. To increase the half-life of ivermectin in the blood, sustained or slow-release formulations of the drug should be introduced. This issue has been extensively studied recently [284,315–317], showing that relatively stable mosquitocidal plasma levels of the drug can be safely sustained in animals for months. Although such techniques seem to be very promising, their safety and efficacy need to be validated in humans. As ivermectin is readily metabolized in the liver by the 3A subfamily of cytochrome P450 (CYP3A) enzymes system [318], it is also postulated that an increase in the plasma levels of ivermectin can be achieved by the use of inhibitors to these specific enzymes, such as ketoconazole [319–322].

Ivermectin is well-tolerated in most mammals, has minimal side effects, and can be administered even by non-medical personnel with appropriate training, as long as it is used at recommended doses. The Mazzotti reaction and other early adverse side effects should always be considered after ivermectin administration, but neurological dysfunction and other systemic symptoms may occur following an ivermectin overdose [23,323,324]. It has been documented that >100 times the normal dose of ivermectin may result in the accumulation of the drug in the brain, leading to coma and even death [23]. Ivermectin is classified by the FDA as a Pregnancy Category C drug [24], and therefore, pregnant women are typically excluded from receiving this drug due to potential adverse effects on the fetus, but potential benefits may warrant its use in pregnant women despite the potential risks. However, the available data regarding the safety of ivermectin in pregnancy are limited and ambiguous [325,326]. Thus, more studies are needed to carefully evaluate the risk ivermectin may pose during pregnancy. With respect to the potential brain-damaging effects of ivermectin, Mealey et al. [327] have found that the drug induces neurotoxic reactions in collies carrying a deletion mutation of the MDR1 gene. Mutations of this gene lead to an incomplete synthesis of the P-glycoprotein (P-gp) [327], which plays a pivotal role in the process by which the blood-brain barrier limits the uptake of drugs into the brain. Therefore, the lack of the P-gp causes an increase in the level of ivermectin which explains why the drug displays severe neurotoxic effects in animals with MDR1 gene deletion.

A growing problem in veterinary medicine is the emergence of drug-resistant parasites. This refers also to ivermectin-resistant parasite strains that developed as a consequence of the MDA-approach of the drug to protect all animals considered “at risk”. In addition, the incomplete understanding of the underlying mechanisms of ivermectin resistance and the lack of diagnostic resistance markers may all have a negative impact on current as well as future parasite control strategies. There are some options to overcome these limitations, which include the combination of ivermectin with albendazole or other antiparasitic drugs in novel treatment regimens, or systematic modification of the multi-functional structure of ivermectin to obtain derivatives with improved activity and selectivity. The sixteen-membered macrocyclic system is necessary for maintaining the high antiparasitic activity of ivermectin and other ivermectin derivatives, as is the presence of the hydroxyl group at the C-5 position of the oxahydrindene (hexahydrobenzofuran) ring (Fig. 1) [328]. The disaccharide unit seems to also be important for retaining the antiparasitic activity, wherein the chemical modification of the C-4' hydroxyl group (Fig. 1) is recognized as one of the promising sites to possibly improve the overall activity profile of the native

structure [328]. Of note, Singh et al. [329,330] have shown that the replacement of the disaccharide unit of ivermectin with rationally selected pharmacophores may lead to molecular hybrids exhibiting potent antimalarial activity. Nevertheless, more work is needed to develop new antiparasitic drug candidates based on the ivermectin molecule.

7. Conclusions

The macrocyclic lactone ivermectin has revolutionized the treatment of roundworm infestations in domestic animals when it was introduced to the animal health market in 1981. The success of the drug is based on its high efficacy against a broad range of nematode and arthropod parasites, its activity at low dosages, and its low toxicity in mammals, as well as on the possibility that the drug can be delivered via diverse routes of administration. Eventually, ivermectin was also introduced for the therapy of a few human parasitic diseases. The use of ivermectin against river blindness was a breakthrough in the treatment and control of this insidious disease that caused blindness in so many people in tropical Africa. In particular, the mass administration of ivermectin from the 1990s had an impressive impact so that in most endemic foci, river blindness is no longer a health problem. Over 40 years of intensive research on ivermectin has led not only to its approval for the treatment of river blindness and strongyloidiasis in humans, and roundworm and arthropod infestations in animals, but also to the effective use of this drug against many other worm-related parasitic diseases in “off-label” practice and experimental therapy. The drug has even the potential as medication for disease other than parasitoses. However, the massive use of ivermectin increases the risk of the development of drug-resistant parasite strains. This problem may be overcome by combining ivermectin with other drugs in treatment regimens. Another possibility is the systematic derivatization of ivermectin to enhance its activity and specificity, a research area which has been explored too little so far.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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11.2. Publikacja II

Michał Sulik, Diana Fontinha, Dietmar Steverding, Szymon Sobczak,
Michał Antoszczak, Miguel Prudêncio, Adam Huczyński

*Unexpected rearrangement of ivermectin in the synthesis of new derivatives with
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Research paper

Unexpected rearrangement of ivermectin in the synthesis of new derivatives with trypanocidal and antiplasmodial activities

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ABSTRACT

Ivermectin is a sixteen-membered macrolactone “wonder drug” of Nobel prize-honored distinction that exhibits a wide range of antiparasitic activities. It has been used for almost four decades in the treatment of various parasitic diseases in humans and animals. In this paper, we describe the synthesis of the first-in-class ivermectin derivatives obtained via derivatization of the C13 position, along with the unexpected rearrangement of the oxahydrindene (hexahydrobenzofuran) unit of the macrolide ring. The structural investigation of the rearrangement has been performed using the single-crystal X-ray diffraction method. The antiparasitic and cytotoxic activities of the newly synthesized derivatives were determined *in vitro* with the bloodstream form of *Trypanosoma brucei brucei*, the hepatic stage of *Plasmodium berghei*, and human leukemia HL-60 cells. The compounds with the highest trypanocidal activity were the C13-*epi*-2-chloroacetamide analogs of native (6b) or rearranged (7h) ivermectin. Both 6b and 7h displayed trypanocidal activities within a similar mid-nanomolar concentration range as the commercially used trypanocides suramin and ethidium bromide. Furthermore, 6b and 7h exhibited a comparable cytotoxic to trypanocidal ratio as the reference drug ethidium bromide. The double-modified compound 7a (C13-*epi*-acetamide of rearranged ivermectin) exhibited the highest activity against *P. berghei* grown in human hepatoma cells, which was 2.5 times higher than that of ivermectin. The findings of this study suggest that C13-*epi*-amide derivatives of ivermectin are suitable leads in the rational development of new antiparasitic agents.

1. Introduction

Ivermectin (IVR, **1**, Fig. 1) is a sixteen-membered macrolactone and the C22,C23-dihydro derivative of avermectin (Fig. 1), which was first isolated from a *Streptomyces avermitilis* strain in Japan in 1967 [1]. A broad range of antiparasitic activities of IVR, combined with its safe use, has led to numerous applications of the drug in the treatment of several parasitic diseases, including river blindness and elephantiasis [2,3]. Mechanistically, IVR potentiates the glutamate-gated chloride ion channels (GluCl), resulting in the hyperpolarization of parasite muscles and nerves [3]. Moreover, IVR has the ability to inhibit the conductance of γ -aminobutyric acid (GABA) channels, while in the asexual blood stages of *Plasmodium falciparum*, the drug has been found to inhibit importin (IMP) α/β , a heterodimer that transports proteins to the

nucleus across the nuclear complex pore [3]. IVR has significantly influenced medical treatments that many scientists call it a “wonder drug” [4], and its discovery, together with that of artemisinin as anti-malarial drug, was awarded the Nobel Prize in Physiology/Medicine in 2015 [5]. IVR is used in human and veterinary medicine as a mixture of two forms – B1a with an ethyl group at the C26 position, and B1b with a methyl group at this position (Fig. 1) [6]. The mixture consists of at least 80 % of IVR B1a and a maximum of 20 % of IVR B1b [6].

Neglected tropical diseases (NTDs) are a group of about 20 different infections that mainly affect people living in poverty in tropical and subtropical regions [7]. It is estimated that NTDs are a health and economic threat to approximately one billion people worldwide [7]. However, many NTDs could be easily and cost-effectively controlled, as preventive measures are relatively cheap and bring an estimated benefit

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of US\$ 25 net per US\$ 1 invested in prophylactic chemotherapy [7]. An example of an NTD is African trypanosomiasis (known as sleeping sickness in humans and nagana disease in animals), which is caused by parasites of the genus *Trypanosoma* [8–11]. The infection is transmitted through the bites of tsetse flies (*Glossina* sp.) that have acquired the parasite from infected humans or animals [8–11]. The disease develops in two stages, termed the hemo-lymphatic and neurological stages [8–11]. If left untreated, the disease can lead to behavioral changes, sensory disturbances, coma, and subsequent death [8–11]. Thanks to preventive as well as new therapeutic interventions introduced by the World Health Organization (WHO), the number of sleeping sickness cases has been significantly reduced in recent years [7,8]. However, due to the limited number of drugs available to treat African trypanosomiasis and the emergence of drug-resistant trypanosomes, it is necessary to develop new therapeutics to fight the infection [12].

Another parasitic disease that, although not belonging to the group of NTDs, poses a real threat to global health care is malaria. According to WHO data, in 2021, malaria caused the death of over 600,000 people, making it the deadliest parasitic disease [13]. Malaria is caused by parasites of the genus *Plasmodium*, which are transmitted in their sporozoite form by infected female *Anopheles* mosquitoes [13,14]. Injected sporozoites asymptomatically infect and replicate inside hepatocytes, generating thousands of merozoites that are subsequently released into the bloodstream, where they cyclically infect erythrocytes causing the disease [15]. The initial symptoms of malaria include fever, chills, and headache, but after progression, it can lead to fatigue, confusion, difficulty in breathing, or even death [13,16]. Preventive measures that are currently employed to reduce the spread of the disease include vector control, prophylactic chemotherapies, and the introduction of the RTS,S/AS01 and the R21/Matriguard malaria vaccines [13,17]. Chloroquine, primaquine and artemisinin-based combination therapy medicines are mainly used to treat the disease, but due to the growing problem of drug resistance, the search for new lead structures for anti-malarial drugs is a top priority [13,16,18].

In addition to the licensed uses of IVR, the drug has been investigated in experimental therapies against sleeping sickness and malaria, as well as other diseases [3]. IVR has been shown to be effective at micromolar concentrations against *T. cruzi* and *T. evansi* [19,20]. The use of IVR in mice infected with *T. brucei* was found to be efficacious, resulting in a prolongation of the average survival time of infected animals [21]. This drug was also found to be potent in controlling the spread of tsetse flies, which are the vectors of African trypanosomes [22]. The antimalarial properties of IVR have also been extensively studied [23]. IVR was able to reduce *P. berghei* infection in human hepatoma cells, with efficacy similar to that of primaquine – the only licensed liver-stage antiparasitic drug [24,25]. Furthermore, IVR exhibited also strong mosquitocidal and larvicidal properties [26]. However, to the best of our knowledge, there are no or only scarce reports in the scientific literature [27,28] on the antitrypanosomal or antiparasitic activity of IVR derivatives, respectively.

Having access to the C13-*epi*-amine precursor of IVR (Scheme 1), we now describe the synthesis of a series of amide derivatives modified at position C13 (Scheme 2). We have further extended a library of new compounds with first-in-class analogs with a rearranged oxahydrindene

(hexahydrobenzofuran) unit of IVR (Scheme 2 and 3). We also report on the trypanocidal and antiparasitic activities of the IVR derivatives using the bloodstream form of *T. brucei brucei* and the hepatic cell-infective form of luciferase-expressing *P. berghei*.

2. Results and discussion

2.1. Analogs design and synthesis

Most of the chemical transformations of avermectin derivatives, including IVR (form B1a), described in the scientific literature concern the modification of either the hydroxyl group at position C5 or the sugar moiety, especially the C4' hydroxyl group (Fig. 1) [29]. It has been shown that C4'-substituted avermectin derivatives exhibited improved bioactivities as well as better pharmacokinetic profiles than the starting compounds [30–34], while the hydroxyl group at position C5 is considered to be crucial for the biological properties [35]. This clearly shows that a rational modification of avermectin derivatives may be a source of promising bioactive compounds. On the other hand, there is less evidence for the effects of chemical modification of other functional groups of IVR and other avermectin derivatives, including position C13 (Fig. 1) [27–29,36–39]. Recently, it has been demonstrated that the replacement of the IVR sugar moiety with other structural motifs may significantly affect the antimalarial activity of the obtained derivatives [27,28]. Of note, selected sulfonamide-like derivatives of C13-*epi*-aminovermectin exhibited higher biological activity than IVR against *Tetrahymena cinnabarinus* and *Aphis craccivora* [36]. On the basis of this information, we designed and synthesized a series of new amide derivatives of IVR with an inverted configuration at position C13 to expand the structural diversity at this position and generate attractive candidates for biological investigations.

Starting from IVR, a key C13-*epi*-amine precursor was obtained according to protocols published previously [28,36,38,39] with or without minor modifications (Scheme 1). Briefly, IVR was treated with a solution of sulfuric acid in methanol to remove the sugar moiety, which resulted in the formation of aglycone 2, with a 84 % yield (Scheme 1). To mask the C5 functionality, 2 was treated with *tert*-butyldimethylsilyl chloride in the presence of imidazole to give 3 with a 91 % yield (Scheme 1). A complete regioselectivity of this reaction towards the C5 hydroxyl group was observed. Next, the C13 hydroxyl group was oxidized to ketone 4 using Dess-Martin periodinane with a 82 % yield, followed by a reductive amination of the C13 ketone group with ammonium acetate to the amine 5 with a 51 % yield (Scheme 1). The reductive amination was stereoselective and gave only one diastereoisomer, which was confirmed by X-ray analysis (see section 2.2 for details). Gently heating the reaction mixture in the initial phase (imine formation) and extending the reaction time increased its overall yield, compared to that in the original protocol [38]. The spectroscopic data of 2–5 were in agreement with those published previously for the same compounds [28,38,39].

Amine 5 was then used in the synthesis of new amides of IVR in a reaction with various acyl chlorides in the presence of triethylamine, or using carboxylic acids and HATU as the coupling agent (Scheme 2). After removing the silyl protection group at position C5 with *para*-

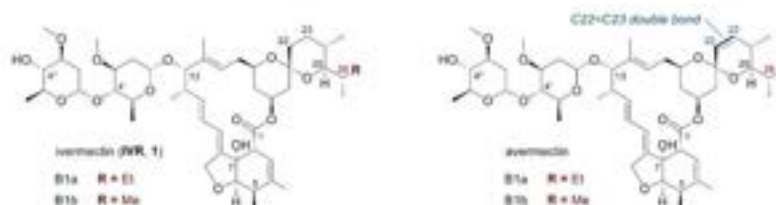
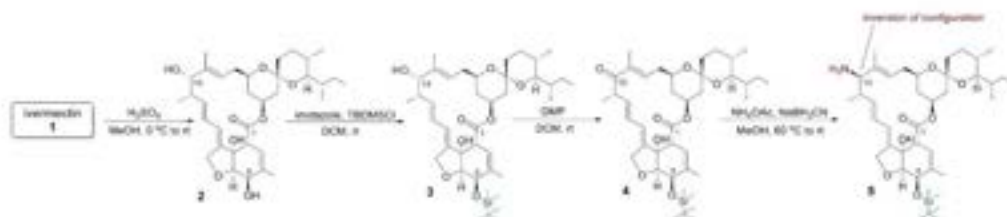
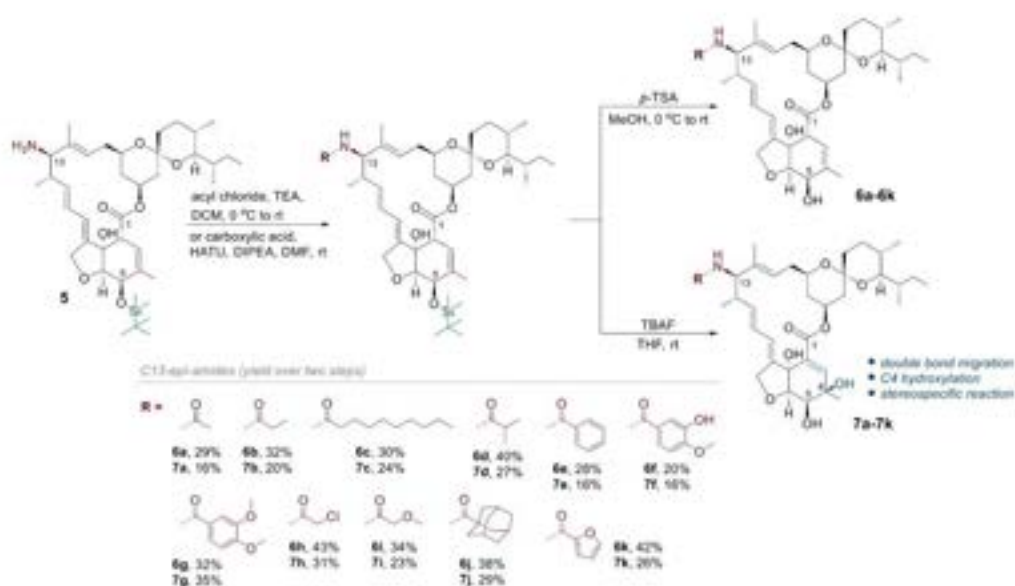


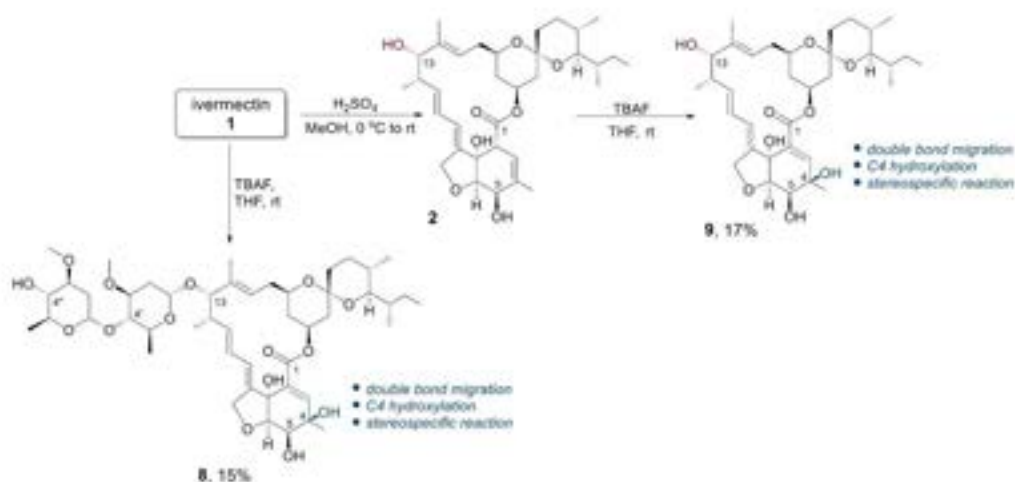
Fig. 1. Chemical structure of ivermectin (left) and avermectin (right).



Scheme 1. Synthesis of the C13-epi-amine precursor of ivermectin.



Scheme 2. Synthesis of singly and doubly modified derivatives of ivermectin.



Scheme 3. Synthesis of rearranged ivermectin and its aglycone.

toluenesulfonic acid (*p*-TSA), a series of singly modified IVR derivatives **6a–6k** was synthesized (Scheme 2). Interestingly, when TBAP was used, instead of *p*-TSA, to deprotect the hydroxyl group from the silyl ether, we observed the formation of the C13-*epi*-amide analogs of rearranged IVR (compounds **7a–7k**, Scheme 2).

The structure of the unexpectedly rearranged IVR was confirmed by the single-crystal X-ray diffraction (sXRD) method. The X-ray structure analysis (see section 2.2 for details) clearly showed that during the reaction the double bond migrated and an additional hydroxyl group was introduced at position C4. Trying to rationally explain this transformation, we have found an interesting study by Buckley et al. [40]. The authors have documented the hydroxylating properties of TBAP, with atmospheric oxygen playing a pivotal role in the reaction and acting as an electrophile [40]. Despite extensive experimentation, including varying the solvent (CHCl_3 , CH_2Cl_2 , MeOH) and reagent (TBAB, TBAI, KF), the rearrangement was exclusively accomplished for TBAP in THF. For IVR and its derivatives, apart from TLC analysis, the progress of the reaction can be easily monitored by observing the color of the reaction mixture turning red (Supplementary material, Figure S1). Carrying out the reaction in an “open vessel” or in an insert (argon) atmosphere, we observed a rapid change in the color of the reaction mixture to red only in the “open vessel” variant, which confirmed the key role of atmospheric oxygen in the rearrangement of IVR. On the other hand, the addition of hydrogen peroxide resulted in the inhibition of reaction progress (Supplementary material, Figure S1). Based on all these observations, we proposed the mechanism of the rearrangement of IVR (Fig. 2). Once the rearrangement reaction conditions were established, the series of derivatives was extended to include the rearranged IVR (compound **8**) and its aglycone **9** (Scheme 3).

The purity and structure of the synthesized derivatives of IVR were determined using spectroscopic (^1H NMR, ^{13}C NMR) and spectrometric (HRMS) methods. In the ^{13}C NMR spectra of IVR derivatives, the signals of the highest analytical significance were assigned to the newly introduced carbonyl group of the amide moiety at position C13. Depending on the type of substituent used, the signal from the amide group appeared in the range of 177.6–157.9 ppm, while the signals from the lactone group at the C1 position appeared in a narrow range of 173.4–173.6 ppm or 168.5–169.0 ppm for C13-*epi*-amide derivatives of native or rearranged IVR, respectively. The HRMS analysis confirmed the formation of the desired products, with $[\text{M}+\text{Na}]^+$ as the main peak (intensity 100 %). The NMR spectra of all novel IVR derivatives are included in the Supplementary material (Figures S2–S49).

2.2. X-ray diffraction analysis

To elucidate the structure of IVR derivatives and establish possible structure-activity relationship (SAR), X-ray diffraction analysis is required. Thus, we characterized derivatives **6a** and **7a** using the sXRD method. This analysis confirmed the (*R*)-stereochemistry at position C13 of the compounds, at which acetamide groups were introduced, and the structure of the rearranged oxahydrindene ring in **7a** (Fig. 3).

In-room conditions, derivative **6a** crystallizes in the monoclinic $P2_1$ space group with two molecules in the asymmetric part of the unit cell ($Z' = 2$, molecules labeled as A and B, Fig. 3). The appearance of two separate IVR conformations can be attributed to the labile *sec*-butyl group at position C25, engaged in $\text{CH}\cdots\text{C}$ intermolecular interactions. The *sec*-butyl group exhibits positional disorder over two equivalent

positions, which can be characterized by the torsion angle C28–C27–C26–C25, with values of 112.13° and 66.53° in molecule A and 178.15° and 129.35° in molecule B. The structure of **6a** is porous (the solvent accessible volume calculated with the program Mercury [41] for probe radius 1.2 Å and grid spacing 0.2 Å is equal to 2 % of the total unit-cell volume), which degrades the quality of the single crystals due to the presence of highly disordered, non-stoichiometric amount of solvent (acetone) trapped in the pores.

Interestingly, the rearrangement of the IVR molecule does not significantly influence the conformation as observed in **7a** (Fig. 4). The sXRD experiments revealed that the colorless block crystals precipitated from the solution are an acetone solvate of **7a** crystallized in the orthorhombic $P2_12_12_1$ space group. The co-crystallized acetone molecule is disordered over two positions with a site occupancy factor (SOF) of 0.5.

All IVR derivatives share similar structural features – a rigid sixteen-membered lactone ring, a spiroketal comprising two six-membered rings, and a cyclohexene ring fused to a five-membered cyclic ether with two or three hydroxyl groups (Fig. 4) [42,43]. Although the introduction of an additional hydroxyl group at position C4 does not significantly influence the lactone ring and spiroketal conformation, the rearrangement does modify the cyclohexene ring. The hydroxyl group (O4A) at position C4 assumes an axial position, while other substituents maintain their positions – C1 and O5A at position C5 are in equatorial positions while O6A and O7A at positions C6 and C7, respectively, are in axial positions. The migrated double bond in **7a** (from C3–C4 to C2–C3 with distances 1.325(4) Å and 1.493(4) Å, respectively) additionally flattens the cyclohexene with respect to already distorted half-chair conformation observed in **6a** in which C3–C4 and C2–C3 are equal to 1.35(3) Å and 1.50(2) Å in molecule A, and 1.29(3) Å and 1.56(2) Å in molecule B, respectively.

2.3. Biological activity

2.3.1. Trypanocidal activity

The trypanocidal and cytotoxic activities of the newly synthesized IVR derivatives were determined with bloodstream forms of *T. b. brucei* and human myeloid HL-60 cells *in vitro*. The antiproliferative activity of the compounds was evaluated with the resazurin cell viability assay as previously described [44].

IVR (compound **1**) itself displayed moderate antitrypanosomal activity with a 50 % growth inhibition (GI_{50}) value in the mid-micromolar range, while aglycone **2** was about twofold more trypanocidal (Table 1). The trypanocidal activity of IVR against bloodstream forms of *T. b. brucei* was within the range previously reported for trypanostigmine forms of *T. cruzi* ($\text{EC}_{50} = 10.4$ (7.0–15.5) μM [20]). The rearranged IVR and its aglycone (compounds **8** and **9**) were slightly less trypanocidal than their counterparts (Table 1). All four parent compounds **1**, **2**, **8**, and **9** exhibited similar cytotoxic activity against HL-60 cells with GI_{50} values between 31 and 35 μM (Table 1). Therefore, the selectivity (ratio of cytotoxic to trypanocidal activity) of these four compounds was poor, ranging between 2 and 6 (Table 1).

With the exception of **6h** and **7h**, all other IVR derivatives (**6a–6g**, **6i–6k**, **7a–7g**, and **7i–7k**) displayed similar antitrypanosomal activity with GI_{50} values ranging between 3.0 and 19.1 μM (Table 1). There was also not much difference in the trypanocidal activity between singly (series 6) and doubly (series 7) modified derivatives. Compounds **6h** and

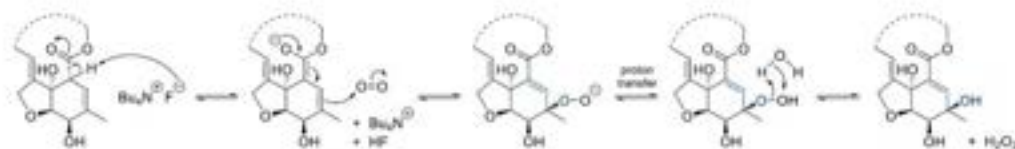


Fig. 2. Proposed mechanism of the rearrangement of the oxahydrindene (hexahydrobenzofuran) ring of ivermectin.

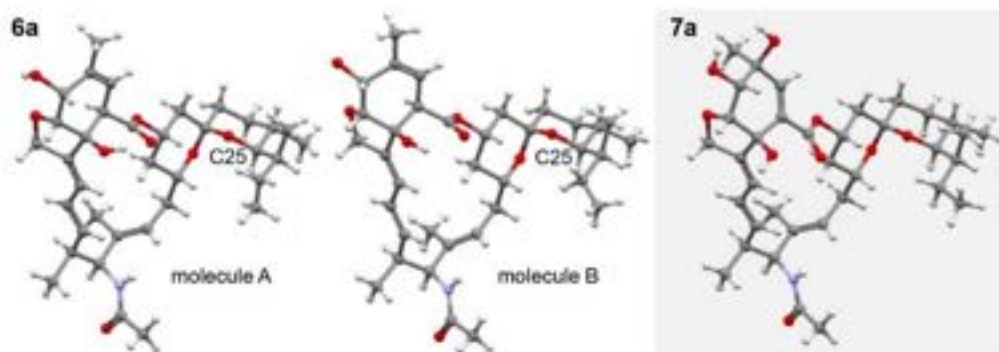


Fig. 3. Ball-and-sticks models of **6a** (molecules A and B) and **7a** from the sXRD experiments. For compound **6a**, only one position of the disordered acetate group at position C25 is shown, while in **7a** the co-crystallized acetone is omitted for clarity. Atom color code: C – gray, O – red, N – blue, and H – white.

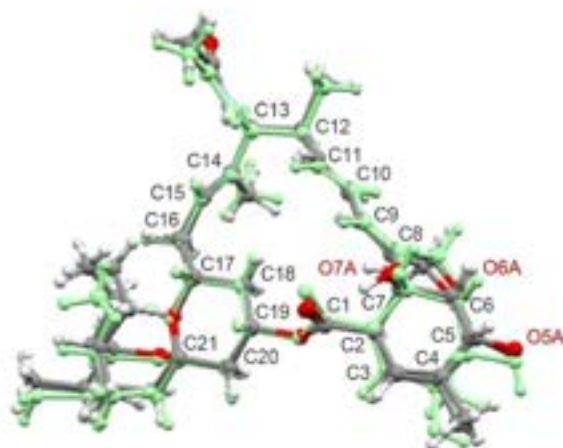


Fig. 4. Molecules A and B of **6a** and molecule **7a** highlighted in green superimposed with respect to the 14-membered lactone ring (atom labels are provided).

7h, however, exhibited substantially higher antitrypanosomal activity than any of the other derivatives, with GI_{50} values in the mid-nanomolar range (Table 1). Importantly, the trypanocidal activity of both **6h** and **7h** was within the potency range of suramin and ethidium bromide (commercially known as benznidazole) (Table 1), two drugs used in the treatment of sleeping sickness and nagana disease, respectively. All the derivatives with weak antitrypanosomal activity exhibited similar cytotoxicity against HL-60 cells with GI_{50} values ranging between 30 and 35 μ M (Table 1). By comparison, compounds **6h** and **7h** were about 10 times more cytotoxic (Table 1). It follows that the selectivity indices of all other derivatives were <11 whereas those of **6h** and **7h** were 48 and 67, respectively (Table 1). Therefore, **6h** and **7h** have a similar trypanocidal and selectivity profile as the anti-nagana drug ethidium bromide. Although **6h** and **7h** display similar trypanocidal activity to suramin, the anti-sleeping sickness drug is nonetheless superior as it is non-toxic to human HL-60 cells.

2.3.2. Antiplasmodial activity

To further evaluate the antiparasitic potential of IVR derivatives, all four parent compounds **1**, **2**, **8**, and **9**, and two corresponding derivatives **6a** and **7a**, which have been obtained in crystalline form, were screened for antiplasmodial activity against the hepatic infectious stage

of *P. berghei* in vitro. To this end, Huh7 cells, a human hepatoma cell line, were infected with luciferase-expressing *P. berghei* parasites in the presence of a range of seven concentrations of the six compounds (Fig. 5). Assessment of the cell confluency revealed that none of the compound concentrations employed was toxic to the host cells, as the cell confluency values obtained were similar to that of the cells exposed to the drug vehicle (DMSO) alone. Infection values were then used to determine the half-maximal inhibitory concentration (IC_{50}) values of each compound (Table 2).

Compound **6a** was less active than the parent compound **1**. The corresponding rearranged IVR molecule **8** exhibited also lower activity. On the other hand, this modification resulted in an improved antiplasmodial activity of compounds **9** and **7a**, when compared to that of compounds **2** and **6a**, respectively. Overall, the double modification in **7a** yielded the highest activity against *P. berghei* grown in Huh7 cells, with an IC_{50} value of 0.38 μ M for **7a**, which was about 2.5 times lower than that of IVR (IC_{50} = 0.91 μ M).

2.3.3. Structure-activity relationship considerations

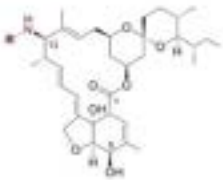
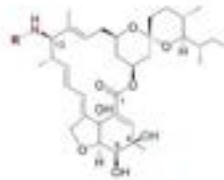
For the newly synthesized IVR derivatives, no consistent SAR was observed. While the rearranged parent compounds **8** and **9** displayed lower trypanocidal activity than the native parent compounds **1** and **2**, such a trend was only confirmed for the compound pair **1/8** for the plasmodicidal activity (Tables 1 and 2). Moreover, the antitrypanosomal activity of the derivative series **6** and **7** gives also an inconsistent picture – while some series **7** derivatives are less trypanocidal than their series **6** counterparts, other series **7** derivatives display higher trypanocidal activity than their series **6** correspondents (Table 1). Interestingly, doubly modified derivative **7a** was found to be 4 times more plasmodicidal than the corresponding **6a** derivative (Table 2).

From the SAR analysis, it can be concluded that the increased trypanocidal activity of **6h** and **7h** can be attributed to the chlorine atom of the 2-chloroacetamide group. This seems obvious as the structurally related derivatives with an acetamide (compounds **6a** and **7a**), a propanamide (compounds **6b** and **7b**), and an isobutyramide (compounds **6d** and **7d**) group were about 100 times less trypanocidal than **6h** and **7h** (Table 1). The increased trypanocidal activity of **6h** and **7h** may be due to the greater electronegativity (EN) of the $-CH_2Cl$ group (EN = 2.47 [45]) compared to that of the $-CH_3$, $-CH_2CH_3$, and $-CH(CH_3)_2$ groups, which all have similar EN values (EN = 2.27, 2.28, and 2.28, respectively [45]). Therefore, it would be interesting to see whether a more electronegative group (e.g., $-CF_3$; EN = 3.46 [45]) can further increase the trypanocidal activity of C13- α -amide derivatives of IVR.

3. Conclusions

Synthesis of a series of novel C13- α -amide derivatives of native or

Table 1
GI₅₀ values and ratios of ivermectin derivatives for *T. b. brucei* and HL-60 cells.

No.	R or compound	<i>T. b. brucei</i> GI ₅₀ (μM) ^a	HL-60 GI ₅₀ (μM) ^a	Selectivity GI ₅₀ ratio ^b	No.	<i>T. b. brucei</i> GI ₅₀ (μM) ^a	HL-60 GI ₅₀ (μM) ^a	Selectivity GI ₅₀ ratio ^b
Native oxahydrindene ring					Rearranged oxahydrindene ring			
1	ivermectin	14.6 ± 6.4	31.3 ± 1.8	2.1	8	20.0 ± 3.8	32.0 ± 0.7	1.6
2	splyoxate	6.1 ± 3.5	35.2 ± 1.9	5.8	9	12.1 ± 1.9	31.5 ± 1.7	2.6
C13-epi-amides of native ivermectin					C13-epi-amides of rearranged ivermectin			
								
6a		5.5 ± 2.4	32.0 ± 1.9	5.8	7a	7.5 ± 1.1	33.8 ± 1.0	4.5
6b		7.4 ± 5.1	32.1 ± 0.5	4.3	7b	8.1 ± 3.6	35.2 ± 2.0	4.3
6c		9.3 ± 5.2	30.0 ± 1.7	3.2	7c	4.1 ± 0.6	32.7 ± 1.8	7.8
6d		10.2 ± 7.7	33.8 ± 1.7	3.3	7d	6.6 ± 1.9	33.5 ± 1.3	5.1
6e		8.6 ± 5.0	30.8 ± 1.6	3.6	7e	4.3 ± 0.6	30.2 ± 1.2	7.0
6f		3.9 ± 0.8	32.0 ± 3.0	8.2	7f	4.1 ± 0.7	32.6 ± 2.8	8.0
6g		5.8 ± 3.3	32.2 ± 2.7	5.6	7g	5.0 ± 1.1	32.1 ± 1.5	6.4
6h		0.063 ± 0.009	3.0 ± 0.2	47.6	7h	0.064 ± 0.0033	4.3 ± 1.6	67.2
6i		14.4 ± 6.3	33.5 ± 0.7	2.3	7i	18.2 ± 0.4	32.1 ± 1.0	1.8
6j		19.1 ± 7.6	31.2 ± 2.2	1.6	7j	3.0 ± 0.1	30.8 ± 0.4	10.3
6k		10.2 ± 5.1	31.6 ± 1.6	3.1	7k	4.7 ± 0.4	32.8 ± 0.8	7.0
suramin ^c		0.036 ± 0.015	>100	>2778				
ethidium bromide ^c		0.086 ± 0.014	5.8 ± 0.7	67.4				

^a Data shown are mean values ± SD of three independent experiments.

^b GI₅₀ ratio = GI₅₀(HL-60)/GI₅₀(*T. b. brucei*).

^c Reference controls.

rearranged ivermectin (IVR) is described. The practical as well as scalable synthetic approach provides convenient access to material suitable for further development of this class of compounds that are interesting from both medicinal chemistry and cell biology points of view. Of the newly synthesized derivatives, two compounds (6h and 7h) displayed promising antiparasitic activity against *T. b. brucei*. In particular, the antitrypanosomal activity of 6h and 7h is intriguing as their trypanocidal activity and selectivity profile is similar to that of the anti-nagana

drug ethidium bromide. It would be beneficial if the potential carcinogenic ethidium bromide could be replaced by the C13-epi-2-chloroacetamides of either native (compound 6h) or rearranged IVR (compound 7h) for the treatment of animal trypanosomiasis. However, before the compounds can be used as anti-nagana drugs, they would need to be tested in animal models for their *in vivo* efficacy. Given the close relationship between *Leishmania* spp. and *Trypanosoma* spp., it would be interesting to test the IVR derivatives also on those parasites.

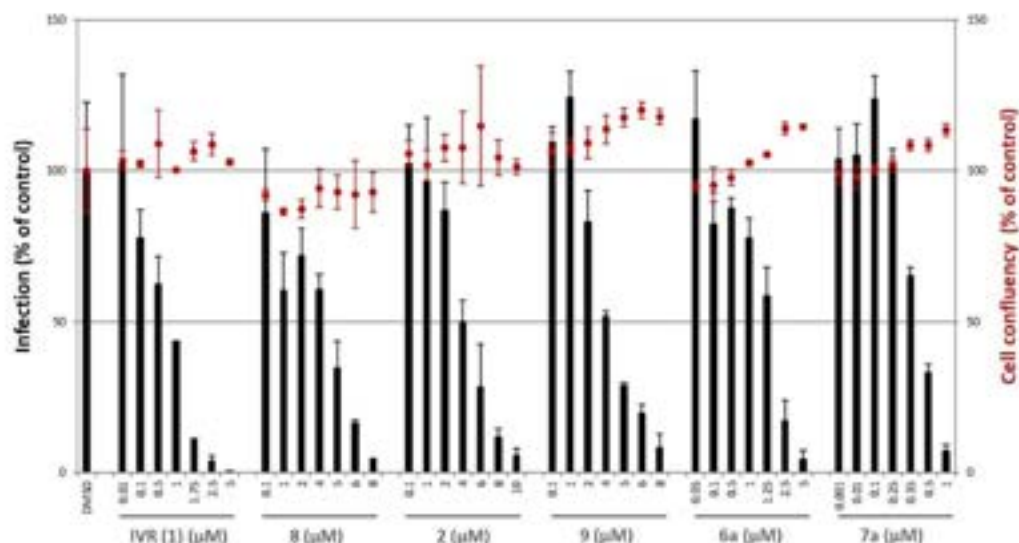


Fig. 5. Dose-dependent response of ivermectin 1, its aglycone 2, and derivatives 6a, 7a, 8, and 9, against the hepatic stage of *P. berghii*. Total parasite load (infection scale, bars) and cell viability (cell confluency scale, dots) are shown. Calculated IC_{50} values are presented in Table 2.

Table 2
IC₅₀ values of selected compounds against the hepatic stage of *P. berghei*

Native oxalhydrindene ring		Rearranged oxalhydrindene ring	
No.	K_{eq} (μM) ^a	No.	K_{eq} (μM) ^a
ivermectin 1	0.911 $\pm$ 0.029	8	4.721 $\pm$ 0.266
splygrose 2	5.452 $\pm$ 1.637	9	2.852 $\pm$ 1.008
6a	1.523 $\pm$ 0.576	7a	0.379 $\pm$ 0.062

* Data shown are mean values $\pm$ SD of two or three independent experiments.

On the other hand, compound **7a** showed better *in vitro* antiparasitic activity against *P. berghei* than **IVR**. The findings of this study suggest that doubly modified **IVR** derivatives may be suitable lead compounds for the rational development of new and effective antiparasitic drug candidates.

4. Experimental

4.1. General procedures

Ivermectin (IVR, form B1a) was purchased from Trimen Chemicals S. A. All reagents, except Dess-Martin periodinane (DMP), which was freshly prepared according to the protocol published by Frigerio et al. [46], were commercially available and purchased from two sources – Merck or Trimen Chemicals S.A., as well as used without further purification. Compounds **2–5** were resynthesized according to protocols published previously by other authors [20,38,39]. A detailed description of the general procedures, measurement parameters, and equipment can be found in the Supplementary material.

4.2. Synthesis of ivermectin aglycone (compound 2)

IVR (4.95 g, 5.66 mmol, 1.0 equiv.) was dissolved in methanol (79.2 mL) and the solution was cooled in an ice bath. Then, concentrated sulfuric acid (0.8 mL) was added dropwise to the reaction mixture. The solution changed its color to greenish and after a few hours to yellow. After 20 h, the reaction mixture was concentrated on a rotary evaporator, diluted with CH_2Cl_2 , and extracted with an aqueous solution of sodium carbonate (0.1 M). The collected organic layers were

subsequently concentrated *in vacuo*. Purification on silica gel using the CombiFlash system (0%–30% EtOAc/CHCl₃) gave the pure aglycone 2 as a clear oil. After twice evaporation to dryness with *n*-pentane, the oily product was completely converted into a white amorphous solid (2.79 g, 84% yield). The spectroscopic data were in agreement with previously published data [28,39].

4.3. Protection of the C5 hydroxyl group of ivermectin aglycone (compound 3)

To a stirred solution of **2** (2.00 g, 3.41 mmol, 1.0 equiv.) in anhydrous CH_2Cl_2 (40 mL), imidazole (2.32 g, 34.07 mmol, 10.0 equiv.) was added. After the imidazole had dissolved, *tert*-butyldimethylsilyl chloride (1.13 g, 7.50 mmol, 2.2 equiv.) was added in one portion. The reaction mixture was stirred at room temperature for 24 h. After this time, the reaction mixture was concentrated on a rotary evaporator to dryness. Purification on silica gel using the Combiflash system (0%–10% $\text{EtOAc}/\text{CHCl}_3$) gave the pure product **3** as a clear oil. After twice evaporation to dryness with *n*-pentane, the oily product was completely converted into a yellow amorphous solid (2.17 g, 91% yield). The spectroscopic data were in agreement with previously published data [28,39].

4.4. Synthesis of C13 ketone of CS-protected ivermectin aglycone (compound 4)

To a stirred solution of **3** (4.90 g, 6.99 mmol, 1.0 equiv.) in anhydrous CH_2Cl_2 (60 mL), *tert*-butyl alcohol (517 mg, 6.99 mmol, 1.0 equiv.) was added. Next, Dess-Martin periodinane (freshly prepared) (4.45 g, 10.49 mmol, 1.5 equiv.) was added to the reaction mixture and stirring was continued for 24 h. The reaction mixture was then diluted with CH_2Cl_2 and extracted with an aqueous solution of sodium thiosulfate (0.5 M). The collected organic layers were subsequently concentrated *in vacuo*. Purification on silica gel using the CombiFlash system (0%–5% $\text{EtOAc}/\text{CHCl}_3$) gave the pure ketone **4** as a clear oil. After twice evaporation to dryness with *n*-pentane, the oily product was completely converted into a white amorphous solid (3.43 g, 82% yield). The spectroscopic data were in agreement with previously published data [39].

4.5. Synthesis of the C13-*epi*-amine of C5-protected ivermectin aglycone (compound 5)

According to Ref. [30], to a stirred solution of 4 (3.43 g, 4.91 mmol, 1.0 equiv.) in methanol (40 mL), ammonium acetate (3.78 g, 49.1 mmol, 10.0 equiv.) was added. The reaction mixture was then heated at 60 °C for an hour. After this time, sodium cyanoborohydride (610 mg, 9.02 mmol, 2.0 equiv.) dissolved in methanol (5 mL) was added dropwise to the reaction mixture. After 24 h, the reaction mixture was concentrated on a rotary evaporator, diluted with CH₂Cl₂, and extracted with an aqueous solution of sodium carbonate (0.1 M). The collected organic layers were subsequently concentrated *in vacuo*. Purification on silica gel using the CombiFlash system (0 %–100 % EtOAc/CHCl₃) gave the pure product 5 as a clear oil. After twice evaporation to dryness with *n*-pentane, the oily product was completely converted into a yellow amorphous solid (1.75 g, 51 % yield).

4.6. General procedure for preparation of C13-*epi*-amide derivatives of native ivermectin (analogi 6a–6e and 6h–6k)

Precursor 5 (250 mg, 0.36 mmol, 1.0 equiv.) was dissolved in CH₂Cl₂ (5 mL) and triethylamine (108 mg, 1.07 mmol, 3.0 equiv.) was added. Next, the respective acyl chloride (0.43 mmol, 1.2 equiv.) was added to the reaction mixture, and stirring was continued at room temperature for 24 h. After this time, the reaction mixture was diluted with CH₂Cl₂ and extracted with an aqueous solution of sulfuric acid (pH = 1) and then with water. The collected organic layers were subsequently concentrated *in vacuo*. Purification on silica gel using the CombiFlash system (0 %–100 % EtOAc/CHCl₃) gave the C13-*epi*-amide products as clear oils.

To remove the C5-protecting group, the appropriate C13-*epi*-amide product was dissolved in methanol (10 mL) and *para*-toluenesulfonic acid hydrate (80 mg, 0.46 mmol, 1.3 equiv.) was added. After 2 h, the organic solvent was evaporated, diluted with CH₂Cl₂, and extracted with an aqueous solution of sodium carbonate (0.1 M). Next, the collected organic layers were subsequently concentrated *in vacuo*. Purification on silica gel using the CombiFlash system (0 %–100 % EtOAc/CHCl₃) gave the pure products 6a–6e and 6h–6k as clear oils. After twice evaporation to dryness with *n*-pentane, the oily products were completely converted into white amorphous solids.

C13-*epi*-acetamide of ivermectin 6a: 65 mg, 29 % yield (over two steps). Isolated as a white amorphous solid, a single spot by TLC. UV-active and strains green with PMA; ¹H NMR (400 MHz, CDCl₃) δ 5.83–5.72 (m, 2H), 5.68 (d, *J* = 9.6 Hz, 1H), 5.42–5.32 (m, 2H), 5.22 (t, *J* = 11.2, 4.8 Hz, 1H), 4.91 (dd, *J* = 10.5, 4.7 Hz, 1H), 4.61 (qd, *J* = 14.5, 1.4 Hz, 2H), 4.41 (d, *J* = 8.8 Hz, 1H), 4.25–4.20 (m, 1H), 3.89 (d, *J* = 6.2 Hz, 1H), 3.57–3.47 (m, 1H), 3.20 (dd, *J* = 4.6, 2.2 Hz, 1H), 3.11 (dd, *J* = 9.0, 1.3 Hz, 1H), 2.71 (ddd, *J* = 17.2, 6.7, 3.7 Hz, 1H), 2.28–2.13 (m, 2H), 2.05 (s, 2H), 1.90 (ddd, *J* = 12.2, 4.9, 1.5 Hz, 1H), 1.82–1.78 (m, 2H), 1.64–0.65 (m, 30H) ppm; ¹³C NMR (101 MHz, CDCl₃) δ 173.5, 169.8, 141.1, 137.8, 136.6, 135.6, 125.9, 119.6, 117.8, 117.4, 97.4, 80.2, 78.9, 77.1, 68.4, 68.2, 67.6, 67.0, 58.1, 45.5, 41.1, 38.1, 36.6, 35.7, 35.4, 34.4, 31.2, 27.9, 27.3, 23.3, 19.9, 19.0, 17.4, 15.6, 12.5, 11.8 ppm; HRMS (ESI⁺) *m/z*: [M+Na]⁺ Calcd for C₃₆H₅₃NNaO₂ 650.3669; Found 650.3686.

C13-*epi*-propionamide of ivermectin 6b: 73 mg, 32 % yield (over two steps). Isolated as a white amorphous solid, a single spot by TLC. UV-active and strains green with PMA; ¹H NMR (401 MHz, CDCl₃) δ 5.88–5.77 (m, 2H), 5.61 (d, *J* = 9.8 Hz, 1H), 5.47–5.36 (m, 2H), 5.27 (ddd, *J* = 16.4, 11.7, 4.9 Hz, 1H), 4.93 (dd, *J* = 10.0, 5.4 Hz, 1H), 4.66 (q, *J* = 14.4 Hz, 2H), 4.47 (d, *J* = 9.4 Hz, 1H), 4.27 (d, *J* = 6.0 Hz, 1H), 3.94 (d, *J* = 6.2 Hz, 1H), 3.56 (td, *J* = 9.9, 5.2 Hz, 1H), 3.25 (dd, *J* = 4.4, 2.1 Hz, 1H), 3.16 (d, *J* = 7.8 Hz, 1H), 2.81–2.71 (m, 1H), 2.37–2.15 (m, 5H), 2.03–0.66 (m, 34H) ppm; ¹³C NMR (101 MHz, CDCl₃) δ 173.5, 173.4, 141.1, 137.9, 136.8, 135.7, 125.8, 119.7, 117.8, 117.3, 97.4, 80.2, 79.0, 76.9, 68.5, 68.2, 67.6, 67.0, 57.6, 45.6, 41.2, 38.2, 36.6,

35.7, 35.4, 34.4, 31.2, 29.9, 27.9, 27.3, 19.9, 18.9, 17.4, 15.6, 12.4, 11.9, 10.1 ppm; HRMS (ESI⁺) *m/z*: [M+Na]⁺ Calcd for C₃₇H₅₅NNaO₂ 664.3820; Found 664.3855.

C13-*epi*-decanamide of ivermectin 6c: 79 mg, 30 % yield (over two steps). Isolated as a white amorphous solid, a single spot by TLC. UV-active and strains green with PMA; ¹H NMR (400 MHz, CDCl₃) δ 5.03–5.66 (m, 2H), 5.49 (d, *J* = 9.8 Hz, 1H), 5.41–5.29 (m, 2H), 5.29–5.17 (m, 1H), 4.88 (dd, *J* = 9.4, 6.0 Hz, 1H), 4.68–4.54 (m, 2H), 4.43 (d, *J* = 9.5 Hz, 1H), 4.22 (dd, *J* = 6.4, 0.9 Hz, 1H), 3.09 (d, *J* = 6.2 Hz, 1H), 3.55–3.46 (m, 1H), 3.20 (dt, *J* = 4.4, 1.6 Hz, 1H), 3.14–3.09 (m, 1H), 2.71 (t, *J* = 10.5, 6.7 Hz, 1H), 2.29–2.10 (m, 4H), 1.99–0.59 (m, 49H) ppm; ¹³C NMR (101 MHz, CDCl₃) δ 173.5, 172.8, 141.1, 137.9, 136.9, 135.7, 125.9, 119.6, 117.8, 117.2, 97.4, 80.2, 78.9, 68.4, 68.2, 67.6, 67.0, 57.7, 45.6, 41.2, 38.1, 37.1, 36.6, 35.7, 35.4, 34.4, 31.8, 31.2, 29.4, 29.34, 29.31, 29.2, 27.9, 27.3, 26.1, 22.6, 19.9, 19.0, 17.3, 15.6, 14.0, 12.5, 11.8 ppm, one signal overlapped; HRMS (ESI⁺) *m/z*: [M+Na]⁺ Calcd for C₄₆H₆₉NNaO₂ 762.4915; Found 762.4936.

C13-*epi*-isobutyramide of ivermectin 6d: 94 mg, 40 % yield (over two steps). Isolated as a white amorphous solid, a single spot by TLC. UV-active and strains green with PMA; ¹H NMR (400 MHz, CDCl₃) δ 5.84–5.70 (m, 2H), 5.51 (d, *J* = 9.7 Hz, 1H), 5.42–5.30 (m, 1H), 5.30–5.09 (m, 2H), 4.87 (dd, *J* = 9.0, 6.4 Hz, 2H), 4.61 (q, *J* = 14.4 Hz, 1H), 4.42 (d, *J* = 9.1 Hz, 2H), 4.22 (d, *J* = 5.5 Hz, 1H), 3.89 (d, *J* = 6.2 Hz, 1H), 3.76–3.63 (m, 1H), 3.55–3.46 (m, 1H), 3.21 (dd, *J* = 4.5, 2.2 Hz, 1H), 3.12 (dd, *J* = 9.3, 1.4 Hz, 1H), 2.72 (ddd, *J* = 13.7, 10.5, 6.7, 3.4 Hz, 1H), 2.44 (t, *J* = 11.2, 5.5 Hz, 1H), 2.27–2.11 (m, 2H), 1.91 (dd, *J* = 11.7, 4.5 Hz, 1H), 1.85–0.54 (m, 35H) ppm; ¹³C NMR (101 MHz, CDCl₃) δ 176.4, 173.5, 141.1, 137.9, 136.9, 135.6, 125.9, 119.6, 117.8, 117.1, 97.4, 80.3, 78.9, 76.7, 68.5, 68.2, 67.6, 67.0, 57.3, 45.5, 41.1, 38.2, 36.6, 36.0, 35.7, 35.4, 34.3, 31.2, 27.9, 27.3, 19.9, 19.0, 19.7, 18.9, 17.4, 15.6, 12.4, 11.9 ppm; HRMS (ESI⁺) *m/z*: [M+Na]⁺ Calcd for C₃₈H₅₇NNaO₂ 678.3976; Found 678.3996.

C13-*epi*-benzamide of ivermectin 6e: 69 mg, 28 % yield (over two steps). Isolated as a white amorphous solid, a single spot by TLC. UV-active and strains green with PMA; ¹H NMR (400 MHz, CDCl₃) δ 7.79–7.66 (m, 2H), 7.52–7.33 (m, 3H), 6.11 (d, *J* = 9.7 Hz, 1H), 5.89–5.69 (m, 2H), 5.47–5.39 (m, 1H), 5.37–5.30 (m, 1H), 5.30–5.11 (m, 2H), 5.01–4.94 (m, 1H), 4.70–4.56 (m, 3H), 4.27–4.20 (m, 1H), 3.88 (dd, *J* = 13.8, 5.6 Hz, 1H), 3.77–3.64 (m, 1H), 3.56–3.45 (m, 1H), 3.22 (td, *J* = 4.4, 2.1 Hz, 1H), 3.08 (dd, *J* = 9.2, 1.1 Hz, 1H), 2.82 (ddd, *J* = 17.2, 6.8, 3.9 Hz, 1H), 2.22 (t, *J* = 8.4 Hz, 2H), 1.98–0.40 (m, 30H) ppm; ¹³C NMR (101 MHz, CDCl₃) δ 173.6, 167.1, 141.3, 137.9, 136.8, 135.4, 134.5, 131.7, 128.7 (2C), 126.8 (2C), 126.1, 119.6, 117.8, 117.5, 97.4, 80.3, 78.9, 68.5, 68.3, 67.6, 67.0, 58.3, 45.6, 41.2, 38.5, 36.6, 35.7, 35.4, 34.4, 31.2, 29.7, 27.9, 27.3, 19.9, 19.1, 17.4, 15.7, 12.4, 11.9 ppm; HRMS (ESI⁺) *m/z*: [M+Na]⁺ Calcd for C₄₃H₅₅NNaO₂ 712.3820; Found 712.3844.

C13-*epi*-2-chloroacetamide of ivermectin 6h: 102 mg, 43 % yield (over two steps). Isolated as a white amorphous solid, a single spot by TLC. UV-active and strains green with PMA; ¹H NMR (401 MHz, CDCl₃) δ 6.72 (d, *J* = 10.0 Hz, 1H), 5.90–5.70 (m, 3H), 5.47–5.30 (m, 4H), 4.99–4.92 (m, 1H), 4.67 (qd, *J* = 14.5, 1.9 Hz, 3H), 4.47 (dd, *J* = 9.7, 2.1 Hz, 1H), 4.30–4.26 (m, 2H), 4.15 (d, *J* = 4.2 Hz, 2H), 4.12–4.02 (m, 2H), 3.97–3.93 (m, 2H), 3.75–3.70 (m, 1H), 3.62–3.58 (m, 1H), 3.29–3.24 (m, 2H), 3.20–3.08 (m, 2H), 2.85–2.76 (m, 1H), 2.46–0.46 (m, 24H) ppm; ¹³C NMR (101 MHz, CDCl₃) δ 173.5, 165.3, 141.5, 137.9, 136.1, 134.8, 126.3, 119.5, 117.9, 117.8, 97.5, 80.3, 79.0, 76.9, 68.5, 68.3, 67.6, 67.0, 58.2, 45.6, 42.9, 41.2, 38.4, 36.6, 35.7, 35.4, 34.3, 31.2, 28.0, 27.3, 19.9, 18.9, 17.4, 15.5, 12.4, 11.9 ppm; HRMS (ESI⁺) *m/z*: [M+Na]⁺ Calcd for C₃₈H₅₃ClNNaO₂ 684.3274, 686.3245; Found 684.3282, 686.3319.

C13-*epi*-2-methoxyacetamide of ivermectin 6i: 80 mg, 34 % yield (over two steps). Isolated as a white amorphous solid, a single spot by TLC. UV-active and strains green with PMA; ¹H NMR (400 MHz, CDCl₃) δ 6.63 (d, *J* = 10.2 Hz, 1H), 5.83–5.74 (m, 2H), 5.39–5.30 (m, 2H), 5.24 (td, *J* = 11.3, 5.6 Hz, 1H), 4.88 (dd, *J* = 9.2, 6.2 Hz, 1H), 4.61 (q, *J* = 13.8 Hz,

2H), 4.44 (d, $J = 9.8$ Hz, 1H), 4.22 (dd, $J = 5.9, 0.7$ Hz, 1H), 3.96–3.85 (m, 3H), 3.52 (dd, $J = 12.3, 6.7$ Hz, 1H), 3.45 (s, 3H), 3.21 (dd, $J = 4.5, 2.2$ Hz, 1H), 3.12 (dd, $J = 8.9, 1.2$ Hz, 1H), 2.72 (ddd, $J = 17.3, 6.8, 3.9$ Hz, 1H), 2.29–2.16 (m, 2H), 2.04–0.53 (m, 32H) ppm; ^{13}C NMR (101 MHz, CDCl_3) δ 173.5, 169.1, 141.0, 137.8, 136.4, 135.4, 125.9, 119.6, 117.8, 117.5, 97.4, 80.3, 78.9, 76.8, 71.8, 68.5, 68.2, 67.6, 67.0, 59.1, 57.0, 45.5, 41.1, 38.4, 36.5, 35.6, 35.3, 34.3, 31.1, 27.9, 27.3, 19.9, 18.9, 17.3, 15.6, 12.3, 11.9 ppm; HRMS (ESI^+) m/z : $[\text{M}+\text{Na}]^+$ Calcd for $\text{C}_{57}\text{H}_{105}\text{NNaO}_5$ 680.3769; Found 680.3794.

C13-*epi*-adamantane-1-carboxamide of ivermectin 6f: 102 mg, 38 % yield (over two steps). Isolated as a white amorphous solid, a single spot by TLC. UV-active and strains green with PMA; ^1H NMR (401 MHz, CDCl_3) δ 5.83–5.73 (m, 2H), 5.61 (d, $J = 9.7$ Hz, 1H), 5.37–5.34 (m, 1H), 5.28 (ddd, $J = 16.3, 11.2, 4.1$ Hz, 2H), 4.86–4.80 (m, 1H), 4.62 (qd, $J = 14.4, 1.2$ Hz, 2H), 4.41 (d, $J = 8.8$ Hz, 1H), 4.25–4.20 (m, 1H), 3.90 (d, $J = 6.2$ Hz, 1H), 3.53–3.44 (m, 1H), 3.24–3.20 (m, 1H), 3.13 (dd, $J = 9.0, 1.3$ Hz, 1H), 2.76–2.67 (m, 1H), 2.20 (t, $J = 8.7$ Hz, 2H), 2.05–1.97 (m, 4H), 1.96–0.58 (m, 43H) ppm; ^{13}C NMR (101 MHz, CDCl_3) δ 177.2, 173.6, 141.1, 138.0, 137.1, 135.7, 125.9, 119.7, 117.9, 117.1, 97.5, 80.4, 79.0, 76.5, 68.6, 68.3, 67.6, 67.1, 56.9, 45.6, 41.2, 41.1, 39.6 (3C), 38.4, 36.6, 36.5 (3C), 35.7, 35.4, 34.3, 31.2, 28.1 (3C), 28.0, 27.3, 19.9, 19.0, 17.4, 15.6, 12.3, 12.2 ppm; HRMS (ESI^+) m/z : $[\text{M}+\text{Na}]^+$ Calcd for $\text{C}_{45}\text{H}_{105}\text{NNaO}_5$ 770.4602; Found 770.4635.

C13-*epi*-furan-2-carboxamide of ivermectin 6k: 102 mg, 42 % yield (over two steps). Isolated as a white amorphous solid, a single spot by TLC. UV-active and strains green with PMA; ^1H NMR (401 MHz, CDCl_3) δ 7.46 (dd, $J = 1.8, 0.8$ Hz, 1H), 7.06 (dd, $J = 3.5, 0.8$ Hz, 1H), 6.48 (dd, $J = 3.5, 1.8$ Hz, 1H), 6.39 (d, $J = 10.1$ Hz, 1H), 5.85–5.72 (m, 2H), 5.45 (ddd, $J = 14.0, 10.4, 5.8$ Hz, 1H), 5.35 (s, 1H), 5.30–5.20 (m, 1H), 4.98 (t, $J = 7.8$ Hz, 1H), 4.69–4.55 (m, 3H), 4.25–4.20 (m, 1H), 3.90 (d, $J = 6.2$ Hz, 1H), 3.55–3.47 (m, 1H), 3.22 (dp, $J = 4.7, 2.4$ Hz, 1H), 3.11–3.05 (m, 1H), 2.83–2.74 (m, 1H), 2.24–2.16 (m, 2H), 2.03–0.52 (m, 32H) ppm; ^{13}C NMR (101 MHz, CDCl_3) δ 173.5, 157.9, 147.7, 143.8, 141.2, 137.8, 136.5, 135.4, 126.0, 119.6, 117.8, 117.7, 114.5, 112.3, 97.4, 80.3, 79.0, 76.9, 68.5, 68.2, 67.6, 67.0, 57.4, 45.6, 41.1, 38.5, 36.6, 35.6, 35.3, 34.3, 31.1, 27.9, 27.3, 19.0, 19.0, 17.3, 15.6, 12.3, 11.8 ppm; HRMS (ESI^+) m/z : $[\text{M}+\text{Na}]^+$ Calcd for $\text{C}_{59}\text{H}_{105}\text{NNaO}_5$ 702.3613; Found 702.3641.

4.7. General procedure for preparation of C13-*epi*-amide derivatives of native ivermectin (analogs 6f–6g)

To a stirred solution of the respective carboxylic acid (0.36 mmol, 1.0 equiv.) in DMF (10 mL), *N,N*-diisopropylethylamine (279 mg, 2.16 mmol, 6.0 equiv.) and 2-(7-*aza*-1*H*-benzotriazol-1-yl)-1,3,3-tetramethylurea hexafluorophosphate (HATU) (176 mg, 0.46 mmol, 1.3 equiv.) were added and the reaction mixture was stirred at room temperature for 15 min. Next, precursor 5 (250 mg, 0.36 mmol, 1.0 equiv.) was added and the stirring was continued for 24 h. After this time, the reaction mixture was diluted with CH_2Cl_2 and extracted with an aqueous solution of sulfuric acid (pH = 1) and then with water. The collected organic layers were subsequently concentrated *in vacuo*. Purification on silica gel using the CombiFlash system (0 %–100 % $\text{EtOAc}/\text{CHCl}_3$) gave the C13-*epi*-amide products as clear oils.

To remove the C5-protecting group, the corresponding C13-*epi*-amide product was dissolved in methanol (10 mL) and *p*-*tert*-toluenesulfonic acid hydrate (80 mg, 0.46 mmol, 1.3 equiv.) was added. After 2 h, the organic solvent was evaporated, diluted with CH_2Cl_2 and extracted with an aqueous solution of sodium carbonate (0.1 M). Next, the collected organic layers were subsequently concentrated *in vacuo*. Purification on silica gel using the CombiFlash system (0 %–100 % $\text{EtOAc}/\text{CHCl}_3$) gave the pure products 6f–6g as clear oils. After twice evaporation to dryness with *n*-pentane, the oily products were completely converted into white amorphous solids.

C13-*epi*-3-hydroxy-4-methoxybenzamide of ivermectin 6f: 53 mg, 20 % yield (over two steps). Isolated as a white amorphous solid, a single spot

by TLC. UV-active and strains green with PMA; ^1H NMR (400 MHz, CDCl_3) δ 7.76–7.64 (m, 1H), 7.32 (tt, $J = 5.5, 2.7$ Hz, 1H), 6.85 (ddd, $J = 11.6, 10.1, 6.3$ Hz, 1H), 6.09–5.99 (m, 1H), 5.06–5.71 (m, 2H), 5.49–5.39 (m, 1H), 5.35 (dd, $J = 3.5, 2.2$ Hz, 1H), 5.30–5.23 (m, 1H), 4.99–4.92 (m, 1H), 4.71–4.55 (m, 2H), 4.28–4.19 (m, 1H), 4.18–4.06 (m, 1H), 3.95–3.77 (m, 4H), 3.51 (ddd, $J = 12.6, 8.3, 6.8$ Hz, 1H), 3.21 (dt, $J = 5.2, 2.6$ Hz, 1H), 3.08 (d, $J = 8.9$ Hz, 1H), 2.85–2.76 (m, 1H), 2.38–2.28 (m, 1H), 2.25–2.11 (m, 2H), 2.01–0.61 (m, 32H) ppm; ^{13}C NMR (101 MHz, CDCl_3) δ 173.6, 166.6, 149.4, 145.6, 141.2, 137.9, 136.9, 135.6, 126.1, 119.7, 117.9, 117.5, 116.3, 113.0, 112.0, 110.2, 110.0, 97.4, 80.3, 78.9, 68.5, 68.3, 67.7, 67.1, 58.2, 56.1, 45.6, 41.2, 38.5, 36.6, 35.7, 35.4, 34.4, 31.2, 27.9, 27.3, 19.9, 19.1, 17.4, 15.7, 12.4, 11.8 ppm; HRMS (ESI^+) m/z : $[\text{M}+\text{Na}]^+$ Calcd for $\text{C}_{42}\text{H}_{127}\text{NNaO}_5$ 758.3875; Found 758.3890.

C13-*epi*-3,4-dimethoxybenzamide of ivermectin 6g: 86 mg, 32 % yield (over two steps). Isolated as a white amorphous solid, a single spot by TLC. UV-active and strains green with PMA; ^1H NMR (400 MHz, CDCl_3) δ 7.46 (d, $J = 2.0$ Hz, 1H), 7.32 (dd, $J = 8.3, 2.1$ Hz, 1H), 6.89 (dd, $J = 8.4, 2.5$ Hz, 1H), 6.13 (d, $J = 9.8$ Hz, 1H), 5.93–5.77 (m, 2H), 5.53–5.44 (m, 1H), 5.42–5.38 (m, 1H), 5.34–5.25 (m, 1H), 5.04–4.98 (m, 1H), 4.74–4.61 (m, 3H), 4.29–4.25 (m, 1H), 3.97–3.87 (m, 9H), 3.59–3.50 (m, 1H), 3.29–3.25 (m, 1H), 3.12 (dd, $J = 9.1, 1.6$ Hz, 1H), 2.91–2.80 (m, 1H), 2.31–2.20 (m, 2H), 2.05–0.56 (m, 30H) ppm; ^{13}C NMR (101 MHz, CDCl_3) δ 173.5, 166.6, 151.9, 149.2, 141.3, 137.9, 136.9, 135.5, 127.1, 126.1, 119.6, 118.6, 117.8, 117.4, 111.0, 110.2, 97.4, 80.3, 79.0, 76.9, 68.5, 68.2, 67.6, 67.0, 58.3, 56.02, 55.99, 45.6, 41.2, 38.5, 36.6, 35.7, 35.4, 34.3, 31.1, 27.9, 27.3, 19.9, 19.1, 17.3, 15.6, 12.4, 11.8 ppm; HRMS (ESI^+) m/z : $[\text{M}+\text{Na}]^+$ Calcd for $\text{C}_{43}\text{H}_{129}\text{NNaO}_5$ 772.4031; Found 772.4055.

4.8. General procedure for preparation of C13-*epi*-amide derivatives of rearranged ivermectin (analogs 7a–7k)

The corresponding C13-*epi*-amide of IVR with protected C5 hydroxyl group (1.0 equiv.) (please see section 4.6 and 4.7 for synthetic details) was dissolved in anhydrous THF. Next, tetra-*n*-butylammonium fluoride (TBAF) (3.0 equiv.) was added in one portion and the reaction mixture was stirred at room temperature for 3 days. After this time, the solvent was evaporated under reduced pressure. Purification on silica gel using the CombiFlash system (0 %–100 % $\text{EtOAc}/\text{CHCl}_3$) gave the pure products 7a–7k as clear oils. After twice evaporation to dryness with *n*-pentane, the oily products were completely converted into white amorphous solids.

C13-*epi*-acetamide of rearranged ivermectin 7a: 37 mg, 16 % yield (over two steps). Isolated as a white amorphous solid, a single spot by TLC. UV-active and strains green with PMA; ^1H NMR (400 MHz, CDCl_3) δ 6.19 (s, 1H), 6.11 (dt, $J = 4.5, 1.9$ Hz, 1H), 5.80 (dd, $J = 14.5, 11.2$ Hz, 1H), 5.65 (d, $J = 9.9$ Hz, 1H), 5.46 (dd, $J = 14.5, 10.6$ Hz, 1H), 5.29–5.22 (m, 1H), 4.92 (ddd, $J = 11.3, 2.3, 1.2$ Hz, 1H), 4.77 (s, 1H), 4.52 (ddd, $J = 22.5, 14.3, 2.2$ Hz, 2H), 4.45 (s, 1H), 4.10 (dd, $J = 7.7, 2.5$ Hz, 2H), 3.61 (tdd, $J = 11.2, 4.3, 2.1$ Hz, 1H), 3.18 (dd, $J = 9.1, 1.5$ Hz, 1H), 2.74 (ddd, $J = 17.1, 6.7, 3.7$ Hz, 1H), 2.43–0.58 (m, 37H) ppm; ^{13}C NMR (101 MHz, CDCl_3) δ 169.9, 168.6, 139.8, 139.1, 136.3, 129.4, 126.6, 122.7, 117.4, 97.4, 82.6, 78.1, 77.2, 73.5, 71.7, 69.2, 68.0, 66.9, 58.1, 40.4, 38.0, 36.5, 35.8, 35.4, 34.7, 31.2, 29.7, 27.9, 27.4, 23.4, 22.8, 18.9, 17.4, 15.5, 12.6, 11.8 ppm; HRMS (ESI^+) m/z : $[\text{M}+\text{Na}]^+$ Calcd for $\text{C}_{36}\text{H}_{103}\text{NNaO}_4$ 666.3613; Found 666.3634.

C13-*epi*-propionamide of rearranged ivermectin 7b: 47 mg, 20 % yield (over two steps). Isolated as a white amorphous solid, a single spot by TLC. UV-active and strains green with PMA; ^1H NMR (401 MHz, CDCl_3) δ 6.17 (s, 1H), 6.10 (dt, $J = 11.0, 2.3$ Hz, 1H), 5.79 (dd, $J = 14.5, 11.2$ Hz, 1H), 5.68 (d, $J = 9.9$ Hz, 1H), 5.45 (dd, $J = 14.5, 10.6$ Hz, 1H), 5.30–5.20 (m, 1H), 4.92–4.85 (m, 1H), 4.77 (s, 1H), 4.57–4.41 (m, 3H), 4.09 (s, 2H), 3.63–3.54 (m, 1H), 3.20–3.06 (m, 2H), 2.78–2.68 (m, 1H), 2.48–0.58 (m, 38H) ppm; ^{13}C NMR (101 MHz, CDCl_3) δ 173.7, 168.6, 139.9, 139.3, 136.4, 136.3, 129.3, 126.5, 122.6, 117.3, 97.3, 82.7, 78.0,

73.4, 71.6, 69.1, 67.9, 66.9, 57.8, 40.4, 38.1, 36.5, 35.8, 35.4, 34.6, 31.2, 29.9, 29.6, 27.9, 27.3, 22.8, 18.8, 17.4, 15.4, 12.4, 11.9, 10.2 ppm; HRMS (ESI⁺) m/z [M+Na]⁺ Calcd for C₂₇H₅₃NNaO₄⁺ 680.3769; Found 680.3785.

C13-*epi*-decanamide of rearranged ivermectin 7c: 65 mg, 24 % yield (over two steps). Isolated as a white amorphous solid, a single spot by TLC. UV-active and strains green with PMA; ¹H NMR (401 MHz, CDCl₃) δ 6.19 (s, 1H), 6.12 (dt, $J = 3.1, 2.2$ Hz, 1H), 5.01 (dd, $J = 14.6, 11.2$ Hz, 1H), 5.54 (d, $J = 9.5$ Hz, 1H), 5.44 (dd, $J = 14.6, 10.5$ Hz, 1H), 5.33–5.22 (m, 1H), 4.90 (dd, $J = 10.0, 3.4$ Hz, 1H), 4.75 (s, 1H), 4.53 (ddd, $J = 14.1, 12.9, 2.7$ Hz, 3H), 4.48–4.44 (m, 1H), 4.11 (s, 1H), 3.60 (td, $J = 10.4, 4.1$ Hz, 1H), 3.20–3.12 (m, 1H), 2.79–2.70 (m, 1H), 2.40–0.40 (m, 53H) ppm; ¹³C NMR (101 MHz, CDCl₃) δ 173.2, 169.0, 140.3, 139.4, 136.9, 136.6, 129.8, 126.9, 123.0, 117.6, 97.7, 82.9, 78.4, 77.5, 73.9, 72.0, 69.5, 68.3, 67.3, 58.1, 40.8, 38.4, 37.5, 36.8, 36.1, 35.8, 35.0, 32.1, 31.6, 29.8, 29.72, 29.67, 29.6, 28.3, 27.7, 26.4, 23.2, 23.0, 19.2, 17.7, 15.8, 14.4, 12.9, 12.1 ppm; HRMS (ESI⁺) m/z [M+Na]⁺ Calcd for C₄₄H₈₉NNaO₄⁺ 778.4865; Found 778.4876.

C13-*epi*-isobutyramide of rearranged ivermectin 7d: 65 mg, 27 % yield (over two steps). Isolated as a white amorphous solid, a single spot by TLC. UV-active and strains green with PMA; ¹H NMR (400 MHz, CDCl₃) δ 6.11 (s, $J = 9.9$ Hz, 1H), 6.03 (dt, $J = 11.0, 2.3$ Hz, 1H), 5.72 (dd, $J = 14.5, 11.2$ Hz, 1H), 5.56 (d, $J = 9.8$ Hz, 1H), 5.37 (dd, $J = 14.6, 10.6$ Hz, 1H), 5.20 (ddd, $J = 16.3, 11.2, 4.9$ Hz, 1H), 4.85–4.76 (m, 1H), 4.60 (s, 1H), 4.44 (ddd, $J = 23.0, 14.5, 2.4$ Hz, 2H), 4.37 (d, $J = 9.8$ Hz, 1H), 4.08–3.97 (m, 2H), 3.52 (ddd, $J = 10.8, 4.7, 2.2$ Hz, 1H), 3.10 (d, $J = 7.9$ Hz, 1H), 2.66 (tt, $J = 10.5, 6.7$ Hz, 1H), 2.43 (tt, $J = 13.7, 6.8$ Hz, 1H), 2.34–0.35 (m, 40H) ppm; ¹³C NMR (101 MHz, CDCl₃) δ 176.8, 168.6, 139.9, 139.2, 136.5, 136.2, 129.3, 126.6, 122.6, 117.2, 97.4, 82.6, 78.0, 76.8, 73.4, 71.6, 69.1, 67.9, 66.9, 57.4, 40.3, 38.2, 36.5, 36.0, 35.7, 35.4, 34.6, 31.2, 27.9, 27.3, 22.8, 19.9, 19.7, 18.8, 17.4, 15.4, 12.4, 11.9 ppm; HRMS (ESI⁺) m/z [M+Na]⁺ Calcd for C₃₉H₆₇NNaO₄⁺ 694.3926; Found 694.3918.

C13-*epi*-benzamide of rearranged ivermectin 7e: 40 mg, 16 % yield (over two steps). Isolated as a white amorphous solid, a single spot by TLC. UV-active and strains green with PMA; ¹H NMR (400 MHz, CDCl₃) δ 7.85–7.70 (m, 2H), 7.53–7.35 (m, 3H), 6.19–6.07 (m, 2H), 5.85–5.74 (m, 1H), 5.48 (dd, $J = 14.6, 10.6$ Hz, 1H), 5.29–5.19 (m, 1H), 4.96–4.89 (m, 1H), 4.70 (s, 1H), 4.66–4.59 (m, 1H), 4.49 (qd, $J = 14.3, 2.3$ Hz, 2H), 4.09–4.00 (m, 1H), 3.57–3.47 (m, 1H), 3.08 (dd, $J = 9.3, 1.4$ Hz, 1H), 2.84–2.75 (m, 1H), 2.27–2.15 (m, 2H), 2.00–0.38 (m, 34H) ppm; ¹³C NMR (101 MHz, CDCl₃) δ 168.7, 167.3, 140.2, 139.0, 136.5, 136.0, 134.5, 131.7, 129.5, 128.7 (2C), 126.9 (2C), 126.8, 122.6, 117.5, 97.4, 82.6, 78.1, 73.6, 71.7, 69.2, 68.0, 66.9, 58.3, 40.4, 38.5, 36.6, 35.7, 35.4, 34.7, 31.2, 29.7, 27.9, 27.3, 22.9, 19.0, 17.4, 15.6, 12.4, 11.8 ppm; HRMS (ESI⁺) m/z [M+Na]⁺ Calcd for C₄₁H₅₉NNaO₄⁺ 728.3769; Found 728.3738.

C13-*epi*-3-hydroxy-4-methoxybenzamide of rearranged ivermectin 7f: 43 mg, 16 % yield (over two steps). Isolated as a white amorphous solid, a single spot by TLC. UV-active and strains green with PMA; ¹H NMR (400 MHz, CDCl₃) δ 7.74–7.61 (m, 1H), 7.35–7.26 (m, 1H), 6.87–6.76 (m, 1H), 6.13–6.00 (m, 2H), 5.76 (dd, $J = 14.4, 11.2$ Hz, 1H), 5.51–5.40 (m, 1H), 5.26–5.16 (m, 1H), 4.94–4.85 (m, 1H), 4.63–4.53 (m, 1H), 4.45 (ddd, $J = 16.5, 14.5, 2.3$ Hz, 2H), 4.08–4.00 (m, 2H), 3.92–3.72 (m, 4H), 3.55–3.45 (m, 1H), 3.10–3.02 (m, 1H), 2.95–2.84 (m, 1H), 2.80–2.70 (m, 1H), 2.27–2.00 (m, 2H), 1.99–0.47 (m, 33H) ppm; ¹³C NMR (101 MHz, CDCl₃) δ 168.6, 166.9, 149.5, 145.7, 140.1, 139.1, 136.5, 136.0, 129.4, 127.5, 126.8, 122.6, 119.5, 117.4, 113.2, 110.3, 97.3, 82.6, 78.1, 73.5, 71.7, 69.1, 68.0, 66.9, 58.3, 56.1, 40.4, 38.5, 36.5, 35.8, 35.4, 34.7, 31.2, 29.6, 27.9, 27.3, 22.8, 19.0, 17.4, 15.5, 12.4, 11.8 ppm; HRMS (ESI⁺) m/z [M+Na]⁺ Calcd for C₄₂H₅₇NNaO₅⁺ 774.3824; Found 774.3834.

C13-*epi*-3,4-dimethoxybenzamide of rearranged ivermectin 7g: 96 mg, 35 % yield (over two steps). Isolated as a white amorphous solid, a single spot by TLC. UV-active and strains green with PMA; ¹H NMR (400 MHz, CDCl₃) δ 7.39 (d, $J = 2.0$ Hz, 1H), 7.29 (dd, $J = 8.3, 2.0$ Hz, 1H),

6.88–6.83 (m, 1H), 6.10–6.02 (m, 2H), 5.77 (dd, $J = 14.5, 11.2$ Hz, 1H), 5.45 (dd, $J = 14.4, 10.6$ Hz, 1H), 5.21 (ddd, $J = 16.1, 11.1, 4.8$ Hz, 2H), 4.89 (dd, $J = 10.2, 4.7$ Hz, 1H), 4.59 (d, $J = 9.4$ Hz, 1H), 4.52–4.38 (m, 3H), 4.07–3.99 (m, 2H), 3.92–3.80 (m, 8H), 3.49 (dd, $J = 9.8, 5.1$ Hz, 1H), 3.12–3.01 (m, 1H), 2.81–2.71 (m, 1H), 2.26–2.13 (m, 2H), 2.00–0.53 (m, 30H) ppm; ¹³C NMR (101 MHz, CDCl₃) δ 168.7, 166.9, 151.9, 149.2, 140.1, 139.2, 136.6, 136.0, 129.3, 127.0, 126.8, 122.5, 118.8, 117.4, 110.9, 110.2, 97.3, 82.6, 78.1, 73.4, 71.6, 69.2, 67.9, 66.9, 58.4, 56.0 (2C), 40.4, 38.5, 36.5, 35.7, 35.4, 34.6, 31.2, 29.6, 27.9, 27.3, 22.8, 19.0, 17.3, 15.5, 12.4, 11.8 ppm; HRMS (ESI⁺) m/z [M+Na]⁺ Calcd for C₄₃H₅₉NNaO₅⁺ 788.3900; Found 788.4003.

C13-*epi*-2-chloroacetamide of rearranged ivermectin 7h: 75 mg, 31 % yield (over two steps). Isolated as a white amorphous solid, a single spot by TLC. UV-active and strains green with PMA; ¹H NMR (401 MHz, CDCl₃) δ 6.80–6.69 (m, 1H), 6.24–6.12 (m, 2H), 5.83 (dd, $J = 14.5, 11.2$ Hz, 1H), 5.44 (dd, $J = 14.8, 10.6$ Hz, 1H), 5.38–5.31 (m, 2H), 4.98–4.89 (m, 1H), 4.55 (qd, $J = 14.5, 2.9$ Hz, 2H), 4.49–4.44 (m, 1H), 4.17–4.05 (m, 6H), 3.79–3.71 (m, 1H), 3.68–3.58 (m, 1H), 3.23–3.10 (m, 2H), 2.82–2.75 (m, 1H), 2.45–0.61 (m, 30H) ppm; ¹³C NMR (101 MHz, CDCl₃) δ 168.7, 165.5, 140.4, 138.7, 135.8, 135.4, 129.6, 127.0, 122.5, 117.7, 97.4, 82.6, 73.7, 71.7, 69.2, 68.0, 66.9, 58.3, 53.4, 42.9, 40.4, 38.4, 36.6, 35.8, 35.5, 34.7, 31.2, 29.7, 28.0, 27.4, 22.9, 18.8, 17.4, 15.5, 12.5, 11.9 ppm; HRMS (ESI⁺) m/z [M+Na]⁺ Calcd for C₃₉H₆₃ClNNaO₄⁺ 700.3223, 702.3194; Found 700.3244, 702.3289.

C13-*epi*-2-methoxyacetamide of rearranged ivermectin 7i: 55 mg, 23 % yield (over two steps). Isolated as a white amorphous solid, a single spot by TLC. UV-active and strains green with PMA; ¹H NMR (401 MHz, CDCl₃) δ 6.70 (d, $J = 10.3$ Hz, 1H), 6.20–6.11 (m, 2H), 5.89 (dd, $J = 14.4, 11.3$ Hz, 1H), 5.44 (dd, $J = 14.3, 10.7$ Hz, 1H), 5.37–5.26 (m, 1H), 4.89 (dd, $J = 10.5, 3.5$ Hz, 1H), 4.76 (s, 1H), 4.60–4.46 (m, 3H), 4.10 (dd, $J = 14.2, 2.4$ Hz, 2H), 4.03–3.92 (m, 2H), 3.66–3.57 (m, 1H), 3.53 (s, 3H), 3.18 (d, $J = 8.1$ Hz, 1H), 2.75 (ddd, $J = 10.4, 6.9, 3.8$ Hz, 1H), 2.33–2.19 (m, 2H), 2.07–0.57 (m, 32H) ppm; ¹³C NMR (101 MHz, CDCl₃) δ 169.3, 168.7, 140.0, 139.0, 136.2, 136.0, 129.5, 126.6, 122.6, 117.5, 97.4, 82.7, 78.1, 77.0, 73.5, 71.9, 71.6, 69.2, 68.0, 66.9, 59.2, 57.1, 40.4, 38.4, 36.5, 35.8, 35.4, 34.7, 31.2, 28.0, 27.4, 22.8, 18.9, 17.4, 15.5, 12.4, 11.9 ppm; HRMS (ESI⁺) m/z [M+Na]⁺ Calcd for C₃₉H₆₅NNaO₅⁺ 696.3718; Found 696.3740.

C13-*epi*-adamantane-1-carboxamide of rearranged ivermectin 7j: 79 mg, 29 % yield (over two steps). Isolated as a white amorphous solid, a single spot by TLC. UV-active and strains green with PMA; ¹H NMR (400 MHz, CDCl₃) δ 6.13 (s, 1H), 6.10–6.05 (m, 1H), 5.75 (dd, $J = 14.5, 11.2$ Hz, 1H), 5.65 (d, $J = 9.7$ Hz, 1H), 5.34 (dd, $J = 14.5, 10.7$ Hz, 1H), 5.30–5.22 (m, 1H), 4.77 (dd, $J = 9.9, 4.6$ Hz, 1H), 4.70 (s, 1H), 4.53–4.41 (m, 2H), 4.39 (d, $J = 9.2$ Hz, 1H), 4.09–3.99 (m, 1H), 3.52 (td, $J = 9.8, 4.9$ Hz, 1H), 3.14 (dd, $J = 9.0, 1.3$ Hz, 1H), 2.72–2.65 (m, 1H), 2.33–0.47 (m, 50H) ppm; ¹³C NMR (101 MHz, CDCl₃) δ 177.6, 168.7, 140.1, 139.1, 136.8, 136.0, 129.3, 126.7, 122.4, 117.0, 97.4, 82.6, 78.1, 76.5, 71.6, 69.1, 68.0, 66.9, 57.0, 41.1, 40.3, 39.5 (3C), 38.3, 36.5, 36.4 (3C), 35.7, 35.3, 34.6, 31.2, 28.1 (3C), 28.03, 27.96, 27.2, 22.8, 18.8, 17.4, 15.4, 12.3, 12.1 ppm; HRMS (ESI⁺) m/z [M+Na]⁺ Calcd for C₆₂H₉₂NNaO₄⁺ 786.4552; Found 786.4571.

C13-*epi*-furan-2-carboxamide of rearranged ivermectin 7k: 65 mg, 26 % yield (over two steps). Isolated as a white amorphous solid, a single spot by TLC. UV-active and strains green with PMA; ¹H NMR (400 MHz, CDCl₃) δ 7.53–7.48 (m, 1H), 7.44–7.35 (m, 1H), 7.11–7.03 (m, 1H), 6.49 (dd, $J = 3.5, 1.8$ Hz, 1H), 6.46–6.39 (m, 1H), 6.16–6.05 (m, 2H), 5.79 (dd, $J = 14.5, 11.2$ Hz, 1H), 5.47 (dd, $J = 14.5, 10.6$ Hz, 1H), 5.30–5.20 (m, 1H), 4.93 (dd, $J = 9.7, 5.2$ Hz, 1H), 4.72 (s, 1H), 4.56 (d, $J = 10.3$ Hz, 1H), 4.48 (ddd, $J = 22.2, 14.2, 2.3$ Hz, 2H), 4.05 (dd, $J = 9.2, 2.5$ Hz, 2H), 3.59–3.50 (m, 1H), 3.12–3.05 (m, 1H), 2.80–2.72 (m, 1H), 2.38–0.49 (m, 33H) ppm; ¹³C NMR (101 MHz, CDCl₃) δ 168.7, 158.1, 147.6, 144.0, 140.0, 139.1, 136.2, 136.0, 129.4, 126.7, 122.6, 117.7, 114.7, 112.3, 97.4, 82.6, 78.1, 77.1, 73.5, 71.6, 69.2, 68.0, 66.9, 57.5, 40.4, 38.5, 36.5, 35.7, 35.4, 34.6, 31.2, 27.9, 27.4, 22.8, 18.9, 17.4, 15.5, 12.4, 11.8 ppm; HRMS (ESI⁺) m/z [M+Na]⁺ Calcd for

$C_{39}H_{53}NNaO_7$ 718.3562; Found 718.3581.

4.9. General procedure for preparation of rearranged ivermectin and its aglycone (analogs 8–9)

To a stirred solution of IVR or its aglycone **2** (1.0 equiv.) in anhydrous THF, tetra-*n*-butylammonium fluoride (TBAF) (3.0 equiv.) was added and the reaction mixture was stirred at room temperature for 3 days. After this time, the organic solvent was evaporated under reduced pressure. Purification on silica gel using the CombiFlash system (0 %–100 % EtOAc/CHCl₃) gave the pure products **8–9** as clear oils. After twice evaporation to dryness with *n*-pentane, the oily products were completely converted into white amorphous solids.

Rearranged ivermectin 8: 76 mg, 15 % yield. Isolated as a white amorphous solid, a single spot by TLC. UV-active and stains green with PMA; ¹H NMR (401 MHz, CDCl₃) δ 6.13 (q, *J* = 7.9 Hz, 2H), 5.85–5.58 (m, 2H), 5.41–5.26 (m, 2H), 4.90 (d, *J* = 10.0 Hz, 1H), 4.76 (d, *J* = 29.7 Hz, 2H), 4.49 (q, 2H), 4.06 (s, 2H), 3.89 (s, 1H), 3.84–3.58 (m, 5H), 3.51–3.31 (m, 11H), 3.17 (dt, *J* = 31.5, 9.0 Hz, 5H), 2.49–2.39 (m, 1H), 2.26 (dd, *J* = 38.3, 11.9 Hz, 5H), 1.96–0.50 (m, 33H) ppm; ¹³C NMR (101 MHz, CDCl₃) δ 168.9, 138.7, 138.5, 138.4, 134.7, 129.5, 125.4, 123.1, 118.0, 98.4, 97.4, 94.7, 82.8, 81.8, 80.3, 79.3, 78.2, 78.0, 76.6, 75.9, 73.2, 71.6, 69.2, 68.1, 67.10, 67.05, 56.5, 56.31, 56.25, 40.3, 39.6, 36.7, 35.7, 35.4, 34.4, 34.3, 34.1, 31.2, 28.0, 27.2, 22.6, 20.1, 18.3, 17.6, 17.4, 15.1, 12.4, 12.0 ppm; HRMS (ESI⁺) *m/z*: [M+Na]⁺ Calcd for C₃₈H₅₂NaO₇: 913.4920; Found 913.4926.

Rearranged ivermectin aglycone 9: 70 mg, 17 % yield. Isolated as a white amorphous solid, a single spot by TLC. UV-active and stains green with PMA; ¹H NMR (401 MHz, CDCl₃) δ 6.16–6.07 (m, 2H), 5.78–5.63 (m, 2H), 5.32–5.22 (m, 2H), 4.93 (s, 1H), 4.57–4.44 (m, 2H), 4.07 (s, 2H), 3.96 (s, 1H), 3.69 (td, *J* = 11.6, 4.8 Hz, 1H), 3.17 (d, *J* = 8.0 Hz, 1H), 2.48 (p, *J* = 7.4 Hz, 1H), 2.34–2.18 (m, 2H), 2.05–0.54 (m, 33H) ppm; ¹³C NMR (101 MHz, CDCl₃) δ 168.5, 138.5, 138.2, 137.5, 129.5, 125.5, 123.3, 117.1, 97.3, 82.8, 77.8, 77.0, 73.3, 71.6, 69.0, 68.1, 67.1, 40.4, 39.9, 36.6, 35.8, 35.5, 34.5, 31.2, 28.0, 27.4, 22.6, 19.1, 17.4, 14.5, 12.5, 11.7 ppm; two signals overlapped, HRMS (ESI⁺) *m/z*: [M+Na]⁺ Calcd for C₃₄H₅₀NaO₅: 625.3347; Found 625.3366.

4.10. X-ray measurements

For X-ray diffraction, suitable crystals of **6a** and **7a** were grown by slow evaporation of a saturated solution of the IVR derivatives in acetone at room temperature. Single-crystal X-ray diffraction (scXRD) data were collected using a four-circle Oxford-Diffraction SuperNova and Xcalibur both equipped with an EOS-CCD detector. The X-ray radiation source applied for the investigation of **6a** single-crystals was CuKα (λ = 1.54184 Å) while for **7a** single-crystals AgKα (λ = 0.56087 Å) radiation was used.

The collected data were processed and corrected for absorption with CrysAlisPro 171.42.59. The structures were solved by direct methods and refined by full-matrix least-squares on F² using the SHELXS and SHELXL with OLEX2 software package [47–49]. Anisotropic displacement factors were generally applied for non-hydrogen atoms. Hydrogen atoms in **6a** were placed at calculated positions and refined using a riding model, while in **7a** most of their positions (despite solvate molecule and terminal methyl groups: C2B, C12a, C24a, and C2B) were derived from difference Fourier maps. The *U*_{eq} values of the hydrogen atoms were constrained to 1.2 times the *U*_{eq} for carbon atoms and 1.5 for other carrier atoms. The MASK procedure was implemented in **6a** using OLEX2 program to remove the residual electron density located inside void volumes, resulting from the presence of disordered solvent molecules (presumably water molecules). Structural drawings were prepared using the program Mercury CSD 4.0 [50]. Atom numbers were adapted from Ref. [43].

The description of hydrogen bonds can be found in the Supplementary material. The H-bond patterns in molecules **6a** and **7a** are also

shown in the Supplementary material (Figure S50). The final crystal data are in Table S1 (Supplementary material) and have been deposited in the Cambridge Structural Database as supplementary publications CCDC 2294517 (compound **6a**) and 2294516 (compound **7a**). Copies of this information can be obtained free of charge from The Director, CCDC, 12 Union Road, Cambridge, CB2 1EZ, UK; E-mail: deposit@ccdc.cam.ac.uk or <http://www.ccdc.cam.ac.uk>.

4.11. In vitro biological studies

4.11.1. Trypanocidal and cytotoxic assays

Trypanosoma brucei brucei bloodstream form 221a [51] and human promyelocytic leukemia HL-60 cells [52] were employed for the determination of the trypanocidal and cytotoxic activity of test compounds. Cells were seeded at densities of 1×10^4 mL⁻¹ (*T. b. brucei*) and 5×10^4 mL⁻¹ (HL-60) in the presence of various concentrations of test compounds (tenfold dilutions from 100 μM to 1 nM) and 0.9 % DMSO in 200 μL of Raltz medium [53] supplemented with 16.7 % bovine serum in 96-well plates. Controls were incubated in the bovine serum-supplemented medium containing only 0.9 % DMSO. The cultures were incubated at 37 °C in a humidified atmosphere containing 5 % CO₂. After 24 h, 20 μL of a 0.5 mM resazurin solution in PBS was added and the incubation was continued for another 48 h. Then, the absorbance of the cultures was read on a BioTek ELx808 microplate reader using a test wavelength of 570 nm and a reference wavelength of 630 nm. The 50 % growth inhibition (GI₅₀) values (the concentration of a compound required to reduce the growth rate of cells by 50 % compared to the control) were calculated by linear interpolation according to the method described by Huber and Koella [54].

4.11.2. Hepatic stage antiparasitic activity

The antiparasitic activity of test compounds was assessed in vitro with *P. berghei*-infected human hepatoma cells (Huh7 cells), as previously described [27,28]. Briefly, Huh7 cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10 % (v/v) fetal calf serum (FCS), 1 % v/v penicillin/streptomycin, 1 % v/v glutamine, 1 % v/v non-essential amino acids and 10 mM HEPES, pH 7, and incubated at 37 °C and 5 % CO₂. Cells were seeded at 1×10^4 cells per well in a 96-well plate and incubated until the following day. Compound stock solutions were prepared at 10 mM in DMSO and serially diluted in medium (supplemented with 50 μg mL⁻¹ gentamicin and 0.8 μg mL⁻¹ amphotericin B) to achieve the desired test concentrations. As a control, DMSO was diluted in the same medium to mimic the highest percentage of the drug vehicle employed in the assay. One hour prior to infection, the culture medium was replaced by the compound solutions, and the cells were incubated as described above. Luciferase-expressing *P. berghei* sporozoites (PbGFP-Luciferase [55]), obtained from salivary glands dissected from infected female *Anopheles stephensi* mosquitoes, were added to the cells at a 1:1 ratio, and the plates were centrifuged at 1800 × g for 5 min at room temperature. At 46 h post-infection (hpi), the cell confluency was assessed by the AlamarBlue assay (Invitrogen, UK), according to the manufacturer's instructions. Finally, at 48 hpi, the parasite load was assessed in a bioluminescence assay (Firefly Luciferase Assay kit 2.0, Biotium, USA), according to the manufacturer's instructions. Nonlinear regression analysis was employed to fit the normalized results of the dose-response curves, and the half-maximal inhibitory concentration (IC₅₀) values were determined using GraphPad Prism 8.0 (GraphPad software, La Jolla California USA).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ejmech.2023.115951>.

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Supplementary material

Unexpected rearrangement of ivermectin in the synthesis of new derivatives with trypanocidal and antiplasmodial activities

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General procedures

All reagents, except Dess-Martin periodinane (DMP), were purchased from two sources – Merck or Trimen Chemicals S.A., and used without further purification. CDCl_3 spectral grade was stored over 3 Å molecular sieves for several days. Reaction mixtures were stirred using Teflon-coated magnetic stir bars and were monitored by thin-layer chromatography (TLC) using aluminum-backed plates (Merck 60 F₂₅₄). TLC plates were visualized by UV light (254 nm), followed by treatment with phosphomolybdic acid (PMA) (5% in absolute ethanol) and gentle heating. Products of the reactions were purified using CombiFlash® Rf+ Lumen Flash Chromatography System (Teledyne Isco) with integrated ELS and UV detectors. All solvents used in flash chromatography were of HPLC grade (Merck) and were used as received. Solvents were removed using a rotary evaporator.

NMR spectra were recorded on a Varian 400 (^1H NMR at 400 MHz and ^{13}C NMR at 101 MHz) magnetic resonance spectrometer. ^1H NMR spectra are reported in chemical shifts downfield from TMS using the respective residual solvent peak as the internal standard (CDCl_3 δ 7.26 ppm). ^1H NMR spectra are reported as follows: chemical shift (δ , ppm), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, p = quintet/pentet, dd = doublet of doublets, dt = doublet of triplets, dp = doublet of quintets/pentets, ddd = doublet of doublet of doublets, ddt = doublet of doublet of triplets, dtd = doublet of triplet of doublets, dddd = doublet of doublet of doublet of doublets, td = triplet of doublets, tdd = triplet of doublet of doublets, tt = triplet of triplets, qd = quartet of doublets, m = multiplet), coupling constant(s) in Hz, and integration. Significant peaks are reported within the overlapping ~2.00–0.50 ppm region of the ^1H NMR spectra. ^{13}C NMR spectra are reported in chemical shifts downfield from TMS using the respective residual solvent peak as the internal standard (CDCl_3 δ 77.16 ppm). Line broadening parameters were 0.5 or 1.0 Hz, while the error of chemical shift value was 0.1 ppm.

Electrospray ionization (ESI) high-resolution mass spectra were recorded on a QTOF (Impact HD, Bruker Daltonics) mass spectrometer in the positive ion detection mode. Samples were prepared in dry acetonitrile. The mass range for ESI experiments was from $m/z = 300$ to $m/z = 1000$.



Figure S1. Color change of the reaction mixture during the rearrangement of ivermectin. (A) The color of the reaction mixture changed from colorless to orange to red, indicating the progress of ivermectin rearrangement, while (B) the addition of hydrogen peroxide resulted in complete inhibition of the rearrangement and no color change. TLC control clearly confirmed these observations.

Spectroscopic and spectrometric analysis of newly synthesized compounds

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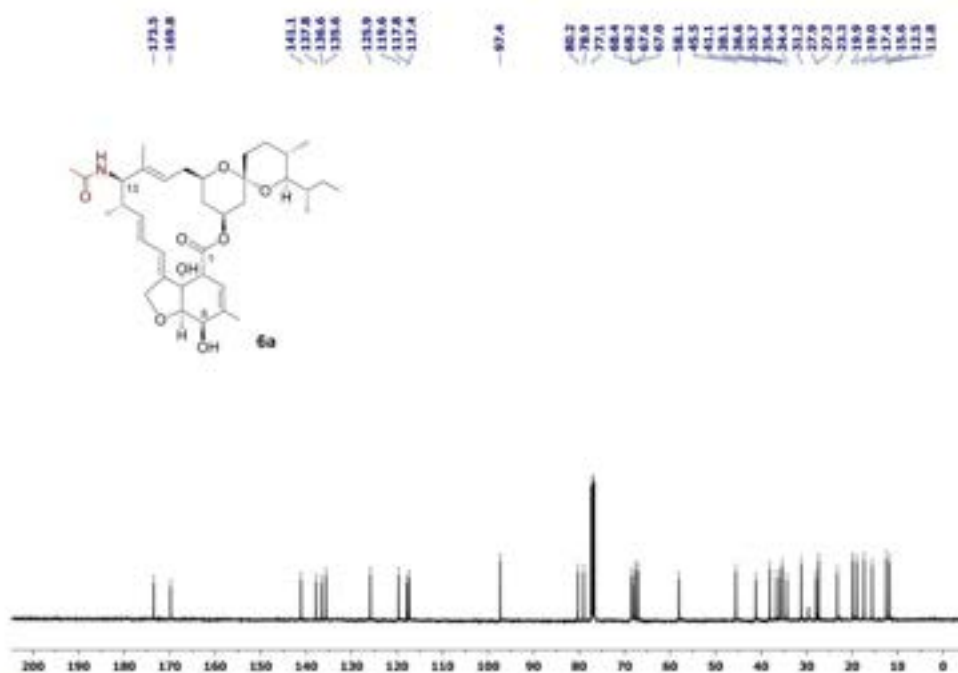


Figure S2. The ^{13}C NMR spectrum of **6a** in chloroform- d .

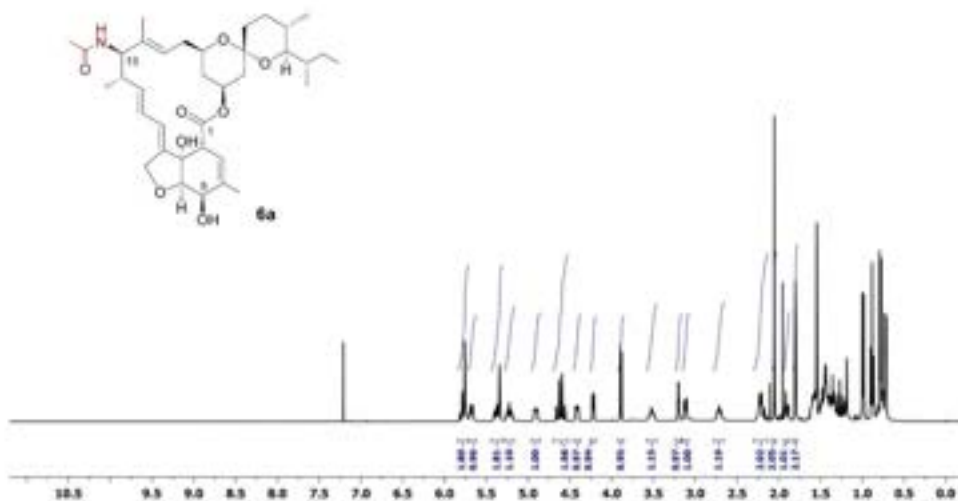


Figure S3. The ^1H NMR spectrum of **6a** in chloroform- d .

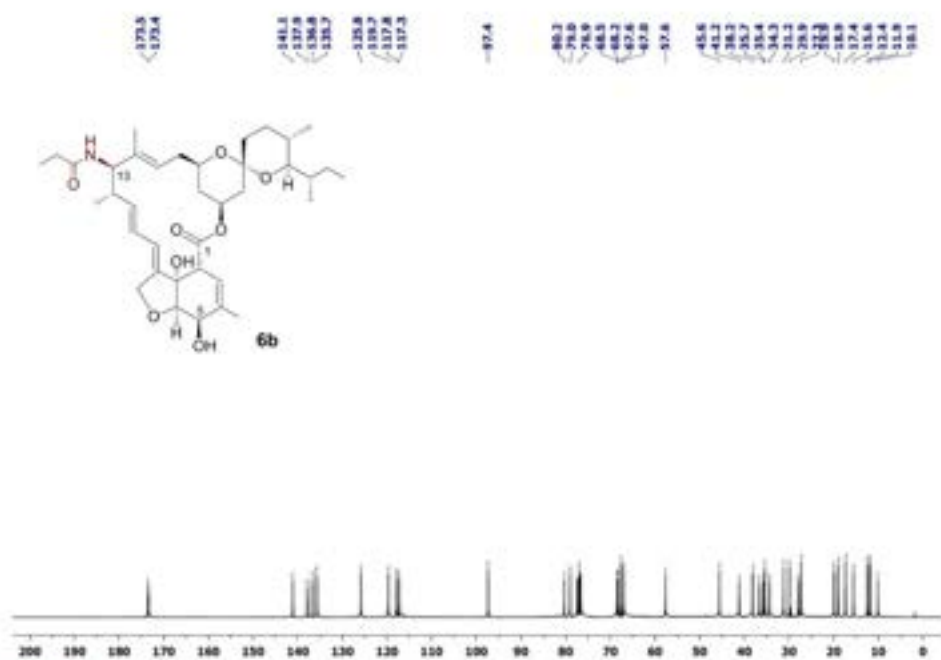


Figure S4. The ^{13}C NMR spectrum of **6b** in chloroform- d .

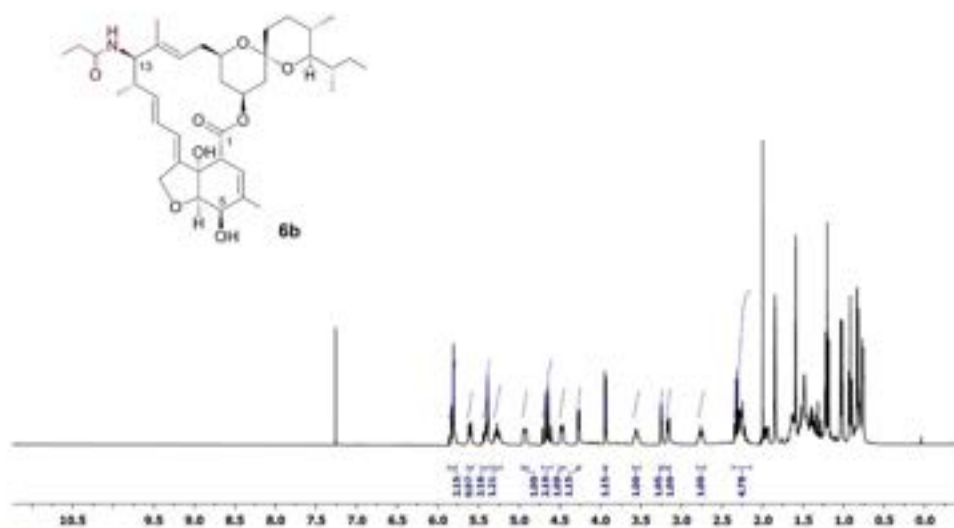


Figure S5. The ^1H NMR spectrum of **6b** in chloroform- d .

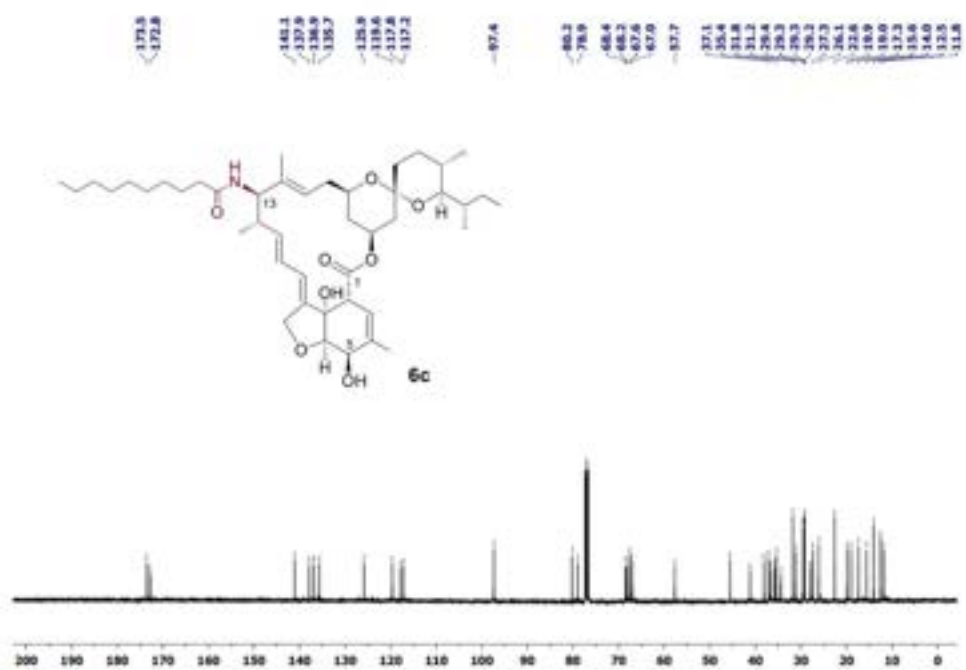


Figure S6. The ^{13}C NMR spectrum of **6c** in chloroform- d .

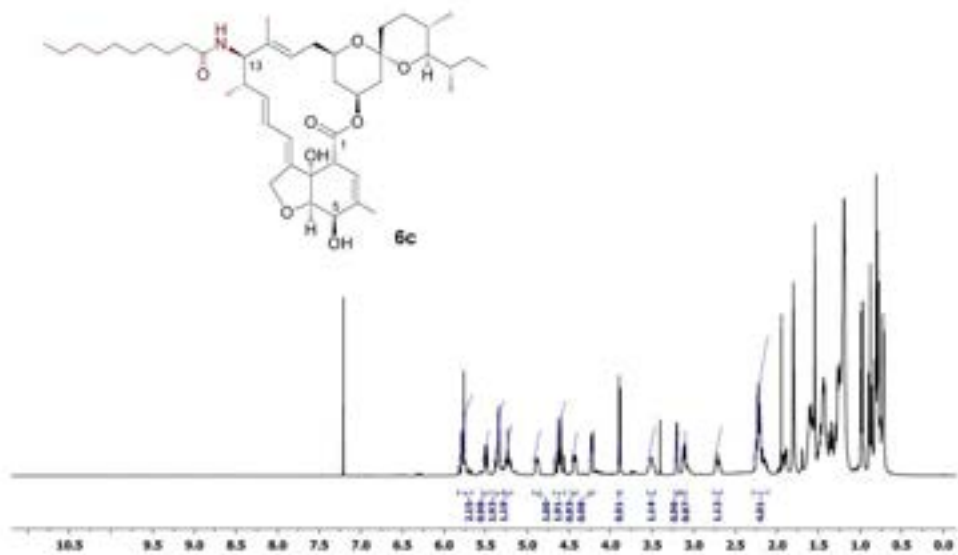
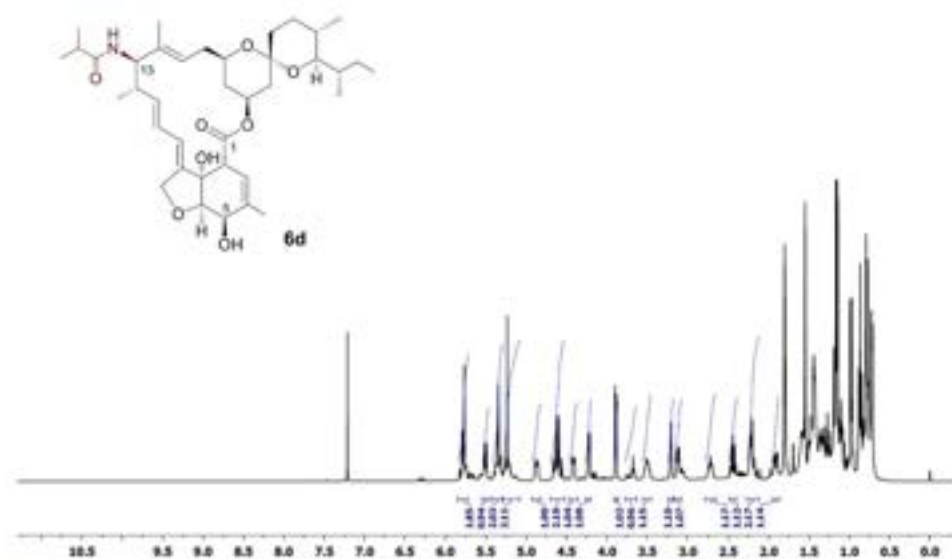
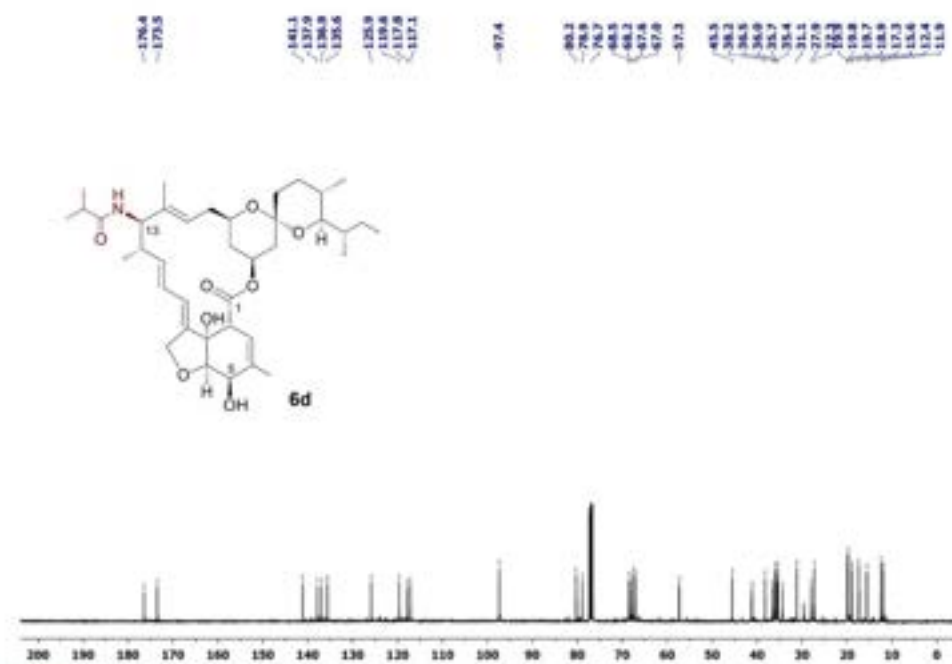
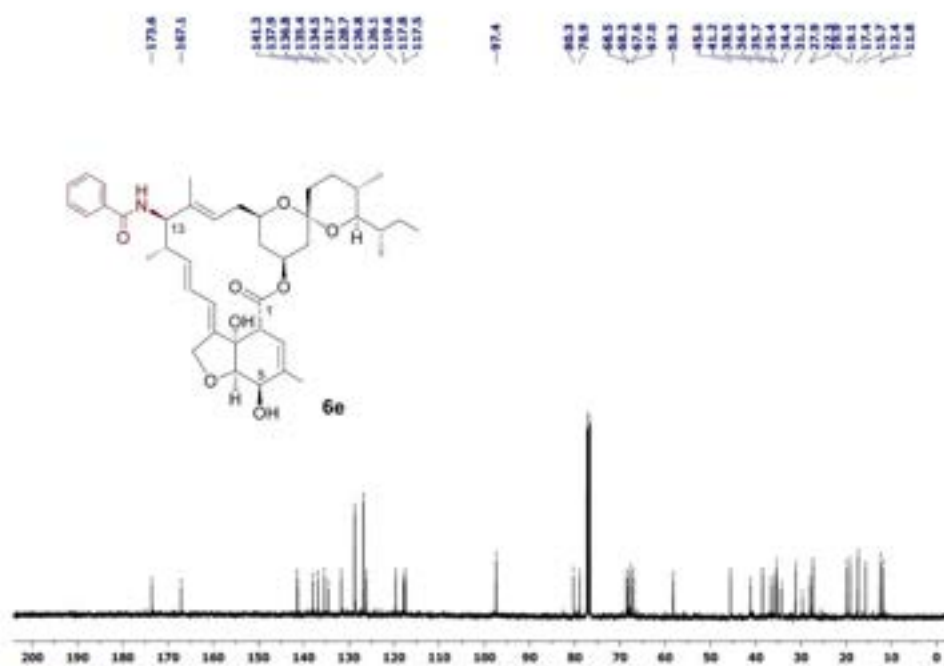
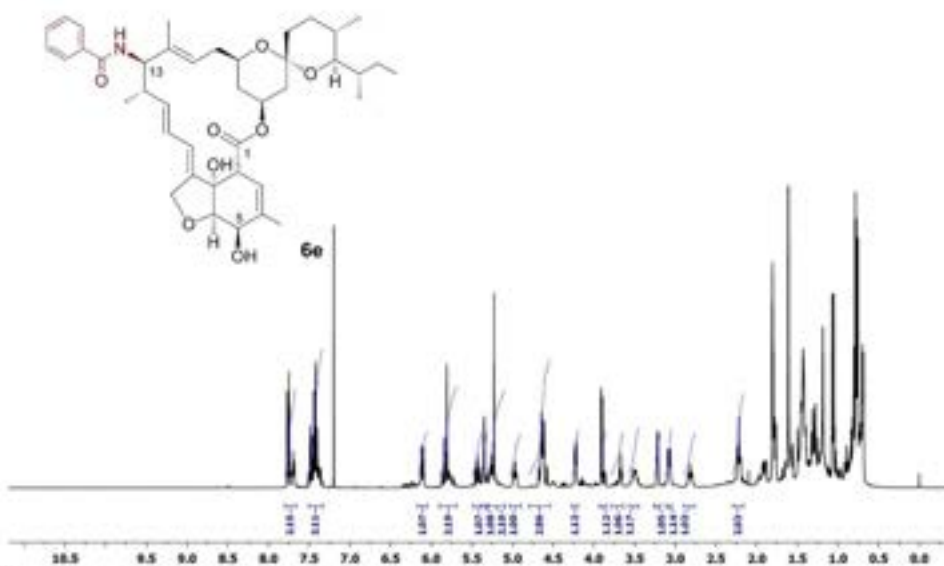


Figure S7. The ^1H NMR spectrum of **6c** in chloroform- d .



Figure S10. The ^{13}C NMR spectrum of **6e** in chloroform- d .Figure S11. The ^1H NMR spectrum of **6e** in chloroform- d .

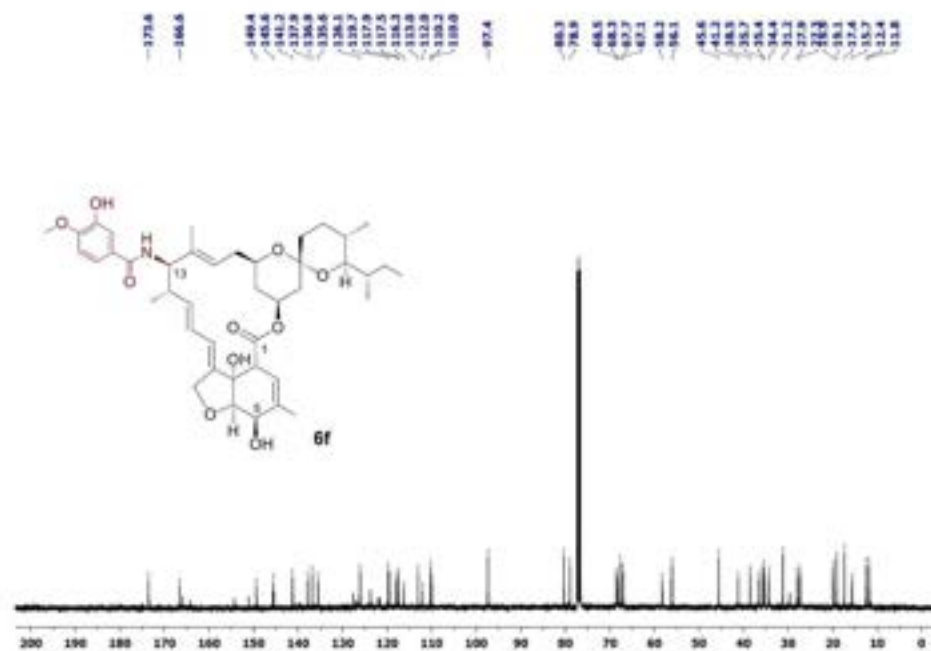


Figure S12. The ¹³C NMR spectrum of **6f** in chloroform-d.

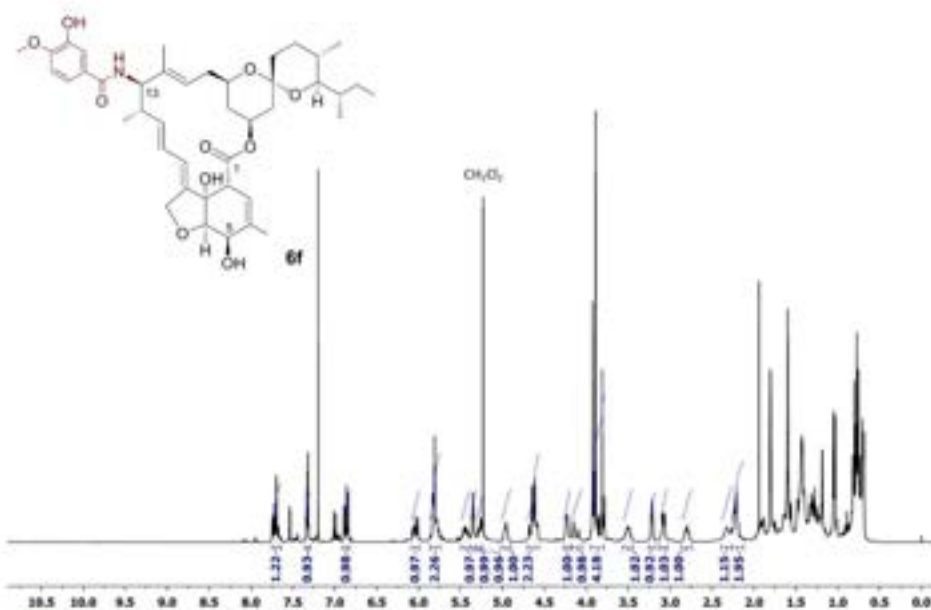


Figure S13. The ¹H NMR spectrum of **6f** in chloroform-d.

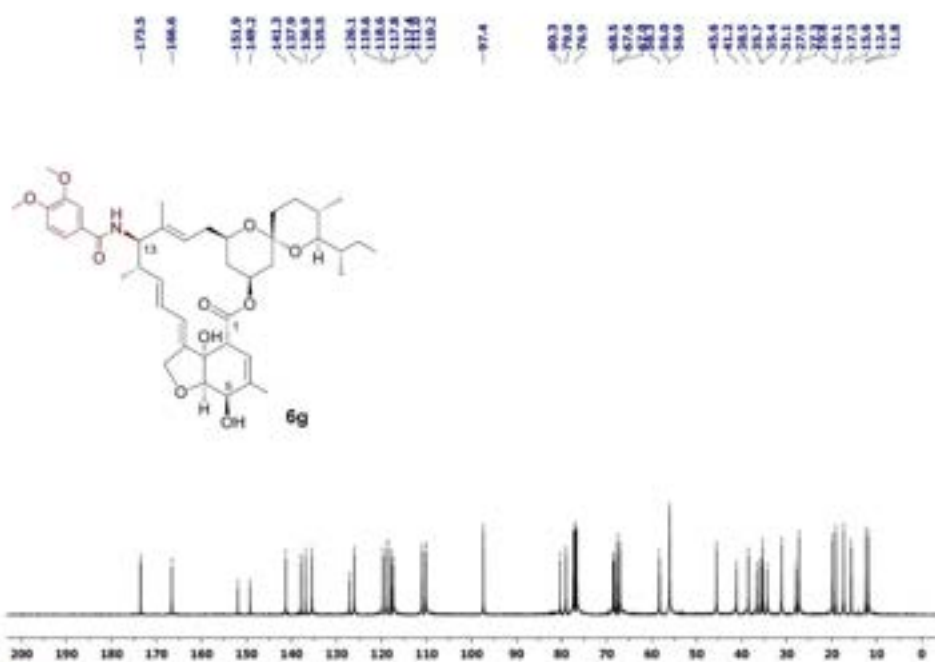


Figure S14. The ^{13}C NMR spectrum of **6g** in chloroform- d

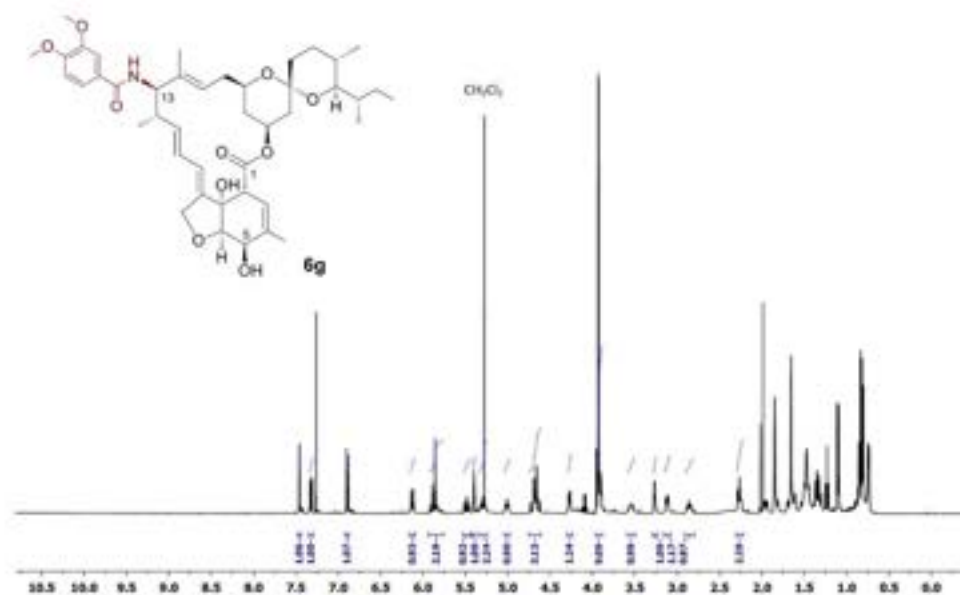


Figure S15. The ^1H NMR spectrum of **6g** in chloroform- d .

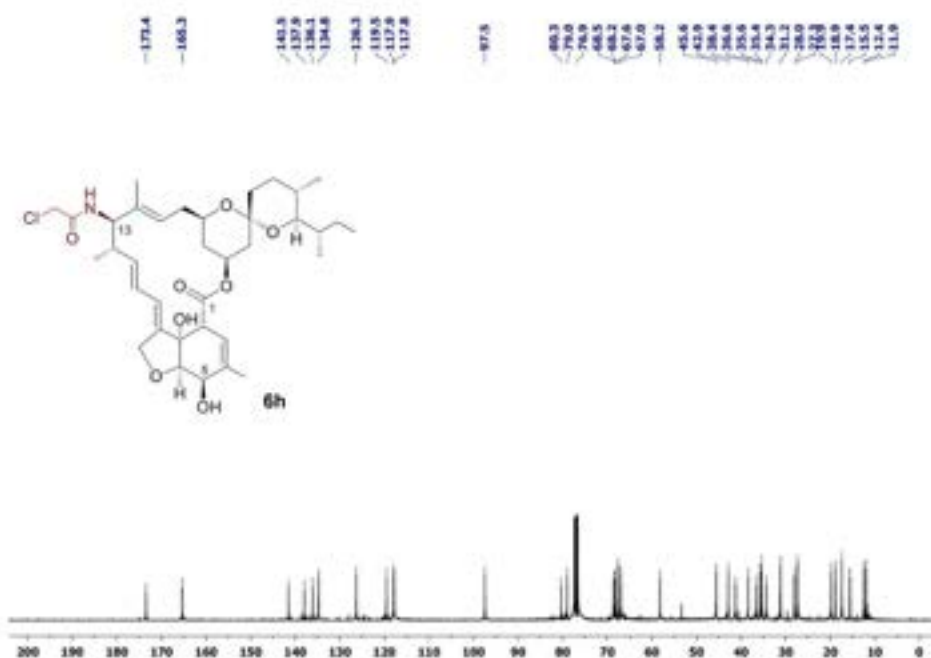


Figure S16. The ^{13}C NMR spectrum of **6h** in chloroform- d .

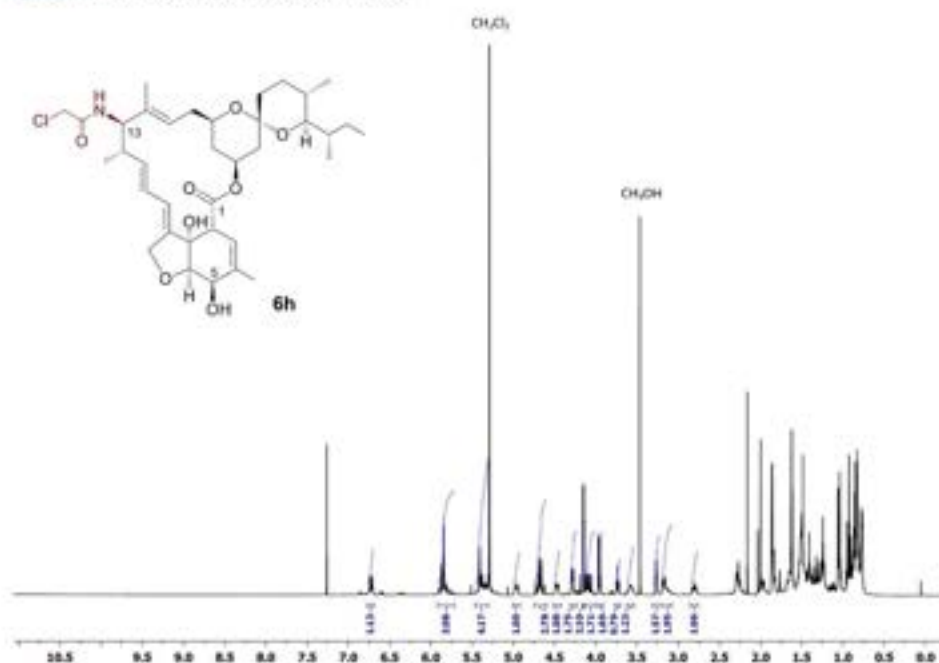
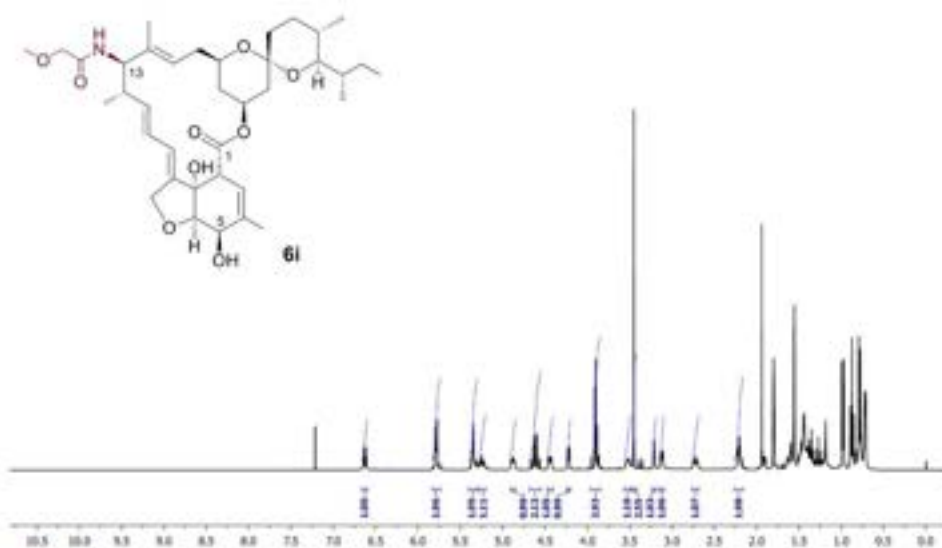
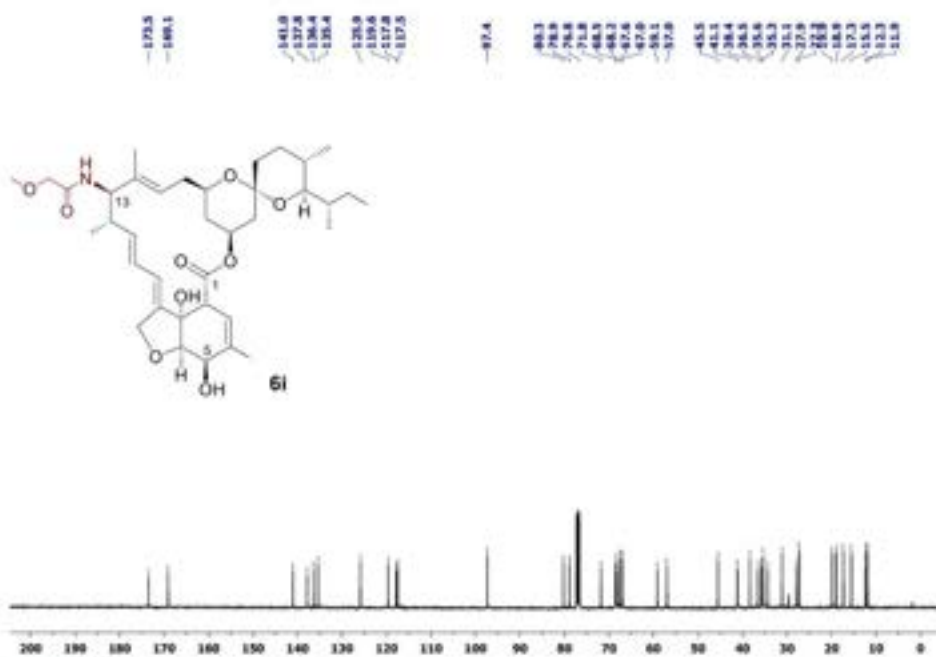


Figure S17. The ^1H NMR spectrum of **6h** in chloroform- d .



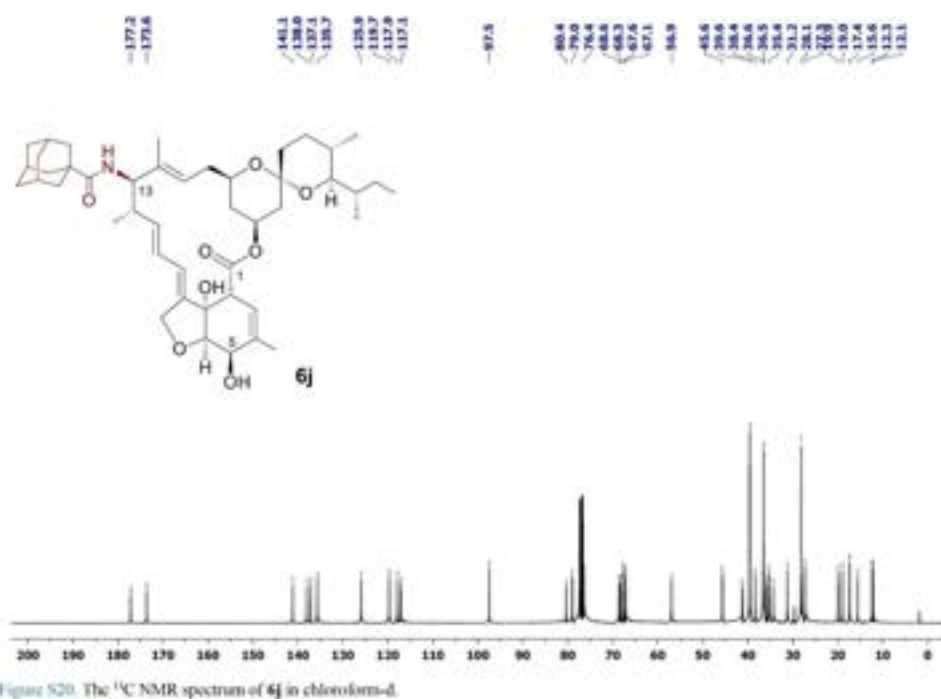


Figure S20. The ^{13}C NMR spectrum of **6j** in chloroform- d .

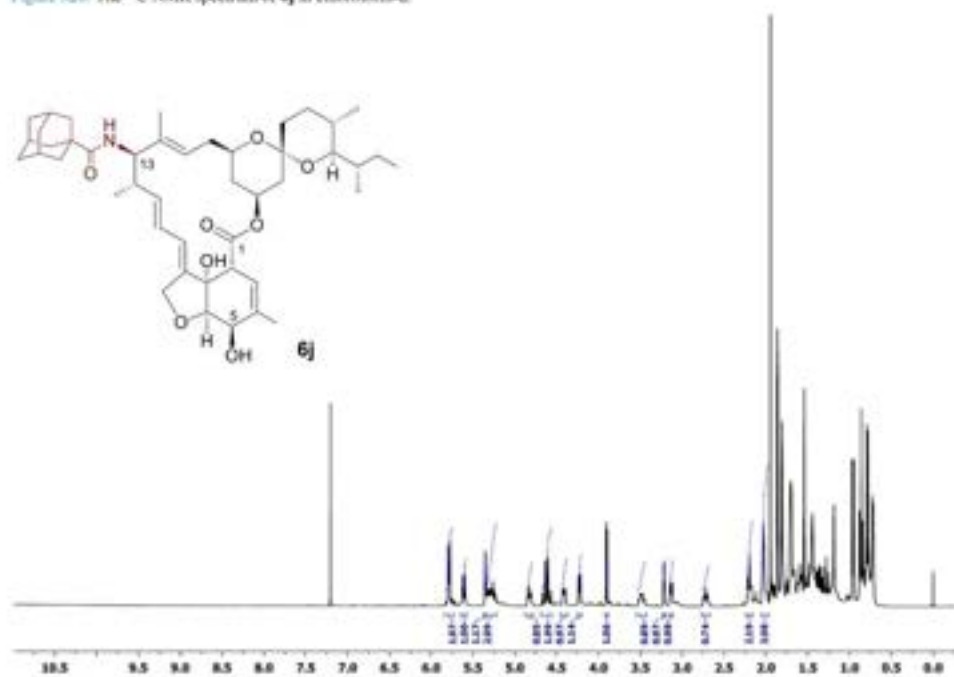
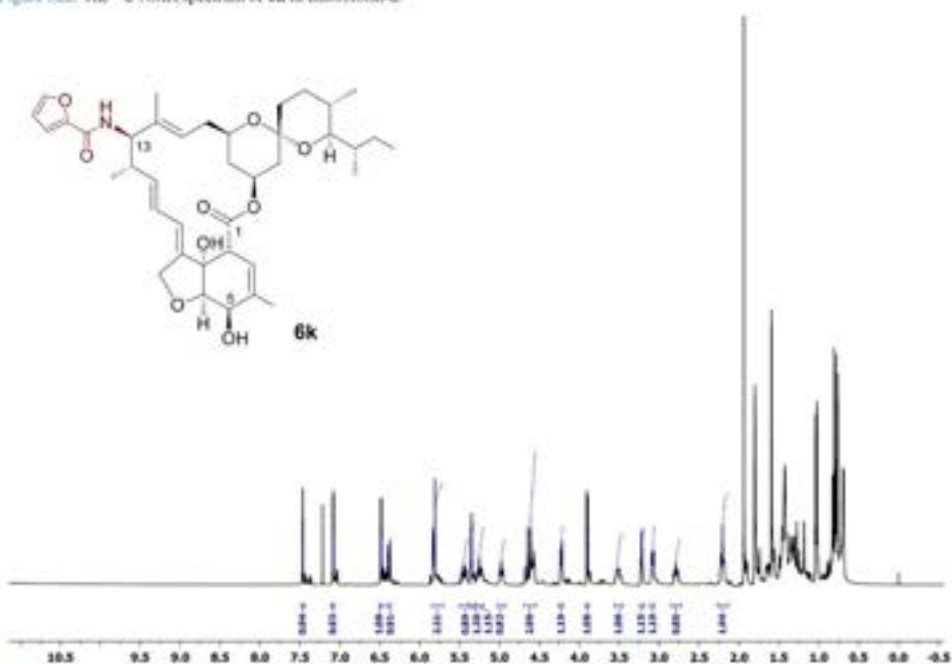
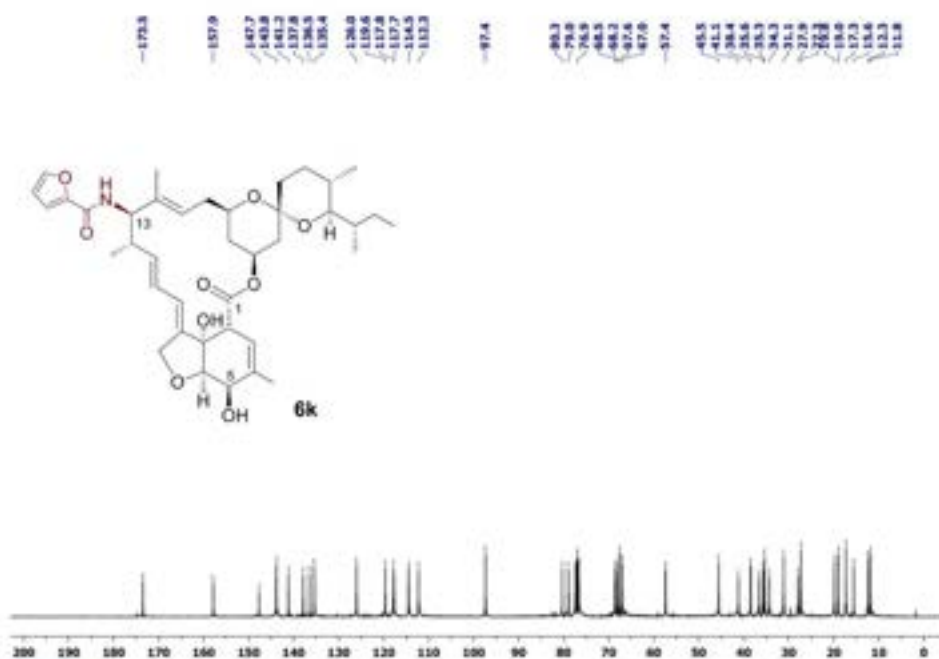


Figure S21. The ^1H NMR spectrum of **6j** in chloroform- d .



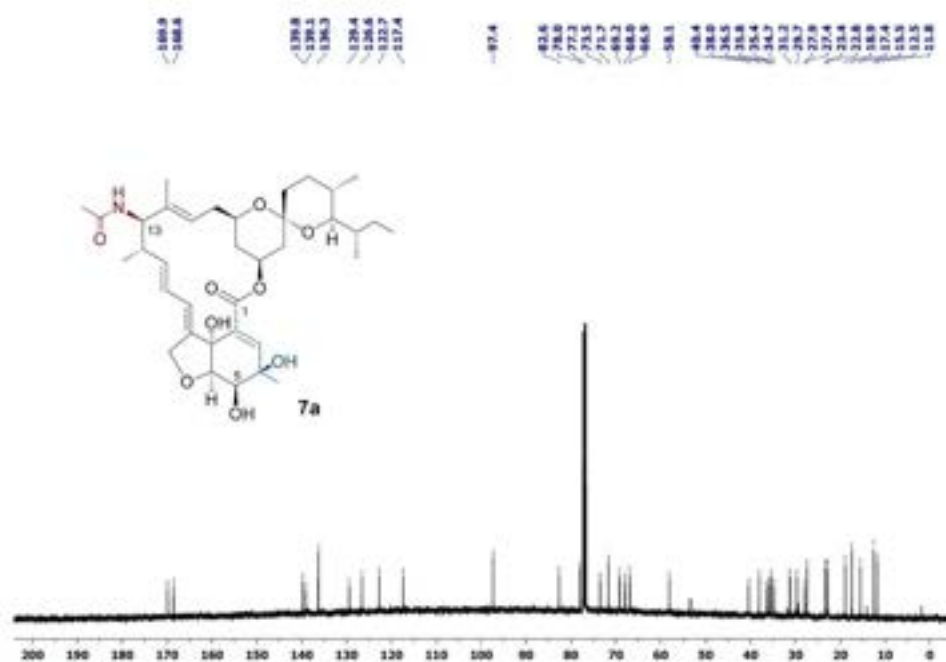


Figure S24. The ^{13}C NMR spectrum of **7a** in chloroform-d.

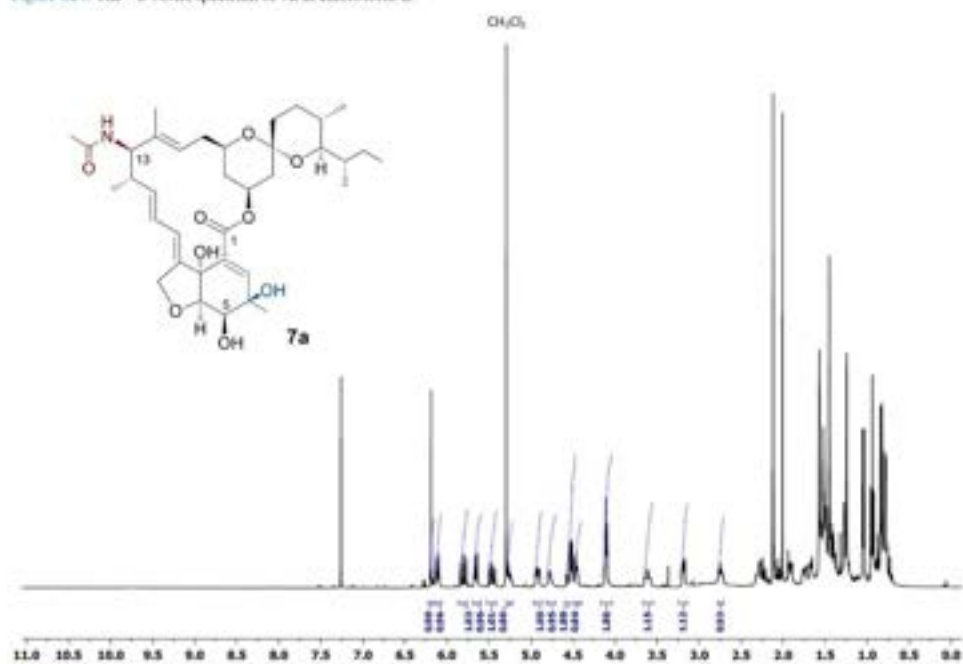
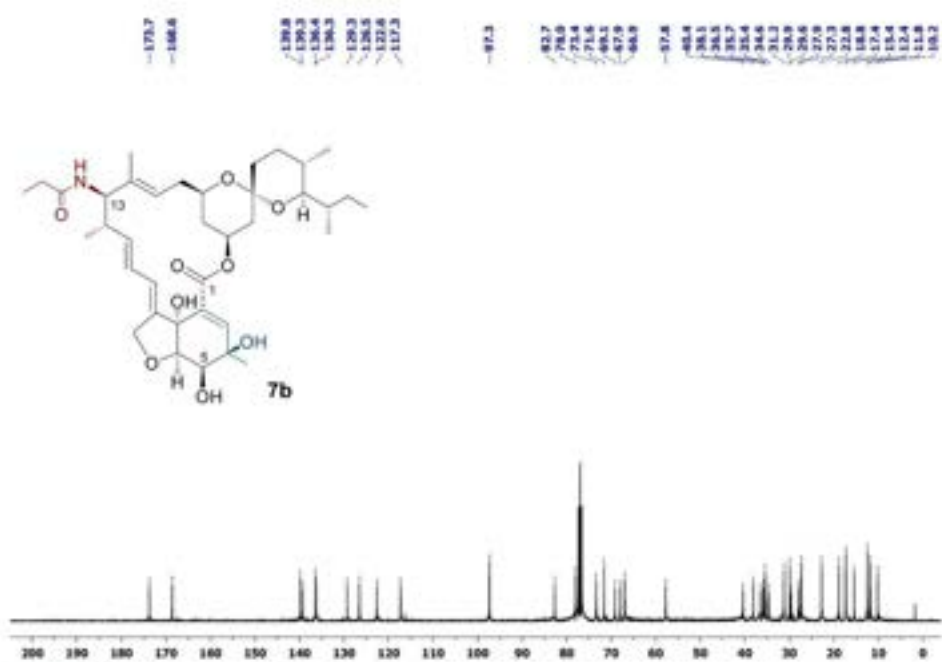
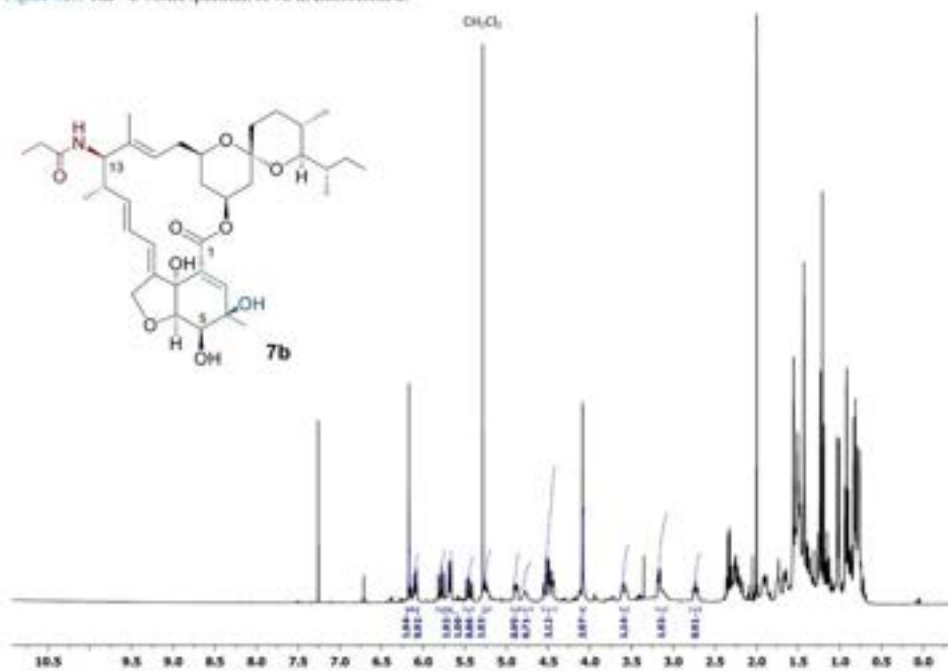


Figure S25. The ^1H NMR spectrum of **7a** in chloroform-d.

Figure S26. The ^{13}C NMR spectrum of **7b** in chloroform- d .Figure S27. The ^1H NMR spectrum of **7b** in chloroform- d .

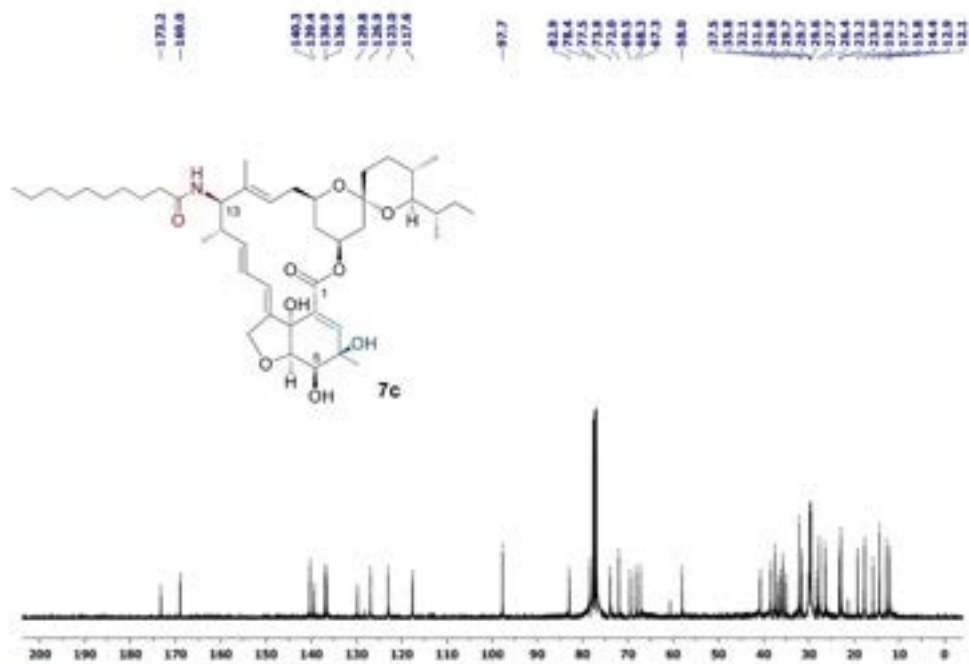


Figure S28. The ^{13}C NMR spectrum of **7c** in chloroform- d .

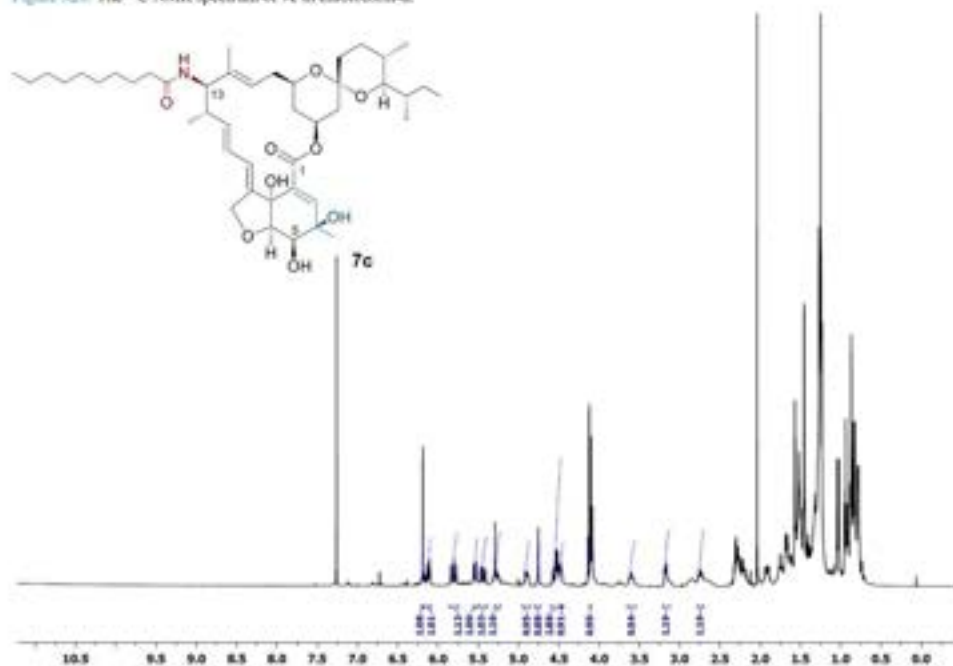
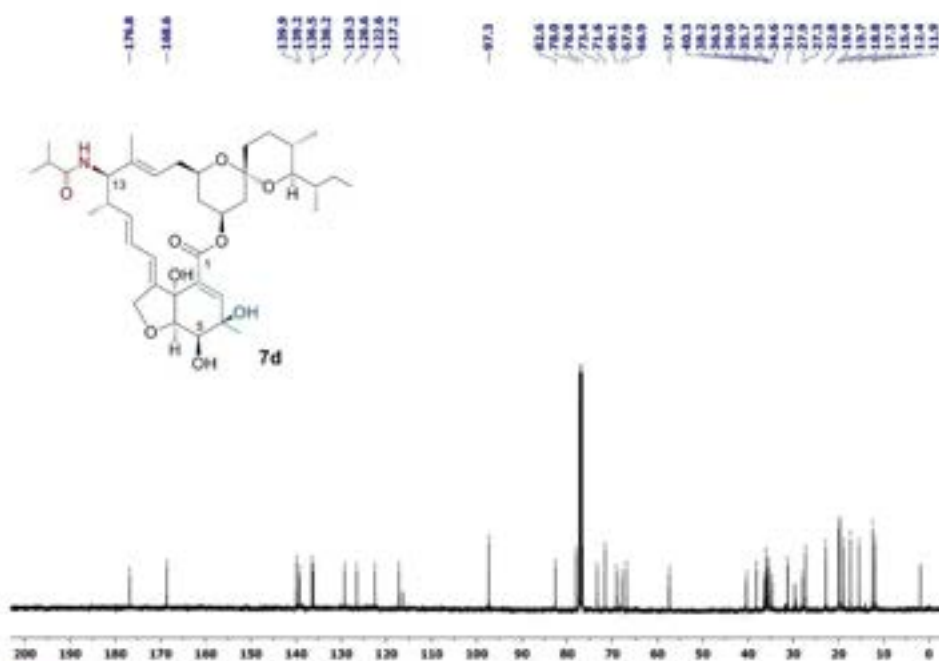
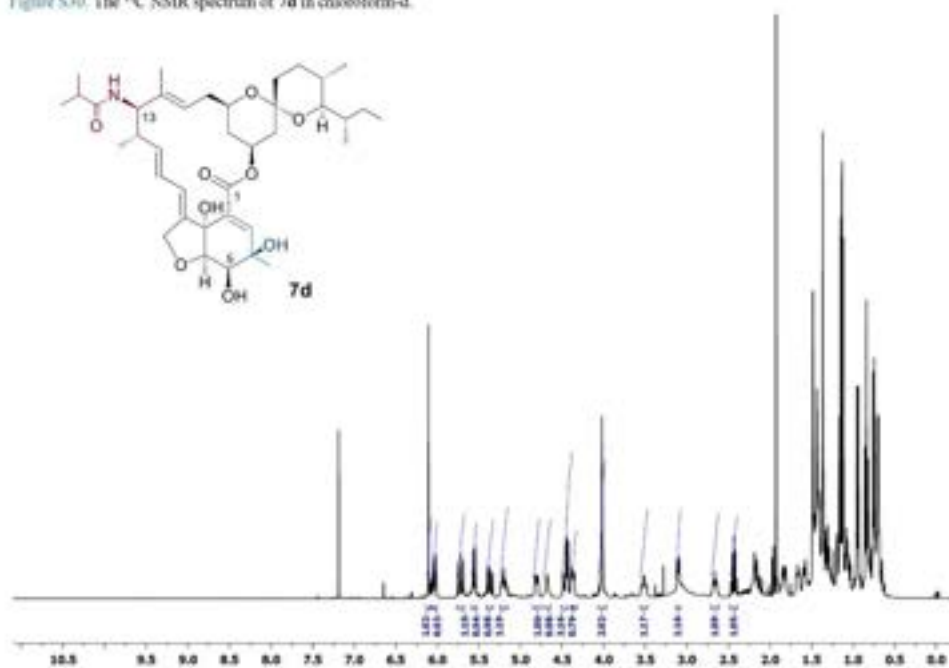


Figure S29. The ^1H NMR spectrum of **7c** in chloroform- d .

Figure S30. The ^{13}C NMR spectrum of **7d** in chloroform- d .Figure S31. The ^1H NMR spectrum of **7d** in chloroform- d .

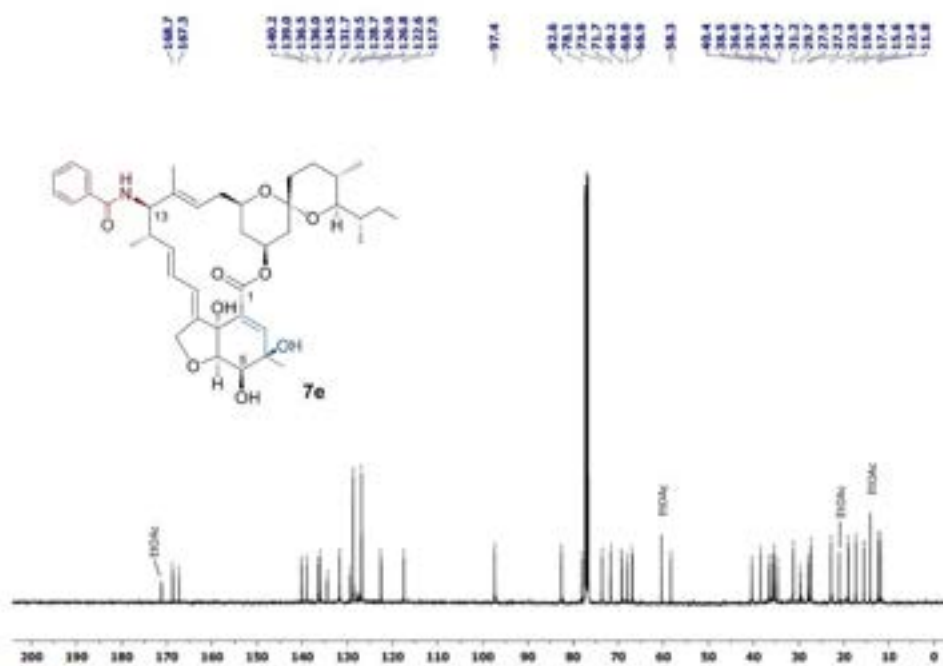


Figure S32. The ^{13}C NMR spectrum of **7e** in chloroform- d .

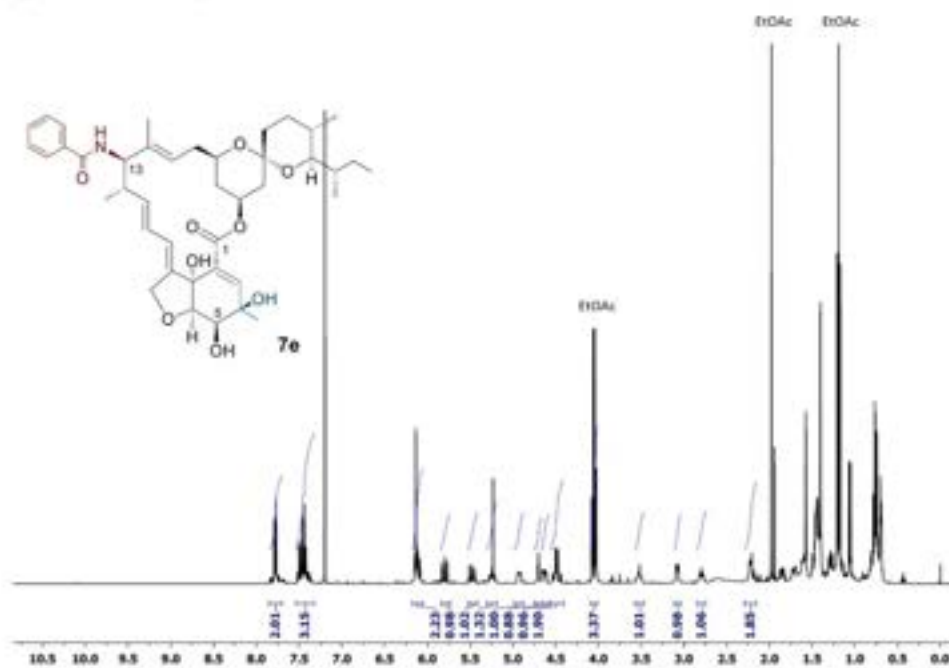
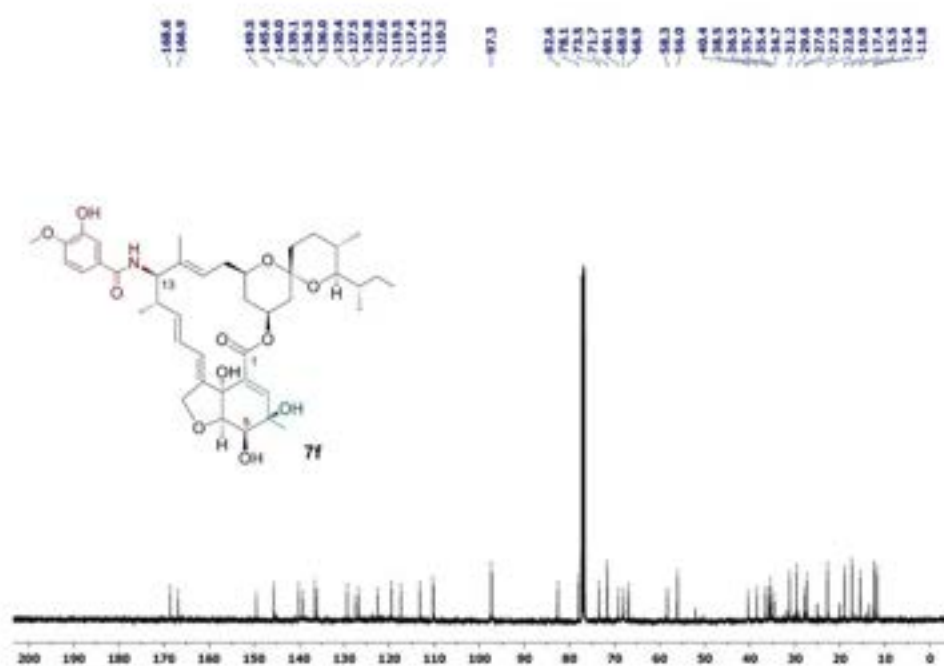
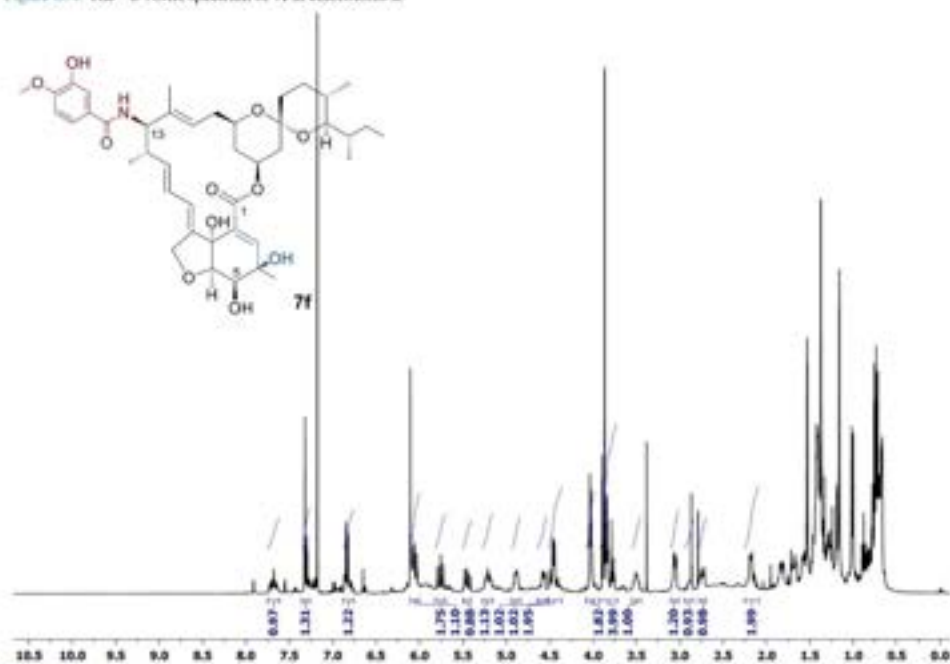


Figure S33. The ^1H NMR spectrum of **7e** in chloroform- d .

Figure S34. The ¹³C NMR spectrum of **7f** in chloroform-d.Figure S35. The ¹H NMR spectrum of **7f** in chloroform-d.

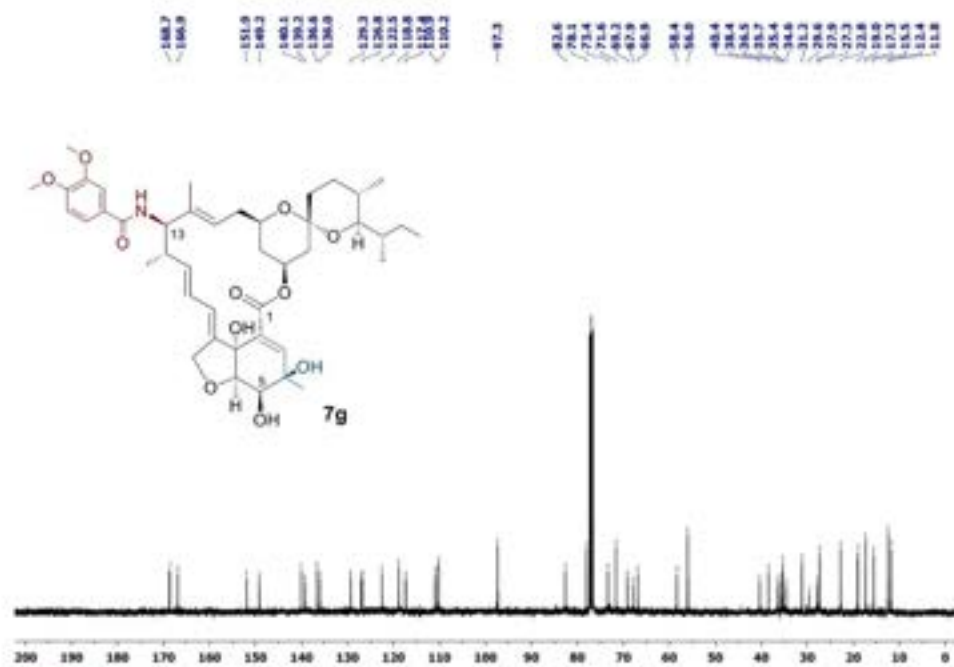


Figure S36. The ^{13}C NMR spectrum of 7g in chloroform-d.

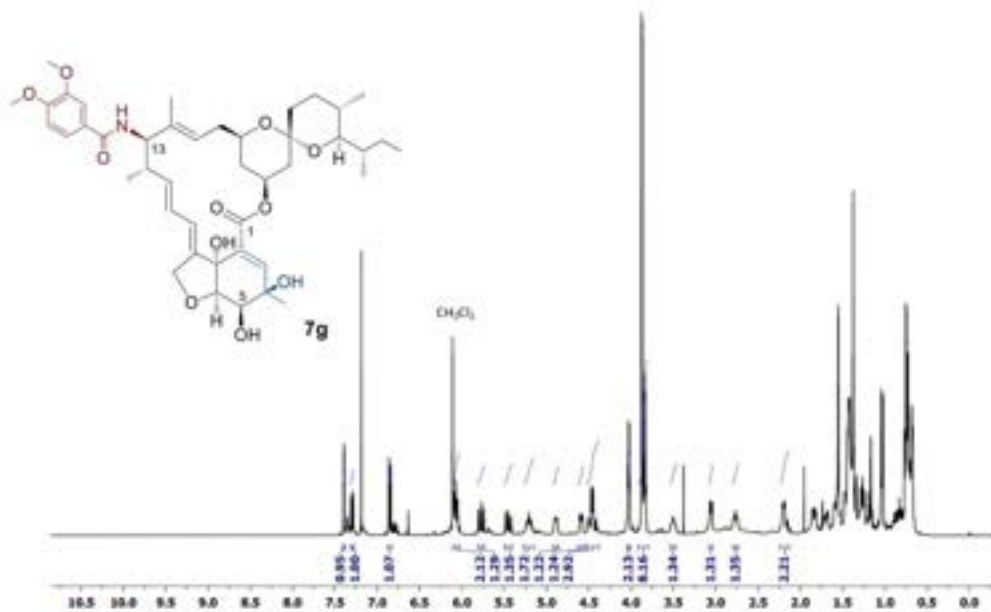


Figure S37. The ^1H NMR spectrum of 7g in chloroform-d.

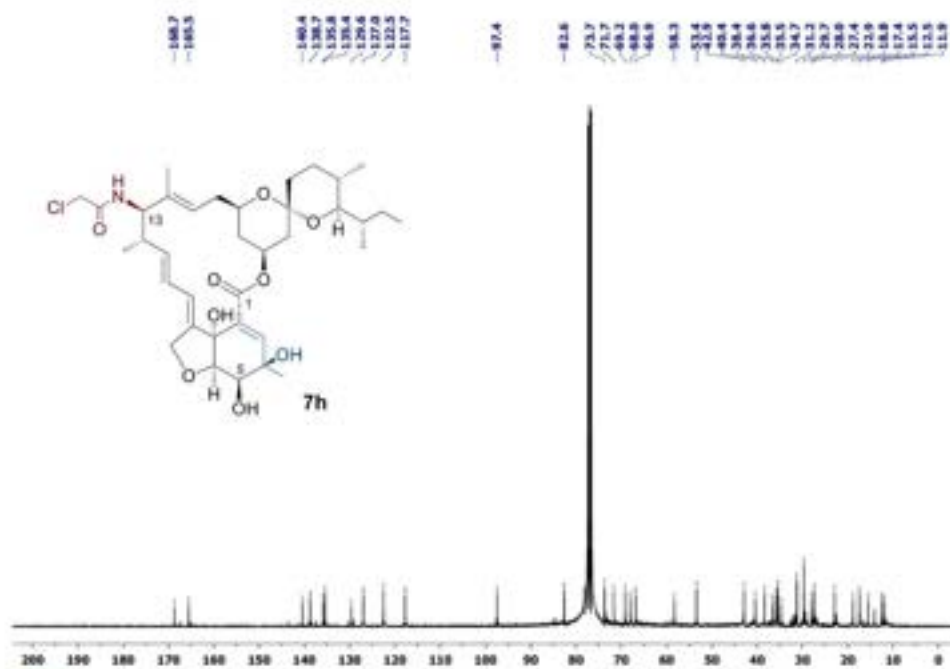


Figure S38. The ^{13}C NMR spectrum of **7h** in chloroform- d .

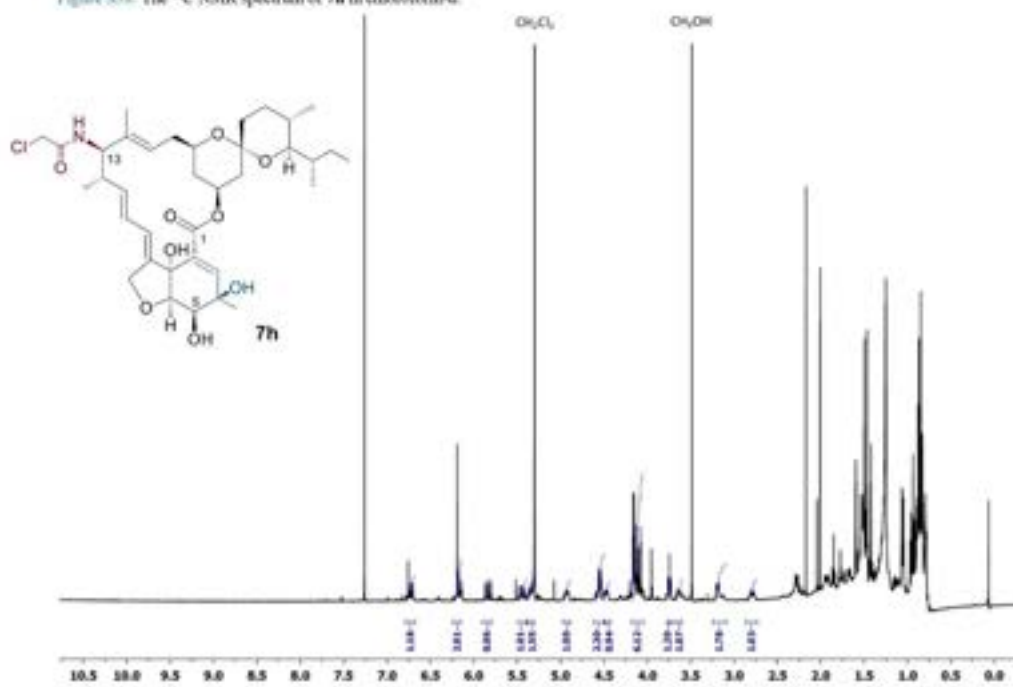


Figure S39. The ^1H NMR spectrum of **7h** in chloroform- d .

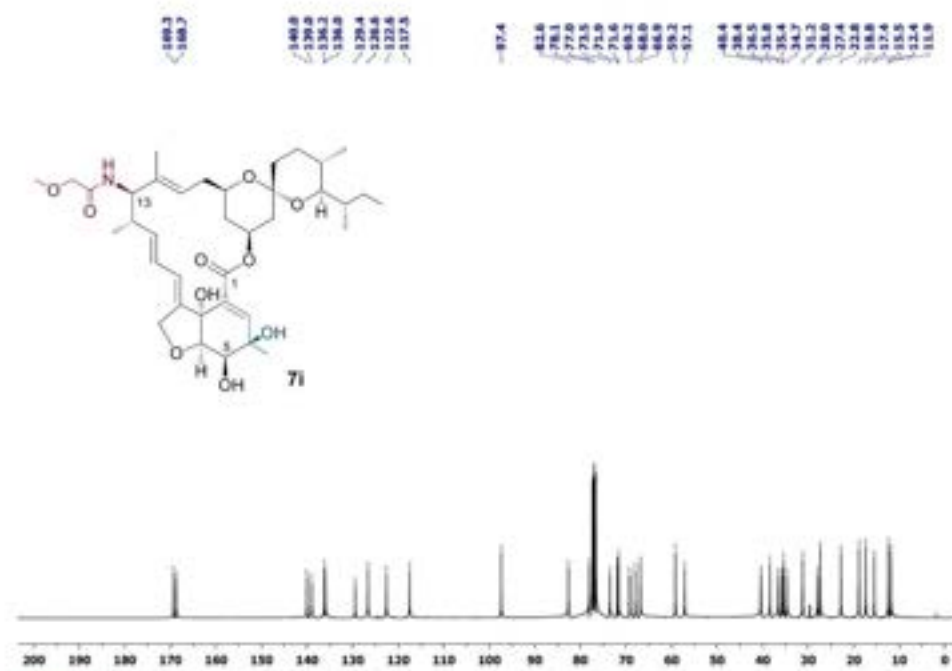


Figure S40. The ¹³C NMR spectrum of 7i in chloroform-d.

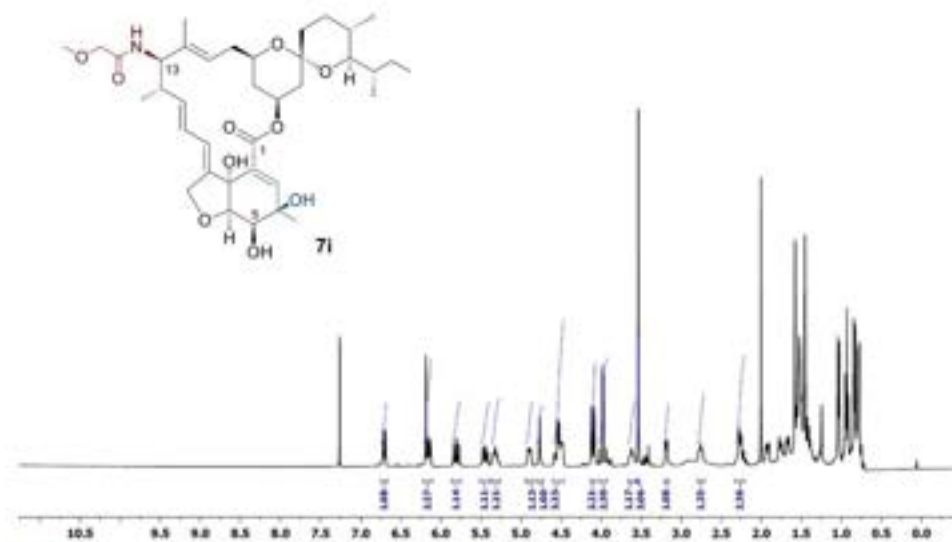


Figure S41. The ¹H NMR spectrum of 7i in chloroform-d.

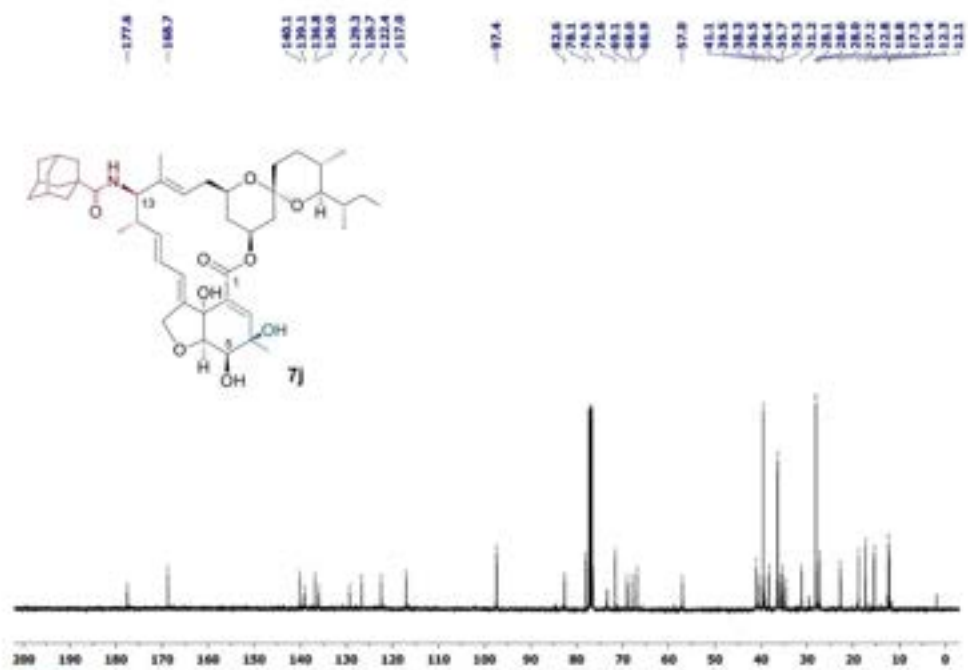


Figure S42. The ^{13}C NMR spectrum of **7j** in chloroform- d .

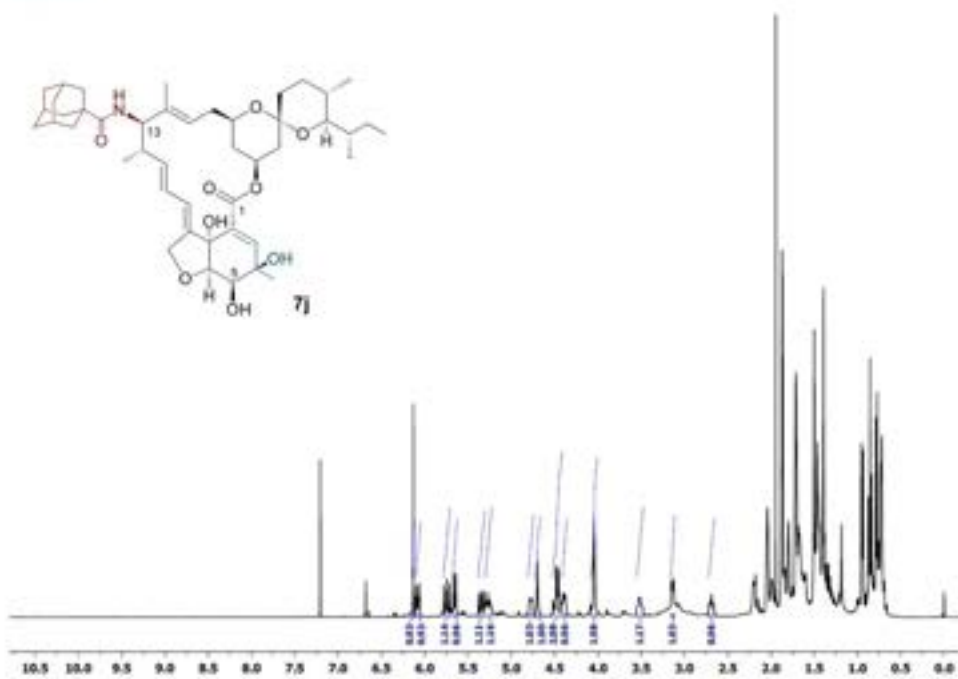


Figure S43. The ^1H NMR spectrum of **7j** in chloroform- d .

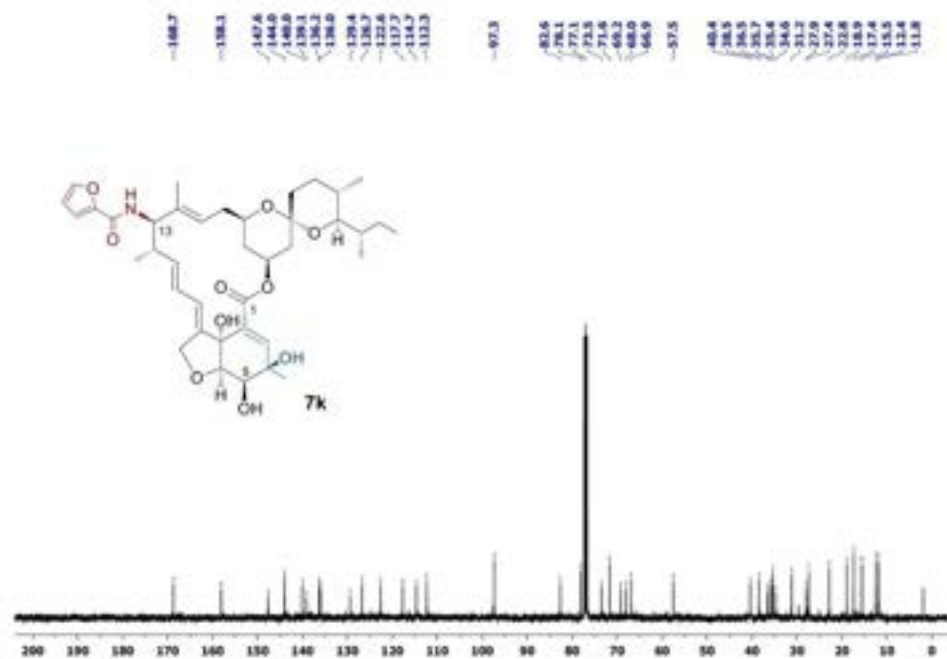


Figure S44. The ^{13}C NMR spectrum of 7k in chloroform-d.

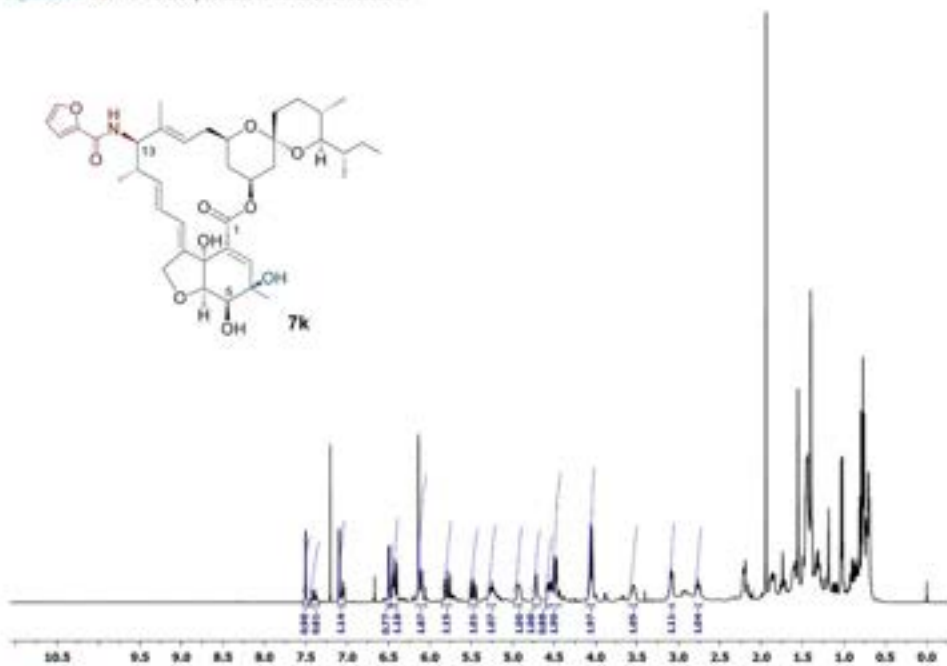


Figure S45. The ^1H NMR spectrum of 7k in chloroform-d.

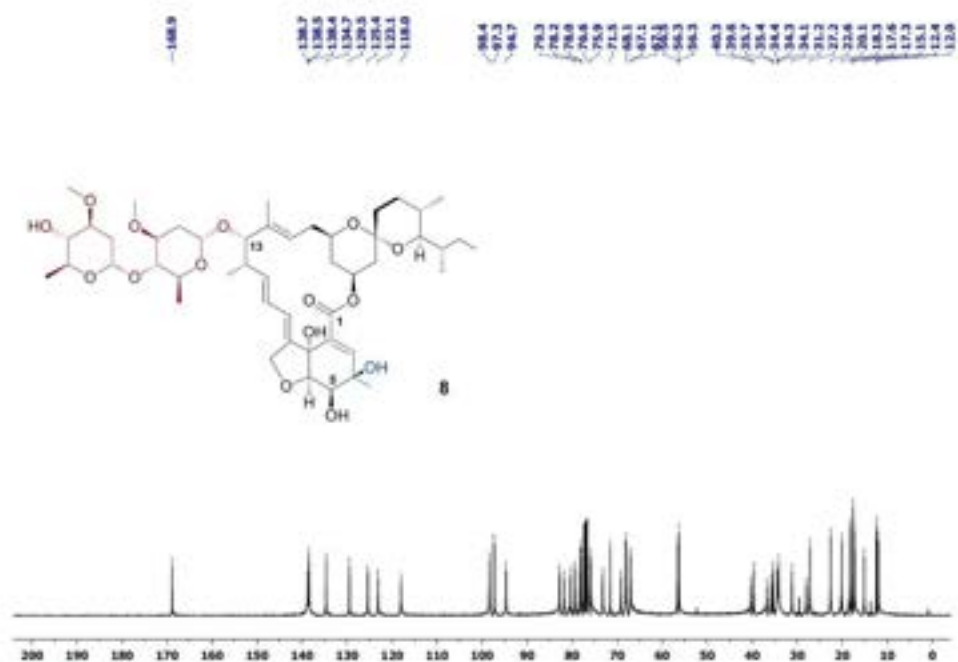


Figure S46. The ^{13}C NMR spectrum of **8** in chloroform- d .

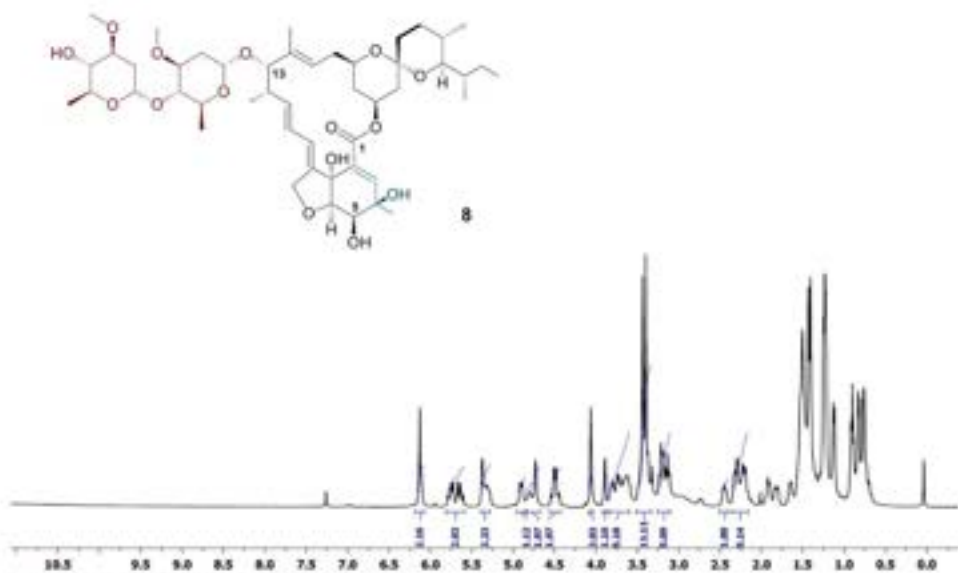


Figure S47. The ^1H NMR spectrum of **8** in chloroform- d .

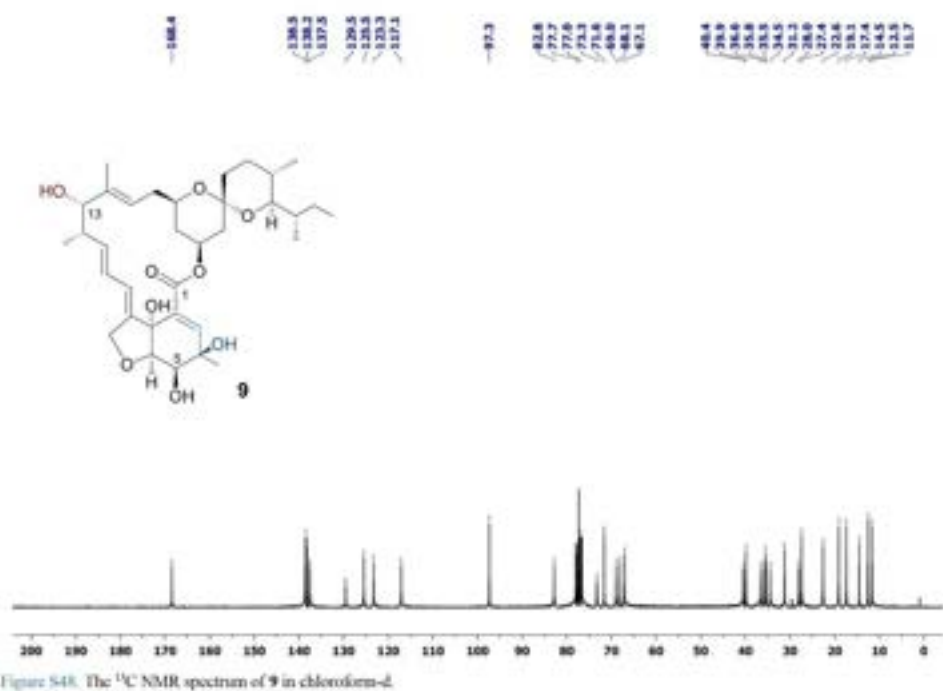


Figure S48. The ^{13}C NMR spectrum of **9** in chloroform- d .

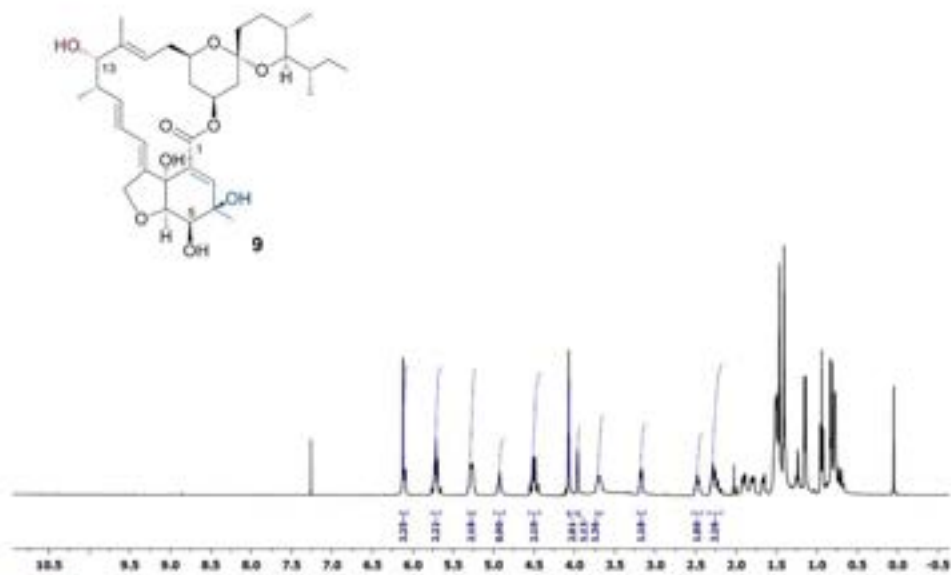


Figure S49. The ^1H NMR spectrum of **9** in chloroform- d .

X-ray analysis**Table S1.** Detailed crystallographic data of ivermectin derivatives **6a** and **7a**.

	6a	7a
Formula	C ₃₆ H ₅₃ NO ₈	C ₃₉ H ₅₉ NO ₁₀
Temperature (K)	293.0(2)	293.0(8)
Formula weight	627.79	701.87
Wavelength (Å)	0.56087	1.54184
Crystal system	Monoclinic	Orthorhombic
Space group	<i>P</i> 2 ₁	<i>P</i> 2 ₁ 2 ₁ 2
Unit cell dimensions (Å, °)	<i>a</i>	33.4076(2)
	<i>b</i>	12.47640(10)
	<i>c</i>	9.55330(10)
	β	90.00
Volume (Å ³)	3864.1(6)	3981.88(6)
<i>Z</i> / <i>Z'</i>	4/2	4/1
Calculated density (g cm ⁻³)	1.079	1.171
Absorption coefficient (mm ⁻¹)	0.048	0.679
<i>F</i> (000)	1360	1520
Crystal size (mm)	0.31 × 0.16 × 0.09	0.102 × 0.084 × 0.033
Θ -range for data collection (°)	3.274 to 29.54	9.258 to 153.358
Min / max indices: <i>h</i> , <i>k</i> , <i>l</i>	-8 ≤ <i>h</i> ≤ 8, -29 ≤ <i>k</i> ≤ 29, -11 ≤ <i>l</i> ≤ 11	-42 ≤ <i>h</i> ≤ 42, -15 ≤ <i>k</i> ≤ 15, -12 ≤ <i>l</i> ≤ 11
Reflect. collected / unique	63329/6070	185654/8310
<i>R</i> _{int} / completeness (%)	0.02135/99.9	0.0490/99.7
Data / restraints / parameters	6070/1706/849	8310/191/619
Goodness-of-fit on <i>F</i> ²	1.009	1.064
Final <i>R</i> ₁ / <i>wR</i> ₂ (<i>I</i> > 2 σ _{<i>I</i>})	0.0659/0.1398	0.0404/0.0950
<i>R</i> ₁ / <i>wR</i> ² (all data)	0.1221/0.1673	0.0489/0.1015

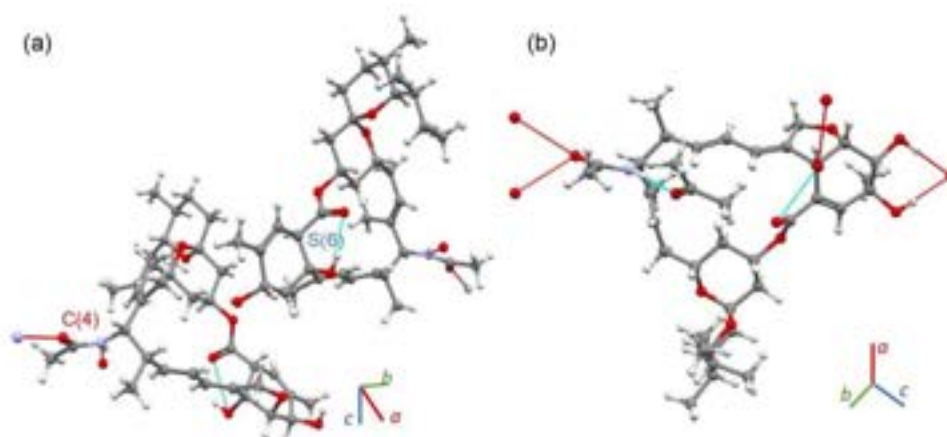


Figure S30. The H-bond patterns in molecules **6a** (a) and **7a** (b). Only one of the positions of disordered *sec*-butyl moiety at the C25 position in **6a** and acetone in **7a** is shown for clarity. Red dashed lines indicate intermolecular- and blue intramolecular-interactions. The tendency to form intramolecular H-bonds with S(6) motif [M.C. Etter, Hydrogen bonds as design elements in organic chemistry, *J. Phys. Chem.* 95 (1991) 4601–4610] between the hydroxyl O7A group at the C7 position and O1A carbonyl at the C1 position is characteristic of both investigated derivatives. However, in **7a** the same hydroxyl group is also intermolecularly bonded to the approaching hydroxyl group at the C7 position, resulting in the disorder of the H7A atom. The presence of the acetamide group in both compounds facilitates the intramolecular interactions – in **6a** leading to chains of the H-bonded molecules extending along the [100] crystal direction via N13...O29A interaction with C(4) descriptor [M.C. Etter, Hydrogen bonds as design elements in organic chemistry, *J. Phys. Chem.* 95 (1991) 4601–4610], while in **7a** the N13 atom is bonded with the acetone O1S carbonyl atom. What is more, the presence of two hydroxyl groups of the cyclohexanetriol located at the C4 and C5 positions in **7a** allows interaction with the C29 carbonyl atom.

11.3. Publikacja III

Michał Sulik, Dagmara Otto-Ślusarczyk, Michał Antoszczak,
Marta Struga, Adam Huczyński

Ivermectin and its synthetic derivatives – A new class of anticancer agents

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Opis wkładu doktoranta w powstanie publikacji: Jestem kierownikiem projektu Diamentowy Grant w ramach którego powstał niniejszy artykuł. Jestem w pełni odpowiedzialny za syntezę oraz oczyszczanie pojedynczo oraz podwójnie modyfikowanych pochodnych iwermektyny opisanych w pracy. Odpowiadałem za koordynowanie badań w tym kontakt z innymi naukowcami. Przeprowadziłem też analizę otrzymanych wyników, w tym tych z badań biologicznych. Odpowiadałem za napisanie manuskryptu.



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Ivermectin and its synthetic derivatives – A new class of anticancer agents

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ABSTRACT

Ivermectin (IVR) is a 16-membered macrocyclic lactone of Nobel prize-honored distinction, showing a broad spectrum of biological activity, especially antiparasitic. We have recently designed a practical and scalable procedure for the synthesis of IVR derivatives with a rearranged oxahydrindene (hexahydrobenzofuran) ring that revealed improved antiparasitic activity compared to that of the native structure. Of note, the compounds that show activity towards parasites, very often are active against cancer cells and vice versa. However, the anticancer potential of IVR has not been studied intensively as yet, and there have been no reports on the effects of its synthetic derivatives against cancer cells. Thus, in the study reported, we thoroughly investigated the anticancer activity of IVR and its derivatives against a panel of four human cancer cell lines. We have identified a number of IVR derivatives with improved cytotoxicity and/or cancer cell-targeting selectivity compared to those of reference compounds. Cell cycle analysis has proved that selected compounds increased the number of cancer cells in the subG1 and G0/G1 phases (PC3, MDA-MB-231 and A549) or S/G2/M phase (HCT-116). The strong proapoptotic effect observed for the most promising IVR derivatives has been associated with a significant increase in caspase-3/7 activation and reactive oxygen species (ROS) production. Finally, these derivatives also showed significant inhibition of interleukin-6 (IL-6) cytokine secretion in cancer cells used. Our results indicate that chemical modification of IVR can lead to synthetic products with enhanced anticancer activity, which may provide an excellent starting point for further development of new IVR-derived agents for the treatment against cancer cells.

1. Introduction

Cancer is one of the leading causes of death in developed countries, affecting people of all social and age groups. According to the World Health Organization (WHO), about 10 million cancer patients died in 2020, and over 19 million new cases of cancer were diagnosed in the same year [1,2]. As these numbers are predicted to continue to increase steadily in the coming decades [2], the current priority is the search for new anticancer drug candidates. In addition to the *de novo* drug development, another way to seek for lead structures with specific biological activity is the repurposing strategy, i.e., the use of drugs that have been already registered in new medical indications [3–5]. Among the drugs that have initially been used to treat non-cancer diseases, but also should be considered potential candidates for selectively targeting cancer cells, is ivermectin.

Ivermectin (IVR, **1**, Fig. 1) is a 16-membered macrocyclic lactone,

widely used in medicine as a mixture of two homologs – B1a, with an ethyl group at position C26, and B1b, with a methyl group at this position (Fig. 1). The mixture contains at least 80 % of homolog B1a and at most 20 % of B1b [6,7]. The drug exhibits a broad spectrum of antiparasitic activities and has been used in the treatment of various diseases, including river blindness (onchocerciasis), elephantiasis or head lice [8]. Importantly, IVR is recognized as a relatively safe drug, with most of the side effects connected with the body's reaction to the death of parasites [9]. With respect to the extraordinary antiparasitic activities of IVR, many scientists call it a “wonder drug” [9], and for its discovery, together with that of artemisinin as a potent antimalarial drug, the Nobel Prize in physiology/medicine was awarded in 2015 [10].

In addition to the antiparasitic effects of IVR, some recent studies have shown its promising activity against cancer cells originating from various organs [4,7]. Mechanistically, IVR has been found to inhibit the synthesis of proteins responsible for multi-drug resistance (MDR) or the

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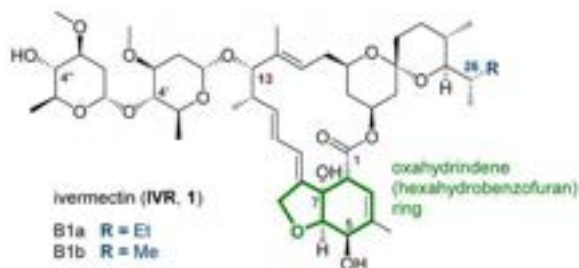


Fig. 1. Chemical structure of ivermectin.

AKT/mTOR pathway [4,11]. The anticancer effects of IVR have also been associated with its ability to increase the reactive oxygen species (ROS) levels, which led to oxidative stress, as well as its effects on a subpopulation of self-renewal cancer cells, commonly known as cancer stem cells (CSCs) [4,11]. Nevertheless, as follows from the limited number of studies published so far [4,11], the exact mechanism of anticancer action of IVR is not fully understood and still requires further investigation.

Moreover, the enormous potential of IVR is related to its very low toxicity – the therapeutic dose used in the treatment of humans and animals is approximately $200 \mu\text{g kg}^{-1}$, while the toxicity in monkeys was noticed at a dose of 24 mg kg^{-1} [12,13]. This indicates that IVR can be considered as a good candidate for repurposing for cancer therapy [4,7]. The promising biological activity of IVR combined with its relatively low toxicity, also makes this compound a good precursor that can be chemically modified to obtain derivatives with improved pharmacological effects compared to that of the starting material. Some authors have confirmed the validity of such an approach, showing that replacement of the sugar motif in the IVR structure with other substituents can significantly affect the antimalarial activity of the obtained analogues [14,15]. Of note, the compounds that show activity against parasites, very often are active against cancer cells and vice versa [16–19]. However, to the best of our knowledge, none of the IVR derivatives have been tested as yet for their anticancer activity.

Taking all these into account, in this study, we evaluated thoroughly the anticancer effects of IVR and a broad library of its derivatives, which showed very promising trypanocidal and antiparasmodial activities

reported in our previous study [20]. The IVR derivatives were obtained by introducing an amide moiety with a reversed absolute configuration at position C13, along with rearrangement of the oxalhydrindene (hexahydrobenzofuran) unit of the macrocyclic ring (Scheme 1) [20]. All compounds were evaluated for their *in vitro* antiproliferative activity against four human cancer cell lines. Moreover, to better understand the mechanism of the anticancer action of these compounds, we performed an in-depth analysis focused on their potential proapoptotic effect, as well as the ability to generate ROS, initiate lipid peroxidation, arrest the cell cycle, and inhibit the release of interleukin 6.

2. Materials and methods

2.1. Compounds

Ivermectin (IVR, homolog B1a) and all other reagents and solvents, except Dess-Martin periodinane (DMP) which was prepared according to the Frigerio et al. protocol [21], were obtained from Merck or Trimex Chemicals S.A. (Poland), and were used as received without further purification. The derivatives of IVR (compounds 4a–4k, 5a–5k and 6–7) were synthesized as shown in Scheme 1 and thoroughly characterized, as reported by us previously [20]. The IVR aglycone (compound 2), together with a key C13-*epi*-amine precursor (compound 3) were resynthesized according to the protocols published by us [20] and others [15,22–24] previously. A detailed description of the general procedures, measurement parameters, and equipment can be found in the Supplementary material. The NMR spectra of all IVR derivatives are included in the Supplementary material (Fig. S1–S48).

2.1.1. Synthesis of aglycone 2

A solution of IVR (1.0 equiv.) in CH_3OH was cooled in an ice bath, followed by a drop by drop addition of concentrated H_2SO_4 . The reaction mixture was stirred overnight, then concentrated to dryness *in vacuo*, diluted with CH_2Cl_2 , and extracted vigorously a few times with an aqueous solution of Na_2CO_3 (0.1 M). The collected organic layers were concentrated on a rotary evaporator, and purified chromatographically using the CombiFlash system (0 %–30 % $\text{EtOAc}/\text{CHCl}_3$) to give aglycone 2 as a white amorphous solid.

2.1.2. Synthesis of *epi*-amine 3

A solution of aglycone 2 (1.0 equiv.), imidazole (10.0 equiv.) and



Scheme 1. Synthesis of ivermectin derivatives with native or rearranged oxalhydrindene ring. Reagents and conditions: (a) H_2SO_4 , CH_3OH , 0 °C to rt; (b) TBAH, imidazole, CH_2Cl_2 , rt; (c) DMP, CH_2Cl_2 , rt; (d) NH_4OAc , NaBH_4/CN , CH_3OH , 60 °C to rt; (e) acyl chloride, TEA, CH_2Cl_2 , 0 °C to rt, or carboxylic acid, HATU, DMF, rt; (f) $p\text{TSA}$, CH_3OH , 0 °C to rt; (g) TBAF, THF, rt [20].

TBDMSCl (2.2 equiv.) in anhydrous CH_2Cl_2 was stirred at room temperature (rt) for 24 h. The reaction mixture was then concentrated to dryness in vacuo, and purified chromatographically using the CombiFlash system (0 %–10 % $\text{EtOAc}/\text{CHCl}_3$) to give C5-protected aglycone.

To accomplish the oxidation of C13 hydroxyl, TBDMS-masked aglycone (1.0 equiv.) was dissolved in CH_2Cl_2 , followed by the addition of *t*-BuOH (1.0 equiv.), and freshly prepared DMP (1.5 equiv.), and the reaction mixture was stirred at rt for 24 h. Next, CH_2Cl_2 was added, and diluted reaction mixture was extracted vigorously with an aqueous solution of Na_2SO_4 (0.5 M). The collected organic layers were concentrated on a rotary evaporator, and purified chromatographically using the CombiFlash system (0 %–5 % $\text{EtOAc}/\text{CHCl}_3$) to give C5-protected C13-ketone of IVR.

Finally, to a solution of C13-ketone (1.0 equiv.) in CH_2OH was added NH_4OAc (10.0 equiv.), and the reaction mixture was stirred at 60 °C for an hour, followed by a dropwise addition of a methanolic solution of NaBH_3CN (2.0 equiv.). Stirring was continued for next 24 h, then the reaction mixture was concentrated in vacuo, diluted with CH_2Cl_2 , and extracted vigorously a few times with an aqueous solution of Na_2CO_3 (0.1 M). The collected organic layers were concentrated on a rotary evaporator, and purified chromatographically using the CombiFlash system (0 %–100 % $\text{EtOAc}/\text{CHCl}_3$) to give epi-amine 3 as a white amorphous solid.

2.1.3. Synthesis of unrearranged amide derivatives 4a–4k

To a stirred solution of epi-amine 3 (1.0 equiv.) in anhydrous CH_2Cl_2 , Et_3N (3.0 equiv.) and a respective acyl chloride (1.2 equiv.) were added, and the reaction mixture was stirred at rt for 24 h. Alternatively, a respective carboxylic acid (1.0 equiv.) was dissolved in DMF, followed by the addition of DIPEA (6.0 equiv.) and HATU (1.3 equiv.). The reaction mixture was then stirred at rt for 15 min, and then, epi-amine 3 (1.0 equiv.) was added and the stirring was continued for 24 h. Next, in both cases, CH_2Cl_2 was added, and diluted reaction mixture was extracted vigorously with an aqueous solution of H_2SO_4 (pH = 1) and water. The collected organic layers were concentrated on a rotary evaporator, and purified chromatographically using the CombiFlash system (0 %–100 % $\text{EtOAc}/\text{CHCl}_3$) to give C5-protected amide derivatives as clear oils.

To remove the TBDMS-masking group, the corresponding amide product (1.0 equiv.) was dissolved in a methanolic solution of pTSA hydrate (80 mg pTSA in 10 mL CH_2OH), and stirred at rt for 2 h. Next, the reaction mixture was concentrated in vacuo, diluted with CH_2Cl_2 , and extracted vigorously a few times with an aqueous solution of Na_2CO_3 (0.1 M). The collected organic layers were concentrated on a rotary evaporator, and purified chromatographically using the CombiFlash system (0 %–100 % $\text{EtOAc}/\text{CHCl}_3$) to give unrearranged amide derivatives 4a–4k as white amorphous solids.

2.1.4. Synthesis of rearranged amide derivatives 5a–5k

To obtain amide derivatives with rearranged oxahydrindene ring, TBDMS-protected amide (1.0 equiv.; please see point 2.1.3 for synthesis procedure) was dissolved in anhydrous THF, followed by the addition of TBAF (3.0 equiv.), and the reaction mixture was stirred at rt for 72 h. The organic solvent was then evaporated in vacuo, and the crude products were purified chromatographically using the CombiFlash system (0 %–100 % $\text{EtOAc}/\text{CHCl}_3$) to give rearranged amide derivatives 5a–5k as white amorphous solids.

2.1.5. Synthesis of rearranged ivermectin 6 and its aglycone 7

To a stirred solution of IVR or 2 (1.0 equiv.) in anhydrous THF, TBAF (3.0 equiv.) was added in one portion, and the stirring was continued at rt for next 72 h. The organic solvent was then evaporated in vacuo, and the crude products were purified chromatographically using the CombiFlash system (0 %–100 % $\text{EtOAc}/\text{CHCl}_3$) to give rearranged analogues 6 or 7 as white amorphous solids.

2.2. Biological activity

2.2.1. Human cell lines

The human cell lines, including A549 (lung cancer), HCT-116 (colon carcinoma), MDA-MB-231 (triple-negative breast adenocarcinoma), PC3 (metastatic prostate cancer), and HaCaT (immortalized keratinocytes), were procured from the American Type Culture Collection (ATCC, Rockville, USA). Culturing conditions varied, with HCT-116 cells cultured in MEM (ThermoSci, USA), PC3 cells in RPMI, as well as A549, MDA-MB-231, and HaCaT cells in DMEM High Glucose (Biowest SAS, France). The growth medium included 10 % fetal bovine serum (FBS, Merck, St. Louis, MO, USA), 20 mM HEPES (Biowest, Nuaille, France), and antibiotics – 100 U mL^{-1} of penicillin and 100 $\mu\text{g mL}^{-1}$ of streptomycin (Gibco, Grand Island, NY, USA). Cells were maintained in a humidified incubator at 37 °C with a 5 % CO_2 atmosphere until reaching a confluence level of 80–90 %.

2.2.2. Cytotoxicity

The assessment of the cytotoxic activity of IVR derivatives was carried out using an MTT assay, which relies on the enzymatic conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide salt (MTT) into insoluble formazan crystals by mitochondrial dehydrogenases within viable cells. The assay involved initial cell plating in 96-well plates at a density of 1×10^4 cells per well, allowing a 24 h adherence period. Subsequently, the cells were exposed to various concentrations of IVR derivatives, ranging from 1 to 100 μM , with untreated cells serving as the control group. After a 72 h incubation at 37 °C with 5 % CO_2 , the culture medium was removed, and MTT solution (0.5 mg mL^{-1}) was added to each well. The plates were then incubated for additional 4 h at 37 °C with 5 % CO_2 . Formazan crystals formed were dissolved in a mixture of DMSO and isopropanol (1:1, v/v). The optical density of the solution was measured at a wavelength of 570 nm, using a Multiscan Go spectrophotometer (ThermoSci).

Cell viability was determined as the percentage of MTT reduction in the treated cells compared to the untreated cells (control), with the number of viable cells cultured without the tested compounds set as 100 %. The relative MTT level, expressed as a percentage, was calculated using formula $[A]/[B] \times 100$, where [A] represents the absorbance of the sample being tested, and [B] corresponds to the absorbance of the control sample containing untreated cells. A decreased relative MTT level indicates reduced cell viability.

The IC_{50} values, representing the concentration at which 50 % of cell viability is inhibited, were calculated using GraphPad Prism 8.0.1 software.

2.2.3. Cell cycle analysis

The cells were initially seeded at a density of 1×10^5 in each well of a 6-well plate and left to incubate overnight for optimal attachment. Following this, the compounds studied were administered at IC_{50} concentrations specific to each cell line, and the cells were allowed to incubate for 24 h. After the incubation, both adherent and detached cells were collected and subjected to centrifugation at $400 \times g$ for 5 min at 4 °C. Subsequently, the cells underwent two washes with phosphate-buffered saline (PBS) before being fixed in 500 μL of cold 70 % ethanol at 4 °C overnight. Before analysis, the fixed cells were centrifuged at $850 \times g$ for 5 min at 4 °C and washed again with PBS. The cells were then treated with 50 μL of RNase (100 $\mu\text{g mL}^{-1}$, Merck) and stained with 200 μL of propidium iodide (50 $\mu\text{g mL}^{-1}$) at 37 °C for 30 min in the dark. The staining process was concluded by adding 100 μL of PBS to each sample. Subsequently, the cell cycle analysis was carried out using flow cytometry, specifically a Becton Dickinson instrument. This analytical approach facilitated the identification of cells distributed across various cell cycle stages, encompassing sub-G1, G0/G1, S, and G2/M phases.

The data on the cell cycle distribution after treatment of cancer cells with IVR and its selected analogues detected by flow cytometry are

provided in the Supplementary material (Fig. S49).

2.2.4. Annexin V-FITC/PI binding assay

Apoptosis measurement was carried out using the Annexin V-FITC/PI binding assay. Initially, the cells were seeded in 12-well plates at a density of 5×10^4 cells per well and allowed to incubate for 24 h. After the incubation, the cancer cells were exposed to IVR and its derivatives at concentrations equivalent to their IC_{50} values, whereas HaCaT cells were treated with the respective compounds at concentrations equal to the highest IC_{50} values for cancer cells, with untreated cells serving as the control group. Following 72 h of incubation, the apoptotic effects of the compounds were evaluated in both the cell culture medium and harvested cells. This evaluation involved dual staining with Annexin V-FITC and a propidium iodide (PI) detection kit (BD Biosciences Pharmingen), following the manufacturer's protocol. Subsequently, cell apoptotic rates were measured, distinguishing early apoptosis (Annexin V-FITC positive and PI negative) and late apoptosis or necrosis (Annexin V-FITC positive/negative and PI positive) groups by flow cytometry (Becton Dickinson, San Jose, CA, USA). The tests were conducted in triplicate for statistical reliability.

Representative data on the effects of IVR and its selected analogues on early/late apoptosis or necrosis in PC3, MDA-MB-231, A549 and HCT-116 cancer cells, as well as HaCaT cells detected with Annexin V-FITC/PI staining by flow cytometry are included in the Supplementary material (Fig. S50).

2.2.5. Caspase-3/7 activity assay

Caspase activity was assessed using the Homogeneous Caspase-Glo® 3/7 kit (Promega, Madison, WI, USA), following the manufacturer's instructions. The cells were initially seeded into 96-well plates with white walls (1×10^4 cells per well), then pre-incubated at 37 °C and 5 % CO_2 for 24 h. Subsequently, the cells were exposed to the tested compounds at concentrations equivalent to IC_{50} .

Next, the Caspase-Glo 3/7 reagent was brought to rt and 100 μ L of this reagent was introduced to each well. Subsequently, the plates were incubated at rt for a duration of 2 h. After the 2 h incubation, the luminescence of each sample was measured using a Synergy HTX Multi-Mode Reader (BioTek Instruments, USA). To account for background luminescence, a blank sample containing the culture medium with DMSO, the solvent of the tested compounds, was included. Meanwhile, untreated cells served as the negative control.

2.2.6. DCFH-DA assay

Reactive oxygen species (ROS) production was assessed using a spectrofluorometric method employing 2',2'-dichlorodihydrofluorescein diacetate (DCFH-DA) as the indicator. This technique relies on the conversion of substances into the fluorescent dichlorofluorescein (DCF), induced by ROS [25].

In a black 96-well plate, the cells were initially seeded at a density of 1×10^4 cells per well. After a 24 h incubation, the cells were subjected to the tested compounds at their respective IC_{50} concentrations and allowed to incubate at 37 °C with 5 % CO_2 for additional 24 h. Following this exposure, the cells underwent a PBS wash and were subsequently incubated with 5 μ M of DCFH-DA for 30 min at 37 °C in the dark. A positive control was a sample containing H_2O_2 (1.5 mM), while a negative control was a sample with no reagents. DCF fluorescence was measured using a microplate spectrofluorometer (BioTek Synergy, Bio-Tek Instruments, USA) with excitation and emission wavelengths set at 492 nm and 527 nm, respectively. The level of ROS was quantified in terms of fluorescence intensity (FI).

2.2.7. TBA assay

The quantification of lipid peroxidation products was carried out using the TBA assay, as originally outlined by Buege and Aust in 1978 [26], with modifications detailed by Linden et al. [27]. This particular assay was designed to detect two types of substances: malondialdehyde

(MDA), which is a major product of lipid oxidation, and other compounds that react with thiobarbituric acid (TBA) to form colored complexes. To conduct this assay, A549, HCT-116, MDA-MB-231, PC3, and HaCaT cells were seeded in 12-well plates, with each well containing 5×10^4 cells. The plates were then incubated for 24 h at 37 °C with 5 % CO_2 . After the incubation, the cells were exposed to the tested compounds at their respective IC_{50} values, for 48 h. Untreated cells served as the control group. Following the 48 h incubation, the levels of TBARS were determined. The optical density of the solution was measured at a wavelength of 532 nm using the MultiscanGO system (ThermoSci). The amount of TBARS was quantified in terms of nanomoles of MDA equivalents formed per milligram of cell protein. This calculation was based on the molar extinction coefficient of the MDA-TBA complex, which is $1.56 \times 10^5 \text{ M}^{-1} \times 5 \text{ cm}^{-1}$.

The protein concentration in mg mL^{-1} was determined by the Bradford method in a separate set of cultures that were not used for the TBA assay. The optical density of these solutions was measured at a wavelength of 595 nm using the MultiscanGO system (ThermoSci).

2.2.8. Interleukin-6 assay

The cells were initially plated at a density of 1×10^5 cells per well in 12-well plates containing complete medium and allowed to incubate for 24 h. Subsequently, the cells were exposed to the tested compounds at concentrations corresponding to their IC_{50} values for a duration of 72 h. As a control, untreated cells were included. To assess the level of interleukin-6 (IL-6) in the cell culture supernatant, an Interleukin IL-6 High Sensitivity ELISA kit (Diarcone SAS, Besancon Cedex, France) was employed following the manufacturer's instructions. The absorbance was then quantified at 450 nm using a spectrophotometer (Microplate Spectrophotometer ThermoSci™ MultiscanGO). This experimental procedure was repeated in triplicate.

2.2.9. Statistical analysis

Statistical analysis was performed using GraphPad Prism 9 software (GraphPad Software). The results were reported as mean $\pm$ SD from a minimum of three independent experiments. Statistical significance of differences between values was assessed using analysis of variance with Dunnett's multiple comparison post hoc test, and significance was defined as $p < 0.05$.

3. Results and discussion

3.1. Design and synthesis of ivermectin derivatives

The synthesis of the ivermectin (IVR) derivatives studied in this work, i.e., C13- α -amides of IVR with native or rearranged oxahydrindene ring (compounds 4a–4k or 5a–5k, respectively), together with rearranged IVR (compound 6) and its aglycone (compound 7) (Scheme 1) have been described thoroughly in our previous work [20].

Briefly, IVR was treated with a solution of sulfuric acid in methanol to remove the sugar moiety, which resulted in the formation of aglycone 2 (Scheme 1). The C5 hydroxyl of 2 was then masked in the reaction with *tert*-butyldimethylsilyl chloride in the presence of imidazole (Scheme 1). Next, C13 hydroxyl was regioselectively oxidized to ketone group using Dess-Martin periodinane, followed by a reductive amination of C13 ketone to the key amine precursor 3 (Scheme 1). This precursor was used in the amidation reaction with the appropriate acyl chlorides or carboxylic acids (Scheme 1). A large variety of substituents (linear aliphatic with different carbon chain lengths, branched aliphatic, aromatic, aliphatic-aromatic, and/or those with additional heteroatoms) were selected to determine whether there is a correlation between the structure and the biological activity of the obtained analogues. The silyl group at C5 position could be quantitatively and tracelessly removed with *p*-toluenesulfonic acid, giving a series of singly amide-containing derivatives of IVR with native oxahydrindene ring (compounds 4a–4k, Scheme 1). However, when TBAF was used as a

Table 1
Antiproliferative activity (IC_{50} , μ M) of ivermectin and its derivatives.^a

Compound	Cancer cells								Normal cells HaCaT
	PC3		MDA-MB-231		A549		HCT 116		
	IC ₅₀	SI	IC ₅₀	SI	IC ₅₀	SI	IC ₅₀	SI	
ivermectin 1	8.5 ± 0.2	1.3	8.8 ± 0.3	1.2	9.9 ± 0.9	1.1	9.4 ± 1.2	1.1	10.8 ± 3.1
aglycone 2	17.1 ± 1.2	1.0	12.1 ± 0.1	1.4	8.4 ± 1.3	2.0	16.7 ± 1.9	1.0	16.9 ± 8.1
4a	17.5 ± 3.3	1.6	7.6 ± 0.5	3.7	12.5 ± 0.8	2.3	10.1 ± 0.4	2.8	28.5 ± 6.5
4b	8.7 ± 1.9	1.6	10.9 ± 0.3	1.3	12.3 ± 3.8	1.1	16.7 ± 0.8	0.8	14.2 ± 2.9
4c	7.6 ± 0.6	1.5	9.8 ± 2.3	1.1	19.4 ± 0.9	0.6	9.6 ± 0.6	1.2	11.3 ± 4.1
4d	9.2 ± 0.6	1.1	7.8 ± 1.2	1.3	8.2 ± 0.4	1.2	17.2 ± 2.2	0.6	10.1 ± 2.1
4e	8.2 ± 0.7	1.1	8.5 ± 0.1	1.1	11.8 ± 2.9	0.8	14.1 ± 2.1	0.6	9.1 ± 1.3
4f	8.6 ± 0.3	1.2	13.1 ± 1.2	0.8	16.2 ± 2.3	0.6	4.5 ± 0.6	2.3	10.5 ± 2.8
4g	8.1 ± 1.1	1.3	8.2 ± 0.3	1.3	9.7 ± 1.7	1.1	2.5 ± 0.1	4.3	10.8 ± 2.6
4h	17.6 ± 3.4	1.6	9.5 ± 1.7	3.0	37.9 ± 4.4	0.8	6.5 ± 1.0	4.4	28.9 ± 1.3
4i	8.3 ± 0.4	1.5	10.1 ± 0.4	1.2	11.4 ± 3.4	1.1	14.5 ± 0.8	0.9	12.6 ± 3.4
4j	9.1 ± 0.8	0.7	10.3 ± 3.5	0.6	11.5 ± 3.4	0.6	5.1 ± 1.4	1.2	6.4 ± 0.5
4k	7.8 ± 0.7	1.0	28.5 ± 0.6	0.3	12.5 ± 1.6	0.7	21.7 ± 0.9	0.4	8.2 ± 1.9
5a	25.8 ± 4.1	0.8	45.5 ± 8.1	0.5	29.4 ± 1.1	0.7	15.9 ± 2.9	1.3	21.4 ± 6.4
5b	13.8 ± 3.2	1.5	14.6 ± 6.2	1.4	20.0 ± 1.9	1.0	13.9 ± 3.1	1.4	20.2 ± 1.7
5c	18.3 ± 3.7	0.4	10.2 ± 0.3	0.8	10.2 ± 0.3	0.8	12.7 ± 0.7	0.6	8.2 ± 0.5
5d	16.5 ± 4.6	0.5	27.2 ± 0.1	0.3	22.0 ± 1.2	0.4	31.5 ± 0.2	0.3	8.2 ± 2.1
5e	12.2 ± 0.9	0.5	9.6 ± 0.4	0.7	9.2 ± 0.3	0.7	18.5 ± 4.8	0.3	8.5 ± 0.3
5f	7.7 ± 0.4	1.0	8.2 ± 1.1	0.9	8.5 ± 0.5	0.9	8.4 ± 0.5	0.9	7.7 ± 0.1
5g	7.2 ± 0.4	1.3	7.3 ± 0.5	1.3	9.1 ± 0.7	1.0	16.2 ± 2.4	0.6	9.3 ± 0.6
5h	8.8 ± 0.6	3.2	8.8 ± 0.2	3.2	13.8 ± 0.3	2.0	35.1 ± 1.1	0.8	27.9 ± 1.8
5i	11.3 ± 0.1	2.8	17.3 ± 0.5	1.8	15.6 ± 3.4	2.0	29.3 ± 2.4	1.1	35.2 ± 9.5
5j	9.6 ± 0.3	2.4	15.4 ± 5.1	1.5	13.2 ± 1.3	1.7	9.8 ± 0.1	2.3	22.8 ± 2.7
5k	8.9 ± 0.1	1.7	8.9 ± 0.1	1.7	15.3 ± 0.7	1.0	29.3 ± 2.1	0.5	15.1 ± 2.1
6	7.4 ± 0.2	3.1	8.4 ± 0.3	2.7	9.8 ± 0.4	2.3	14.1 ± 0.4	1.6	22.8 ± 0.3
7	9.3 ± 0.1	1.6	14.8 ± 0.9	1.0	3.9 ± 0.2	3.9	8.9 ± 0.2	1.7	15.3 ± 0.6
doxorubicin ^b	0.6 ± 0.02	0.5	1.83 ± 0.1	0.2	0.63 ± 0.2	0.5	0.6 ± 0.02	0.5	0.29 ± 0.1

^a Data are expressed as mean $\pm$ SD; IC_{50} (μ M), the concentration of the compound that corresponds to a 50 % growth inhibition of the cell line (compared to the control) after culturing the cells for 72 h with the individual compound; SI (selectivity index) was calculated using the formula: $SI = IC_{50}$ for normal cell line (HaCaT)/ IC_{50} for respective cancer cell line (PC3, MDA-MB-231, A549, or HCT-116); PC3, human metastatic prostate cancer cell line; MDA-MB-231, human triple-negative breast cancer cell line; A549, human lung cancer cell line; HCT-116, human primary colon cancer cell line; HaCaT, human immortalized keratinocyte cell line.

^b The selected reference compound commonly used in cancer treatment.

deprotective reagent, the corresponding amide analogues of rearranged **IVR** and an additional hydroxyl at C4 position (compounds **5a–5k**, Scheme 1) were formed. For more reliable structure-activity relationship (SAR) studies, the synthesis of **6** as well as its corresponding rearranged aglycone **7** were also successfully accomplished (Scheme 1).

3.2. Antiproliferative activity

Despite the wide spectrum of biological activities of **IVR**, there are few articles devoted to its anticancer effects [4,7], and there are no reports on the potential of **IVR** derivatives in the fight against cancer cells. Therefore, the antiproliferative activity of **IVR** and its all derivatives was evaluated against four human cancer cell lines, i.e., PC3 (metastatic prostate cancer), MDA-MB-231 (triple-negative breast adenocarcinoma), A549 (lung cancer) and HCT-116 (primary colon cancer), using the MTT colorimetric assay (Table 1). Furthermore, to assess the potential of the compounds to selectively target cancer cells, an immortal keratinocyte cell line from adult human skin (HaCaT) was also included in the antiproliferative activity tests. Doxorubicin was used as a reference anticancer drug.

Firstly, chemically unmodified **IVR** showed good antiproliferative activity against all cancer cell lines tested, with IC_{50} values $< 10 \mu$ M (compound **1**, Table 1). With IC_{50} value of 10.8μ M against non-cancer HaCaT cell line, the calculated values of selectivity indexes (SI) for **IVR** slightly exceeded 1.0 in all cases (Table 1). After removing the sugar moiety, aglycone **2** showed generally lower antiproliferative activity compared to that of reference **IVR**, except for A549 cancer cell line ($IC_{50} = 9.9 \mu$ M, SI = 1.1 for **IVR** versus $IC_{50} = 8.4 \mu$ M, SI = 2.0 for **2**) (Table 1). Noteworthy, the key outcome is that the rearranged analogues of **IVR** and its aglycone (compounds **6** and **7**, respectively) showed significantly

improved antiproliferative activity compared to those of the native structures **1** or **2** (Table 1). The rearranged analogues **6** and **7** were also characterized by a more favorable selectivity of action than the corresponding starting materials (Table 1). Of particular interest in this context seems to be the activity of rearranged aglycone **7** against A549 cell line ($IC_{50} = 3.9 \mu$ M, SI = 3.9), which is superior to those of both unrearranged **IVR** and its aglycone **2** (Table 1).

Secondly, the introduction of an amide moiety at the C13 position had an ambiguous effect on the antiproliferative activity, depending on the cell line used and the type (chemical nature) of amide residue. Of note, amides **4f–4g** significantly inhibited the proliferation of cancer cell lines, especially HCT-116 colon cancer cell line (Table 1). With IC_{50} values in the low micromolar concentration range of 2.5–6.5 μ M and SI values of 2.3–4.4 (Table 1), these derivatives were up to four times more antiproliferative active and selective than chemically unmodified **IVR**.

Thirdly, for **IVR** amides, additional rearrangement of the oxahydrindene ring generally resulted in reduced antiproliferative activity. However, for the compound pairs **4f/5f** and **4g/5g**, an improvement in the antiproliferative potential was found against PC3, MDA-MB-231 and A549 cancer cell lines after modification of the oxahydrindene ring, while the values of SI parameters remained comparable with those of the non-rearranged amide analogues (Table 1).

Finally, although doxorubicin showed antiproliferative activity against all cancer cells in a very low micromolar concentration range, it was also highly cytotoxic towards HaCaT cells (Table 1). The calculated SI values for doxorubicin (SI = 0.2–0.5) indicate that it preferentially targets normal cells rather than the cancer cells used in the study. In this context, the advantages of the obtained **IVR** derivatives are their promising antiproliferative activity, together with high selectivity of action. With SI values > 3.0 (Table 1), selected **IVR** analogues should be

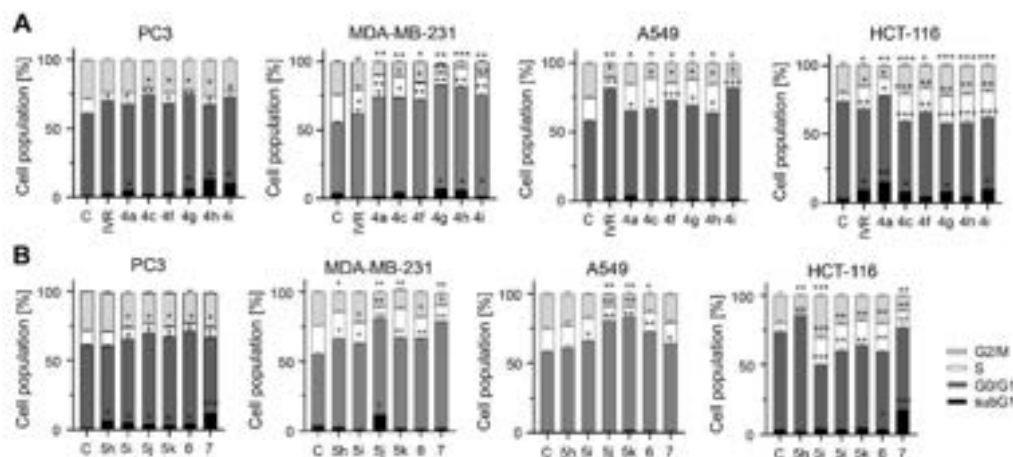


Fig. 2. Flow cytometry analysis of cell cycle parameters after incubation of PC3, MDA-MB-231, A549 and HCT-116 with (A) chemically unmodified ivermectin and its unarranged C13-*epi*-analogues (compounds 4a, 4c, 4f–4i) or (B) a series of rearranged ivermectin analogues (5h–5k, 6, 7) at their IC_{50} concentration for 24 h. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, as compared to the control (C).

considered as highly selective anticancer agents [28].

3.3. Induction of cell cycle arrest

Regulation of the cell cycle progression plays a crucial role in maintaining homeostasis in normal cells and healthy tissues [29]. Alterations in various cellular pathways, including those responsible for regulating the cell cycle machinery, are widely observed in several types

of cancer [30]. Such dysregulation may lead to inhibition of senescence and programmed death of cancer cells and, consequently, to uncontrolled proliferation and tumor growth [31]. Chemo- and radiotherapy aim to interrupt tumor growth, mainly by influencing the cell cycle control and inducing apoptosis [32,33]. Therefore, therapeutic approaches targeting the molecular machinery involved in this process have potential in cancer treatment [34], and the initiation of cell cycle arrest in cancer cells is recognized as a valuable approach to the

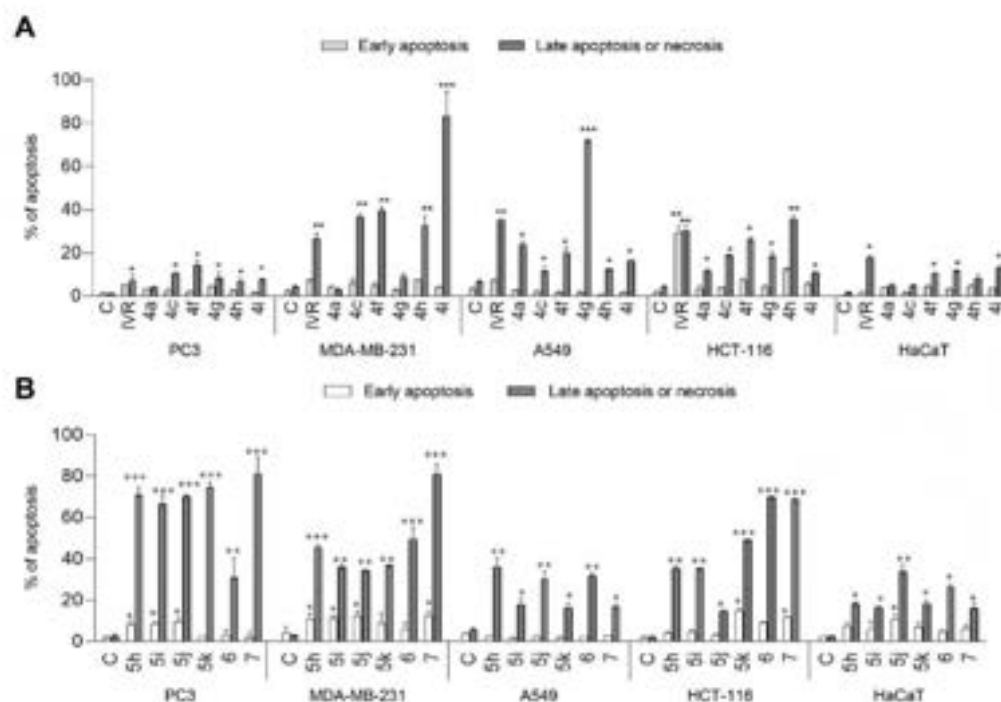


Fig. 3. The effects of (A) chemically unmodified ivermectin and its unarranged C13-*epi*-analogues (compounds 4a, 4c, 4f–4i) or (B) a series of rearranged ivermectin analogues (5h–5k, 6, 7) on early and late apoptosis or necrosis in PC3, MDA-MB-231, A549, HCT-116 and HaCaT cell lines. *** $p \leq 0.0001$, ** $p \leq 0.001$, * $p \leq 0.01$, as compared to the control (C).

development of new anticancer agents [35].

In view of the above, for deeper understanding of the anticancer potential of IVR and its most promising derivatives (4a, 4c, 4f–4i, 5h–5k, 6 and 7) in terms of their antiproliferative activity and selectivity against cancer cells (Table 1), we examined the cell cycle distribution after incubation of cancer cells with the compounds studied, using flow cytometry (Fig. 2).

Some studies have documented that IVR can induce cell cycle arrest, particularly in the G0/G1 phase, in certain cancer cells [36]. Specifically, IVR inhibited the cell cycle progression of C6 and U251 glioma cells, arresting them in the G0/G1 phase [36], while the drug arrested cell cycle in the S phase in cholangiocarcinoma and colon cancer cells [37,38]. We confirmed that chemically unmodified IVR induced cell cycle arrest mainly in the G0/G1 phase in all cancer cells used, particularly in PC3 and A549 cancer cells, while in MDA-MB-231 and HCT-116 cancer cells, the percentage of cell population in the S phase was slightly increased after IVR treatment compared to that in PC3 and A549 cancer cells (Fig. 2A).

Analysis of the cell cycle arrest in PC3, MDA-MB-231 and A549 cancer cells treated with both unrearranged and rearranged IVR derivatives revealed that the compounds increased the G0/G1 cell population by from 3 to 13, 10 to 24, and 3 to 23 times, respectively (Fig. 2). Incubation of PC3 cancer cells with compounds 4a, 4g–4i, 5h, 5i, 5k, 6 and 7 increased the percentage of cell population in the subG1 phase (from 1.3 % to 10.9 %) and slightly decreased that in the S phase (Fig. 2). On the other hand, in HCT-116 cancer cells, the cell cycle arrest in the S/G2/M phase was more frequently observed, with an increase in the cell population by 10–13 times, with the exception of compound 5h (Fig. 2). The derivatives of IVR induced the G0/G1 phase arrest in MDA-MB-231, A549 and PC3 cancer cells, and S/G2/M in HCT-116 cancer cells (Fig. 2). Collectively, our findings suggest a potential therapeutic role of IVR derivatives in modulating cell cycle dynamics in specific cancer types.

3.4. Induction of apoptosis

Anticancer agents can trigger various mechanisms of cell death [39]. Apoptosis is the predominant form of programmed cell death of cancer cells that is often induced by treatment with chemotherapeutics [40]. Therefore, apoptotic pathways are considered to be extremely important targets in the context of designing and development of new anticancer agents. Some previous studies have shown that apoptosis could be the main form of cancer cell death induced by IVR, including colorectal cancer, ovarian cancer, leukemia and esophageal squamous cell carcinoma [38,41–43]. To thoroughly investigate the potential of IVR and its derivatives to induce apoptosis, the Annexin V-FITC/PI binding bioassay was used, evaluating potential proapoptotic effects of selected compounds (4a, 4c, 4f–4i, 5h–5k, 6 and 7) against cancer cells (PC3, MDA-MB-231, A549, HCT-116) or non-cancer cells (HaCaT) (Table 1).

Briefly, cancer cells were treated with the compounds studied at their respective IC_{50} concentrations. Nevertheless, given the remarkable selectivity of IVR derivatives for targeting cancer cells (Table 1), the cytotoxic concentrations of the compounds were significantly lower against normal cells compared to those towards cancer cells. Thus, HaCaT cells were exposed to these compounds concentrations corresponding to the lowest IC_{50} values observed for cancer cells, such as 7.6 μ M for 4a/4c, 8.8 μ M for 5h, and 3.9 μ M for 7 (Table 1).

As shown in Fig. 3, the tested compounds induced late apoptosis/necrosis significantly stronger in cancer cells compared to the control (untreated cells). Moreover, the apoptosis-activating effects were stronger in cancer cells compared to normal HaCaT cells (Fig. 3). The analogues of rearranged IVR (5h–5k, 6 and 7) showed generally stronger proapoptotic activity in all cancer cell lines compared to that of the native oxahydrindene ring (4a, 4c and 4f–4i) (Fig. 3). Specifically, PC3 cancer cells were most susceptible to the proapoptotic action of the rearranged derivatives, resulting in a significantly higher percentage of cells in late apoptosis or necrosis (35 %–80 %, Fig. 3B) than those treated

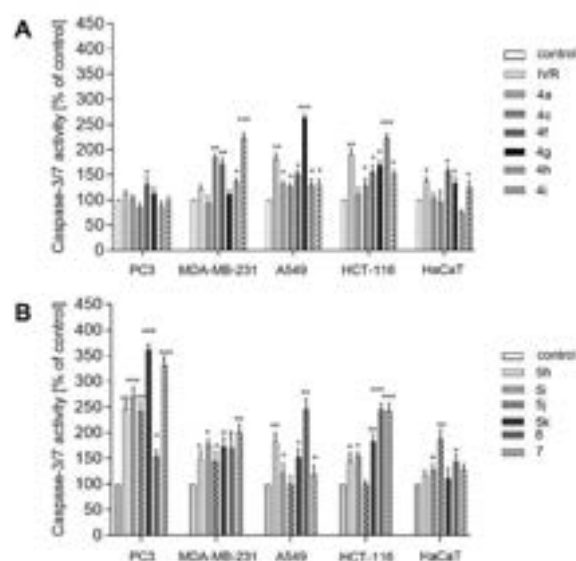


Fig. 4. The effects of (A) chemically unmodified ivermectin and its unrearranged C13-epi-analogs (compounds 4a, 4c, 4f–4i) or (B) a series of rearranged ivermectin analogues (5h–5k, 6, 7) on caspase-3/7 activity. Cells were incubated with the compounds studied at their IC_{50} concentrations for 24 h. Data are expressed as % of control and as the mean $\pm$ SD. *** $p \leq 0.0001$, ** $p \leq 0.001$, * $p \leq 0.01$, as compared to the control.

with IVR or its unrearranged counterparts (2 %–30 %) (Fig. 3A). In MDA-MB-231 cancer cells, the strongest late apoptosis or necrosis-inducing effects were found for compounds 5h, 6 and 7 with the rearranged oxahydrindene ring (46 %, 49 % and 81 %, respectively, Fig. 3B) and unrearranged amide 4i (83 %, Fig. 3A). On the other hand, the strongest late apoptotic or necrotic activity was found to be induced by compound 4g in A549 cancer cells (72 %, Fig. 3A), while in HCT-116 cancer cells, the most promising compounds were again the rearranged derivatives 5k, 6 and 7 (49 %, 69 % and 68 %, respectively, Fig. 3B).

Our study confirmed the proapoptotic activity of chemically unmodified IVR, especially in terms of inducing late apoptosis or necrosis in the MDA-MB-231, A549 and HCT-116 cancer cell lines (Fig. 3A), but in many cases, it was a weaker activator of apoptosis compared to the tested derivatives. Furthermore, it was found that IVR derivatives are generally weaker activators of early and late apoptosis in HaCaT cells compared to cancer cells, which seems to be a good prognostic and confirms their possible therapeutic potential.

3.5. Induction of caspase-3/7 activity

As an imbalance in the survival of cancer cells is associated with such processes as malignant transformation and drug resistance, a key pharmacological strategy is to restore apoptosis in cancer cells [44]. The goals of anticancer interventions may be achieved through a wide range of strategies, including not only cell cycle manipulation, but also regulation of pro- and anti-apoptotic proteins, levels of reactive oxygen species, and control of interleukin-6 concentration [45–49].

Caspase family proteins play a pivotal role in the regulation of programmed cell death [50]. Among them, caspase-3 and caspase-7 are considered key players in the implementation of mitochondrial apoptosis pathways [51]. Their primary function includes the degradation of various regulatory and structural proteins that are essential for the precise control and execution of apoptosis [52]. Therefore, to further investigate the mechanisms of the proapoptotic effect of IVR and its

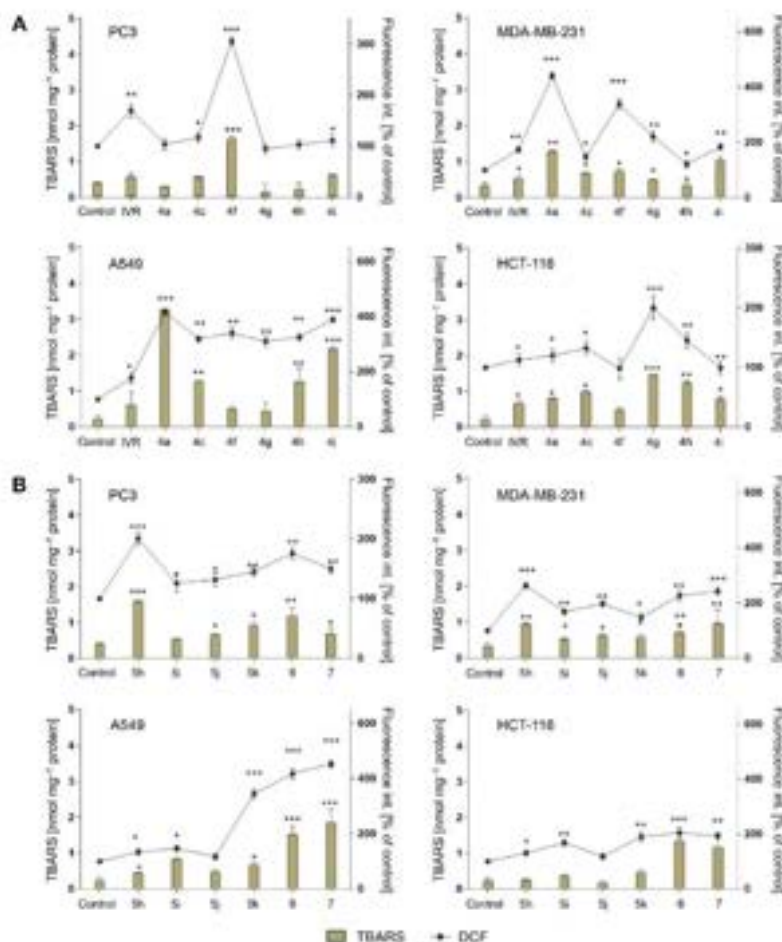


Fig. 5. ROS production and lipid peroxidation in PC3, MDA-MB-231, A549 and HCT-116 cancer cells treated with (A) chemically unmodified ivermectin and its rearranged C13-epi-analogs (compounds 4a, 4c, 4f–4i) or (B) a series of rearranged ivermectin analogues (5h–5k, 6, 7). The results are expressed as mean $\pm$ SD. *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$, as compared to the control.

selected derivatives (4a, 4c, 4f–4i, 5h–5k, 6 and 7) on cancer cells (Fig. 3), the activity of caspase-3/7 was examined after exposing PC3, MDA-MB-231, A549 and HCT-116 cancer cells to the compounds tested at their IC_{50} concentration (Fig. 4).

Our study showed that almost all compounds triggered apoptosis through the caspase-dependent pathway. For chemically unmodified IVR, this is consistent with the reports of other authors who have demonstrated a similar proapoptotic effect of this compound [38,53]. Exposure of all cancer cells to the tested compounds resulted in a significant increase in caspase-3/7 activity (Fig. 4), suggesting their clear effect on enhancing the activation of key apoptotic enzymes in cancer cells. A particularly strong increase in enzyme levels was observed in PC3 cancer cells treated with IVR derivatives with the rearranged oxahydrindene ring, especially compounds 5k and 7 (361 % and 329 %, respectively, Fig. 4B). On the other hand, treatment of PC3 cancer cells with unrearranged derivatives 4a, 4c and 4f–4i showed lower levels of caspase induction (Fig. 4A), which, combined with the low proapoptotic activity of these compounds against prostate cancer cells (Fig. 3A), suggests a non-apoptotic mechanism of their cytotoxic action.

Most of the derivatives strongly increased caspase-3/7 activity in MDA-MB-231, A549 and HCT-116 cancer cells (Fig. 4). Compounds 4g,

4h and 4i caused an >200 % increase in caspase activity compared to control, depending on the cell line used (Fig. 4A). In A549 and HCT-116 cancer cells exposed to compounds 6 and 7, an increase in executive caspase activity of 241 %–246 % was observed (Fig. 4B). Caspase stimulation in non-cancer HaCaT cells incubated with compounds 4a, 4c and 4f–4i was comparable to that in PC3 cancer cells (Fig. 4A). Notably, the rearranged IVR derivatives 5h–5k, 6 and 7 were found to be overall weaker caspase activators in HaCaT cells compared to their activity in all cancer cells used (Fig. 4B), indicating their promising anticancer potential.

3.6. ROS production and lipid peroxidation

Numerous studies have consistently highlighted the importance of modulating the reactive oxygen species (ROS) levels in various types of cancer cells as a fundamental strategy for inducing apoptosis by anti-cancer drugs [47]. In this context, it has been demonstrated that IVR can upregulate the production of ROS in cancer cells, leading to their death [53].

Therefore, to further elucidate the mechanism of cytotoxic action of IVR and its selected derivatives, we examined the effects of the

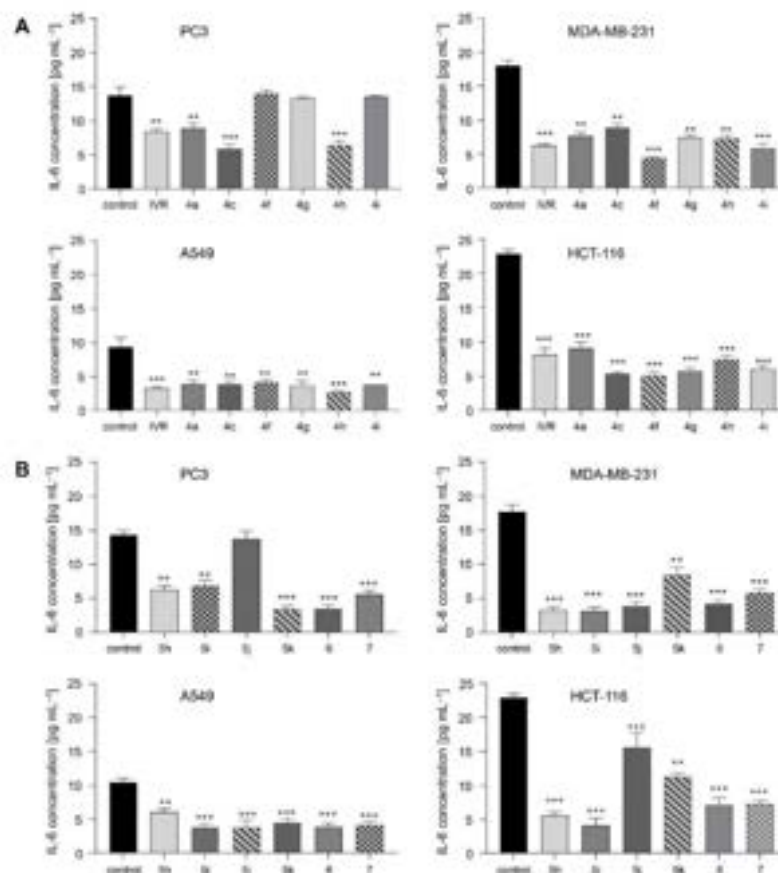


Fig. 6. The effects of (A) chemically unmodified ivermectin and its unrearranged C13-epi-amides (compounds 4a, 4c, 4f–4i) or (B) a series of rearranged ivermectin analogues (5h–5k, 6, 7) on IL-6 levels in PC3, MDA-MB-231, A549 and HCT-116 cancer cells, measured by the ELISA test. Data are expressed as the mean $\pm$ SD. *** $p < 0.0001$, ** $p < 0.001$, * $p < 0.01$, as compared to the control.

compounds on ROS production (Fig. 5). We also evaluated the peroxidation of cell membrane lipids, as a possible consequence of the ROS-producing properties of compounds used (Fig. 5). To evaluate these parameters, both cancer and non-cancer cells were treated with IVR and its unrearranged (4a, 4c, 4f–4i) or rearranged derivatives (5h–5k, 6, 7) at concentrations equal to their respective IC_{50} values. ROS production was measured spectrofluorometrically using 2',7'-dichlorodihydrofluorescein diacetate, while lipid peroxidation was determined by the formation of thiobarbituric acid reactive substances (TBARS) in the TBA assay.

Briefly, the dynamics and levels of ROS and TBARS parameters varied among different cancer types. We confirmed that chemically unmodified IVR upregulated the production of ROS in all cancer cells (Fig. 5A). Importantly, this effect can be further improved with IVR derivatives, depending on the cell line used and the type of chemical modification of the native structure. For instance, all unrearranged derivatives (4a, 4c, 4f–4i), along with selected rearranged analogues 5k, 6 and 7, significantly increased ROS levels in A549 lung cancer cells (Fig. 5). Compared with IVR, overall high levels of ROS were observed in PC3 and MDA-MB-231 cancer cells treated with its rearranged derivatives (Fig. 5B). These compounds were also identified as the most potent activators of late apoptosis or necrosis (Fig. 3B), suggesting that the cytotoxic effect may be achieved through ROS-induced apoptosis.

On the other hand, the reduced concentration of TBARS observed in

HCT-116 cancer cells, particularly for rearranged derivatives 5h–5k (Fig. 5B) compared to their concentration in the other cancer cell lines, may indicate that an effective mechanism of protection against lipid peroxidation exists in these primary colon cancer cells. Nevertheless, ROS production correlated with increased TBARS formation (Fig. 5). For example, compounds 4f and 5h (in PC3 cancer cells), and 4a, 4i, 6 and 7 (in A549 cancer cells) exerted the strongest ROS generation with the highest TBARS concentration among all cancer cells.

3.7. Inhibition of interleukin-6

The occurrence of interleukin-6 (IL-6) within the tumor microenvironment correlates with the advancement of cancer, metastatic potential and resistance to chemotherapy [54]. Abnormal expression and dysregulation of IL-6 are seen in many types of cancer, including prostate, breast, lung and colon cancer [55]. Thus, inhibition of IL-6 activity appears to be a potential therapeutic approach in the treatment of tumors that show pathological overproduction of cytokine [56]. IVR has been found to be involved in the regulation of cellular and humoral immune responses in many diseases, including those based on bacterial, viral and parasitic attack or cancer [57,58]. In addition, IVR inhibits the synthesis of inflammation-related proteins, such as TNF- α , IL-1 and IL-6 [54,59].

In our study, we investigated the ability of IVR and its selected

derivatives (4a, 4c, 4f–4i, 5h–5k, 6 and 7) to inhibit IL-6 production in cancer cell lines (Fig. 6). A significant inhibitory effect of IVR and its unrearranged derivatives 4a, 4c and 4f–4i) on IL-6 production was observed in MDA-MB-231, A549 and HCT-116 cancer cells compared to the control (Fig. 6A). We observed a similar positive effect after treatment of all four cancer cell lines with the rearranged derivatives 5h–5k, 6 and 7, except compound 5j in PC3 and HCT-116 cancer cells (Fig. 6B). Of note, MDA-MB-231 cancer cells were found to be most sensitive to inhibition of IL-6 release after treatment with both unrearranged and rearranged IVR derivatives by 60 %–80 % (Fig. 6). On the other hand, the release of IL-6 was the least strongly inhibited in PC3 cancer cells (Fig. 6).

4. Conclusions

To sum up, for the first time, we thoroughly evaluated the *in vitro* anticancer activity of a number of ivermectin (IVR) derivatives, i.e., a series of its C13- α -amide derivatives with native or rearranged oxahydriodene (hexahydrobenzofuran) ring. Our studies showed that compounds exhibited varying levels of antiproliferative effectiveness on the human cancer cells tested. Importantly, the rearranged derivatives of IVR (compound 6) and its aglycone (compound 7) showed significantly improved ability to inhibit proliferation of cancer cells compared to those of the corresponding native structures. Both analogues 6 and 7 also demonstrated a more favorable cancer cell-targeting selectivity than the reference compounds, including a commonly used anticancer drug doxorubicin. In the group of C13- α -amides of unrearranged and rearranged IVR, we also identified numerous products with improved antiproliferative activity and/or selectivity compared to those of the reference compounds.

IVR derivatives increased the percentage of PC3 (human metastatic prostate cancer), MDA-MB-231 (human triple-negative breast cancer) and A549 (human lung cancer) cancer cells in the subG1 and G0/G1 phases and reduced the cell population in S and G2/M phases, whereas in HCT-116 (human primary colon cancer), the cell cycle arrest was increased in S/G2/M phase. The mechanisms underlying the anticancer effects of the compounds tested were diverse in the cancer cells examined, including induction of apoptosis, generation of reactive oxygen species (ROS), and lipid peroxidation. We showed that IVR derivatives may inhibit cell growth and induce apoptosis via mitochondria-dependent ROS production. Furthermore, most compounds acted as promising interleukin-6 (IL-6) inhibitors in cancer cells.

The results of this study suggest that unrearranged and rearranged derivatives of IVR are suitable lead structures for the rational design of new anticancer agents. Taking into account the promising anticancer activity and selectivity of action of numerous compounds, as well as the practical and scalable synthetic approach ensuring convenient access to the material, further development of these first-in-class derivatives of IVR should be of great interest.

CRediT authorship contribution statement

Michał Sulik: Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Conceptualization. **Dagmara Otto-Ślusarczyk:** Writing – original draft, Validation, Methodology, Investigation. **Michał Antoszczyk:** Writing – review & editing, Writing – original draft, Methodology, Data curation, Conceptualization. **Marta Struga:** Writing – review & editing, Validation, Supervision. **Adam Huczyński:** Writing – review & editing, Supervision, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ejmepr.2024.100176>.

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Supplementary material

Ivermectin and its synthetic derivatives – A new class of anticancer agents

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General procedures

All reagents, except Dess-Martin periodinane (DMP), were purchased from two sources – Merck or Trimem Chemicals S.A., and used without further purification. CDCl_3 spectral grade was stored over 3Å molecular sieves for several days. Reaction mixtures were stirred using Teflon-coated magnetic stir bars and were monitored by thin-layer chromatography (TLC) using aluminum-backed plates (Merck 60 F₂₅₄). TLC plates were visualized by UV light (254 nm), followed by treatment with phosphomolybdic acid (PMA) (5% in absolute ethanol) and gentle heating. Products of the reactions were purified using CombiFlash® Rf+ Lumen Flash Chromatography System (Teledyne Isco) with integrated ELS and UV detectors. All solvents used in flash chromatography were of HPLC grade (Merck) and were used as received. Solvents were removed using a rotary evaporator.

NMR spectra were recorded on a Varian 400 (^1H NMR at 400 MHz and ^{13}C NMR at 101 MHz) magnetic resonance spectrometer. ^1H NMR spectra are reported in chemical shifts downfield from TMS using the respective residual solvent peak as the internal standard (CDCl_3 δ 7.26 ppm). ^1H NMR spectra are reported as follows: chemical shift (δ , ppm), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, p = quintet/pentet, dd = doublet of doublets, dt = doublet of triplets, dp = doublet of quintets/pentets, ddd = doublet of doublet of doublets, ddt = doublet of doublet of triplets, dtd = doublet of triplet of doublets, dddd = doublet of doublet of doublet of doublets, td = triplet of doublets, tdd = triplet of doublet of doublets, tt = triplet of triplets, qd = quartet of doublets, m = multiplet), coupling constant(s) in Hz, and integration. Significant peaks are reported within the overlapping ~2.00–0.50 ppm region of the ^1H NMR spectra. ^{13}C NMR spectra are reported in chemical shifts downfield from TMS using the respective residual solvent peak as the internal standard (CDCl_3 δ 77.16 ppm). Line broadening parameters were 0.5 or 1.0 Hz, while the error of chemical shift value was 0.1 ppm.

Electrospray ionization (ESI) high-resolution mass spectra were recorded on a QTOF (Impact HD, Bruker Daltonics) mass spectrometer in the positive ion detection mode. Samples were prepared in dry acetonitrile. The mass range for ESI experiments was from $m/z = 300$ to $m/z = 1000$.

Spectroscopic and spectrometric analysis of ivermectin derivatives

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Figure S35. The ^{13}C NMR spectrum of 5g in chloroform-d.....	S22

Figure S36. The ^1H NMR spectrum of 5g in chloroform-d.....	S22
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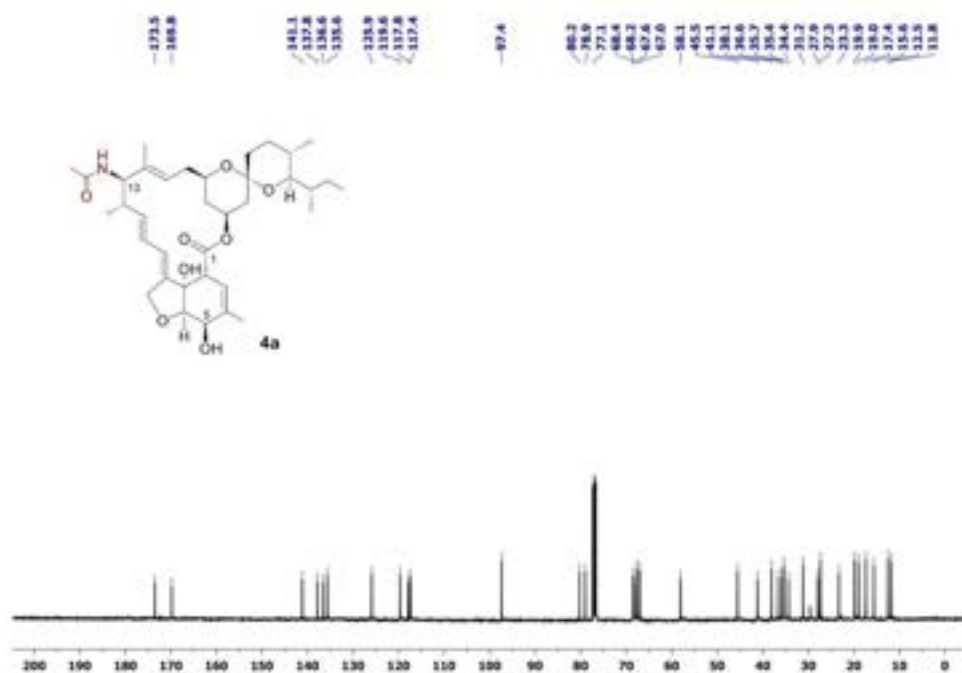


Figure S1. The ^{13}C NMR spectrum of **4a** in chloroform- d .

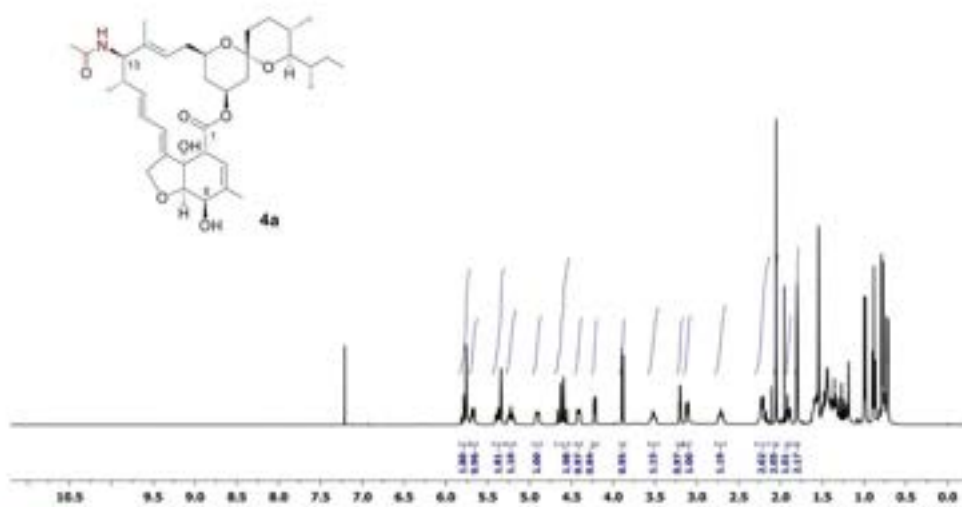


Figure S2. The ^1H NMR spectrum of **4a** in chloroform- d .

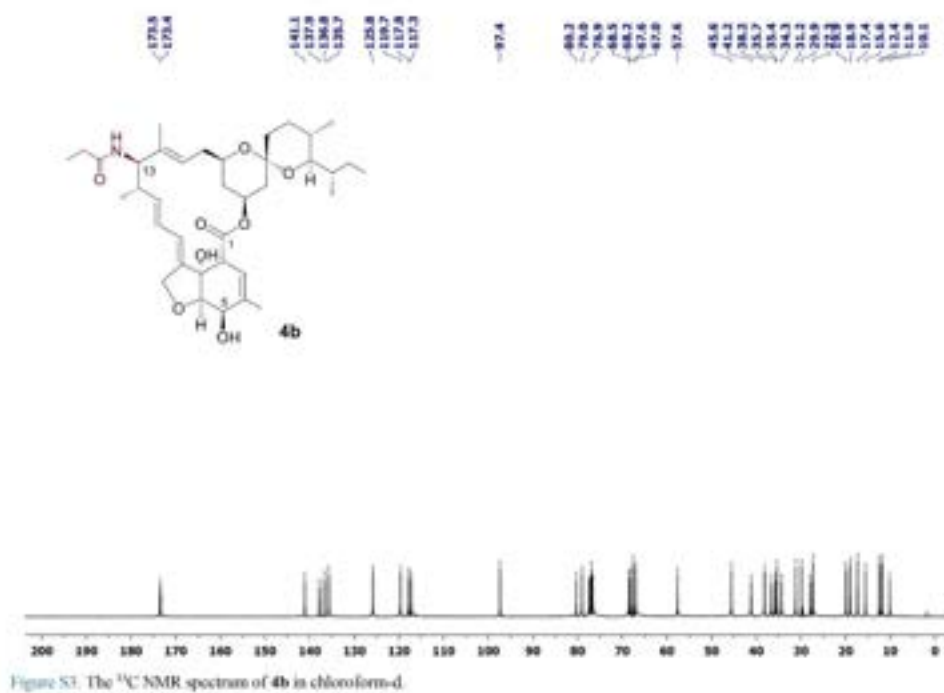


Figure S3. The ^{13}C NMR spectrum of **4b** in chloroform- d .

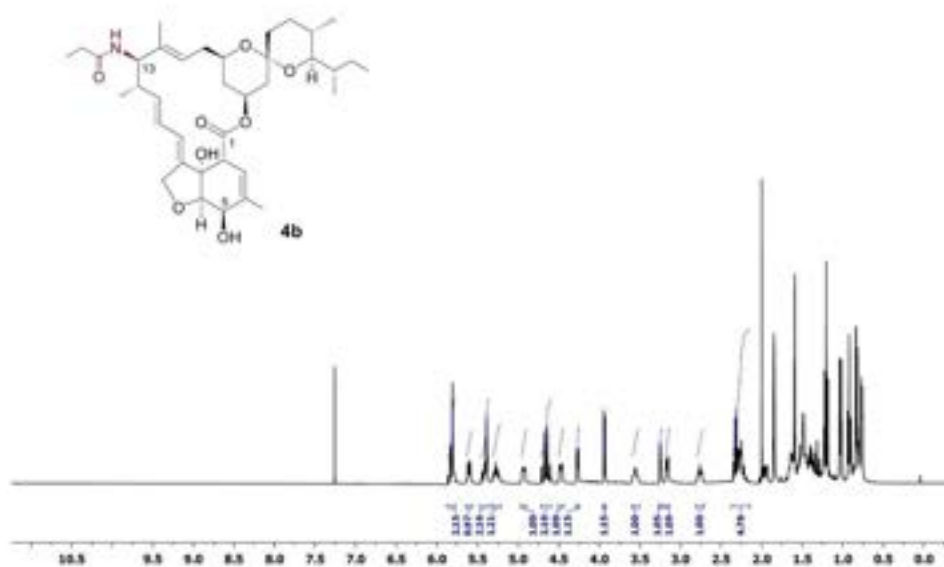
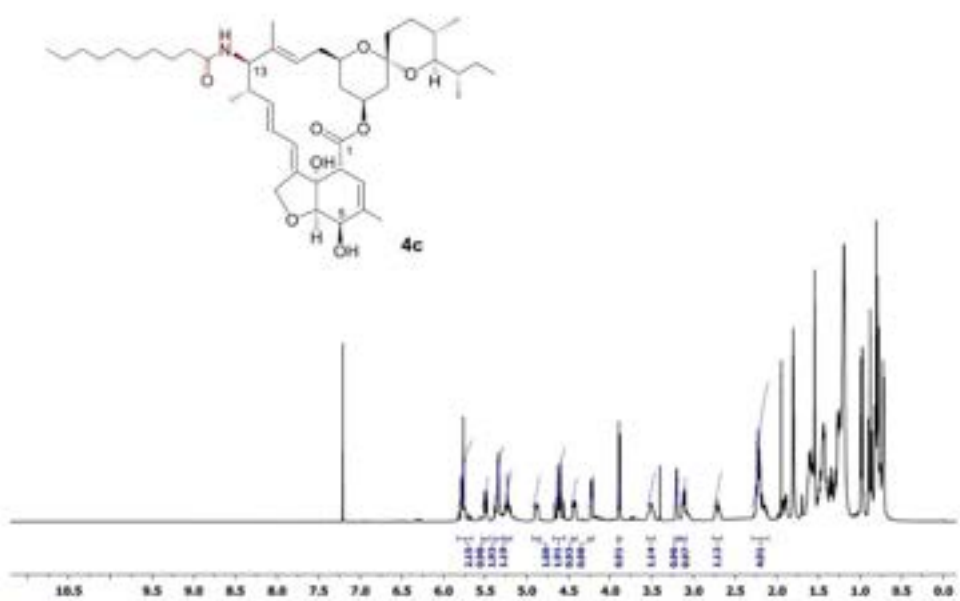
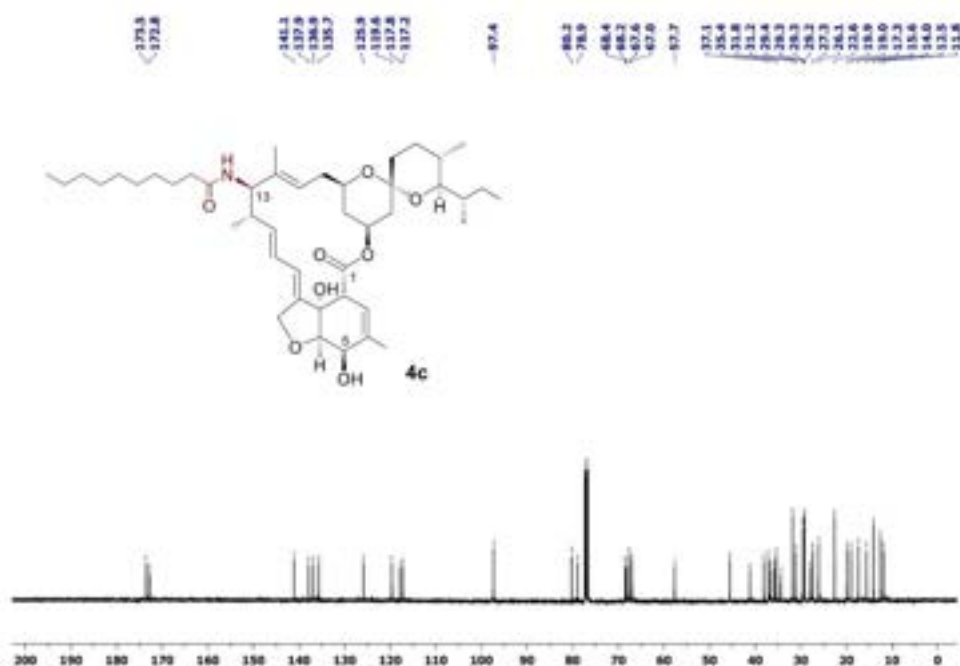
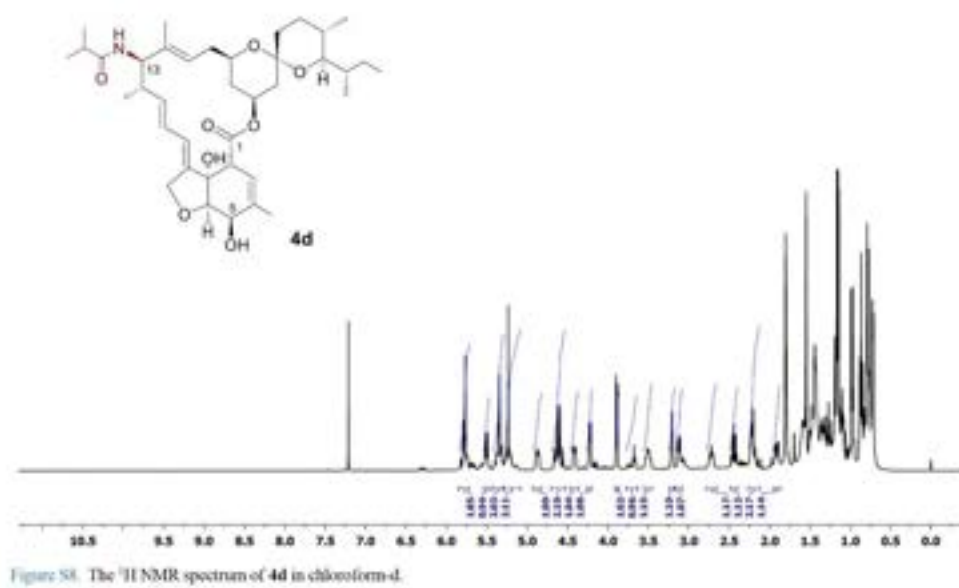
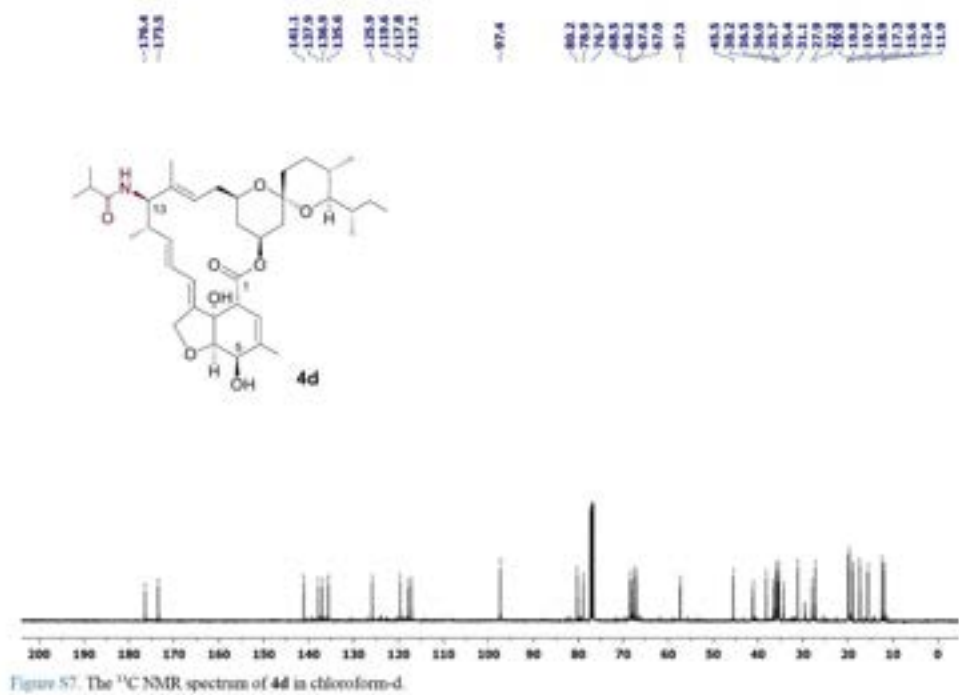
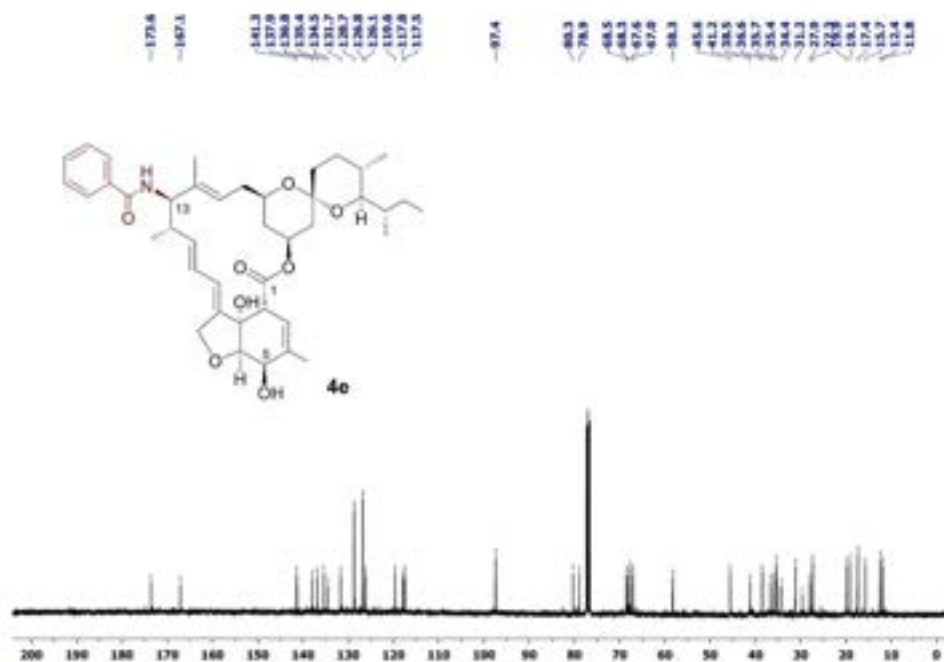
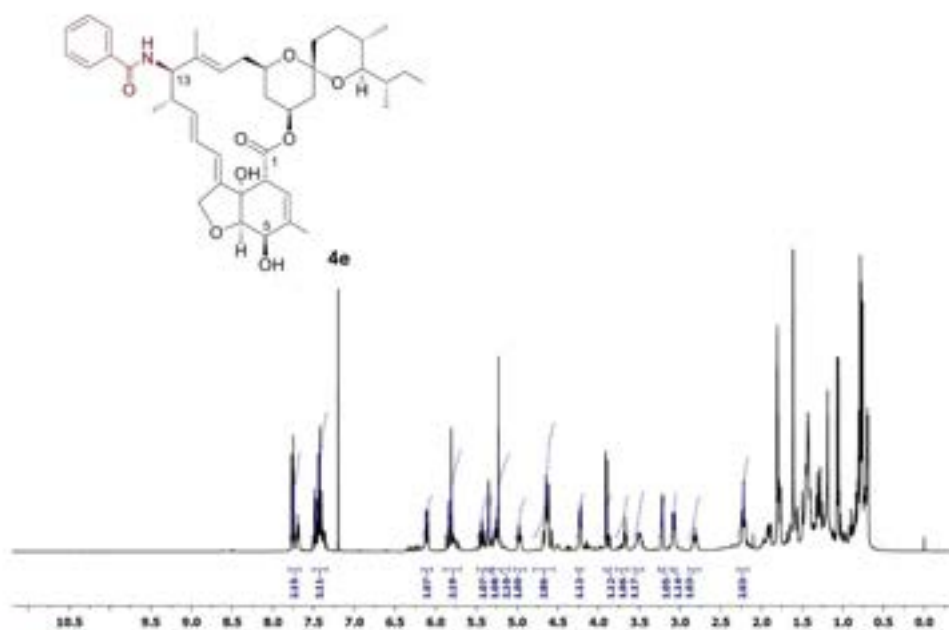


Figure S4. The ^1H NMR spectrum of **4b** in chloroform- d .





Figure S9. The ^{13}C NMR spectrum of **4e** in chloroform- d .Figure S10. The ^1H NMR spectrum of **4e** in chloroform- d .

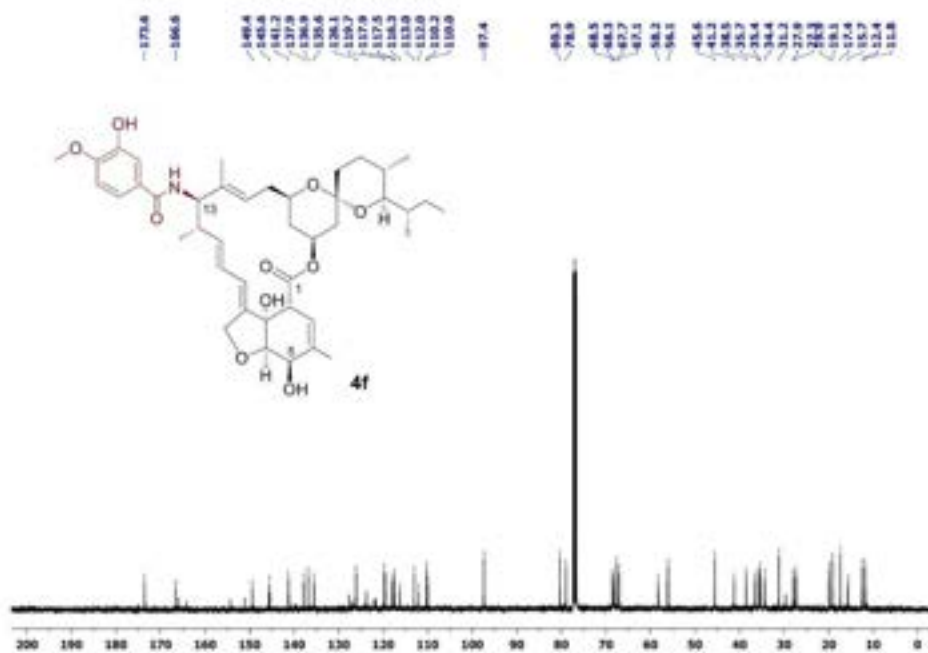


Figure S11. The ^{13}C NMR spectrum of 4f in chloroform-d.

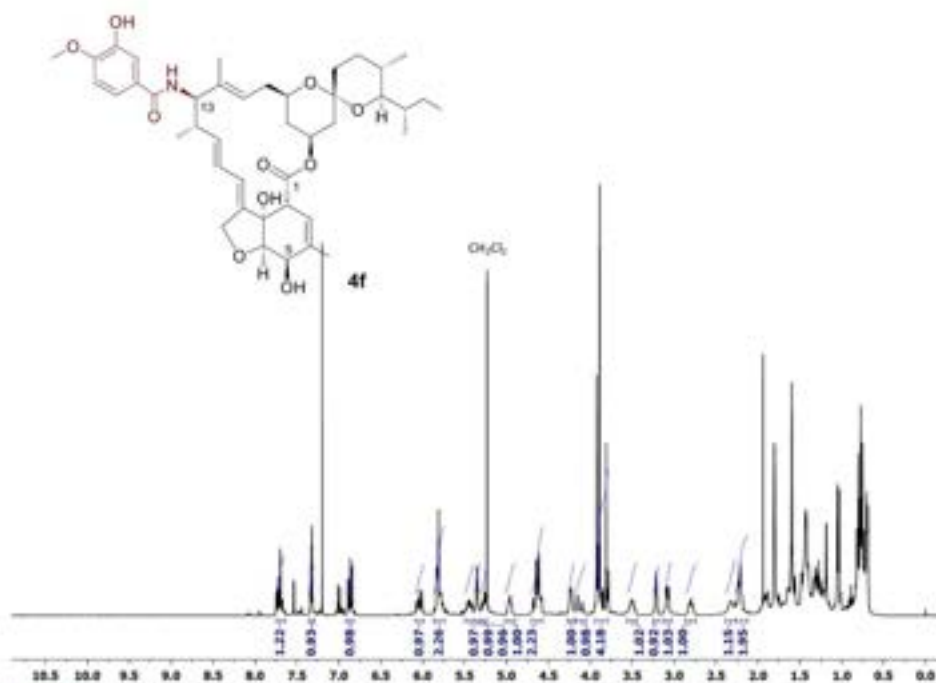
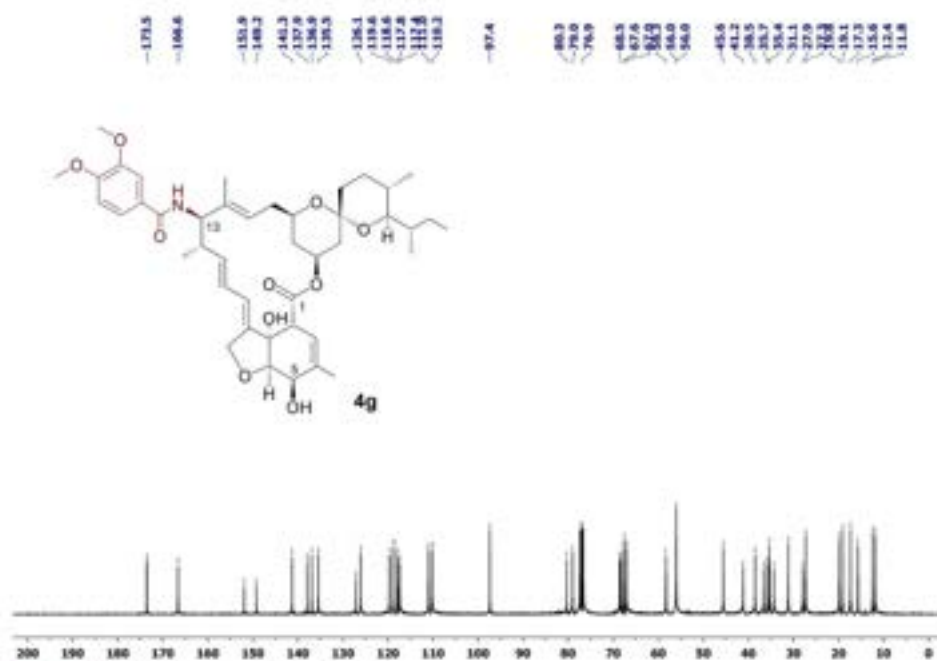
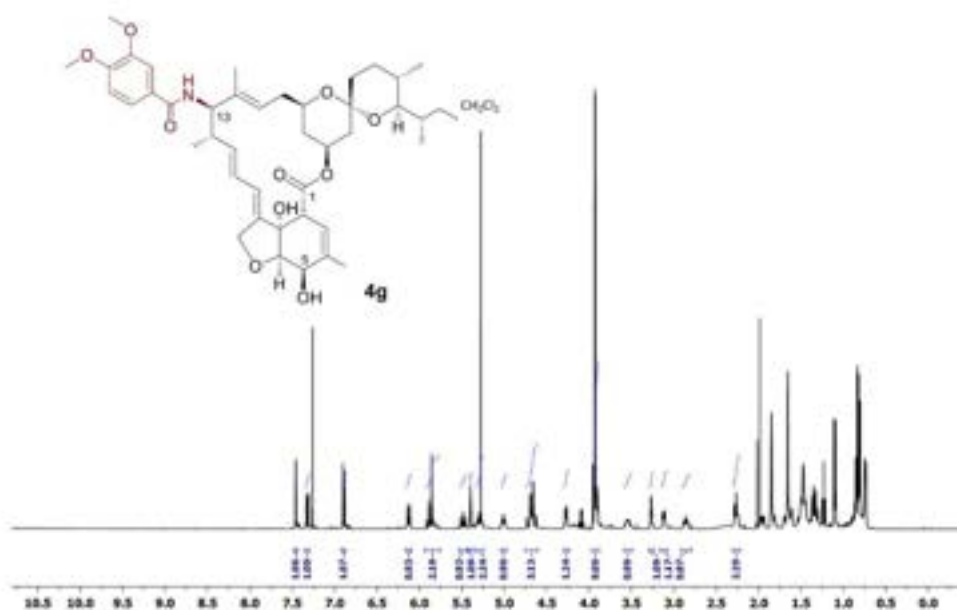


Figure S12. The ^1H NMR spectrum of 4f in chloroform-d.

Figure S13. The ^{13}C NMR spectrum of 4g in chloroform-d.Figure S14. The ^1H NMR spectrum of 4g in chloroform-d.

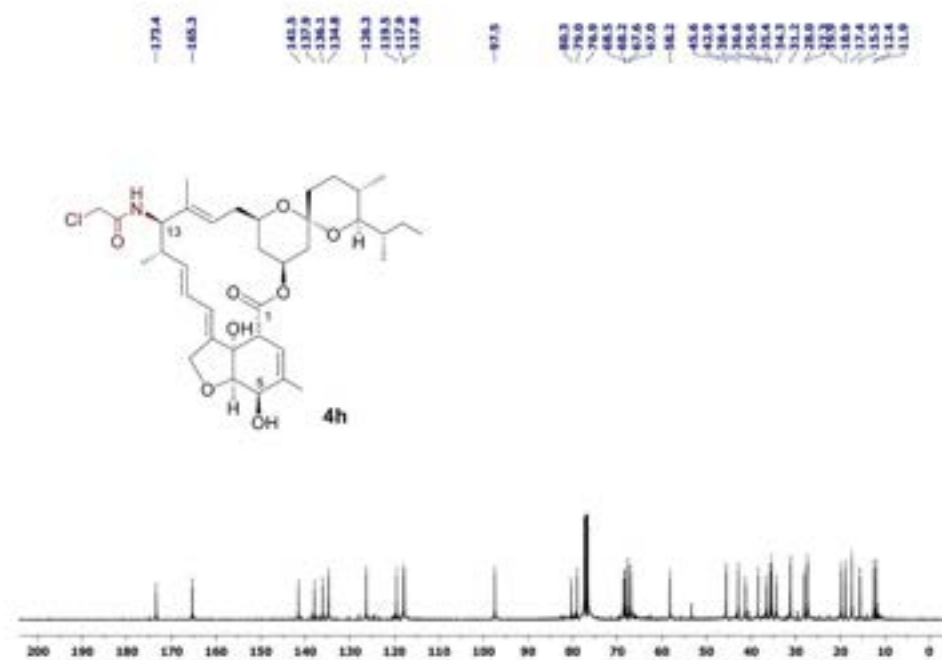


Figure S13. The ^{13}C NMR spectrum of **4h** in chloroform- d .

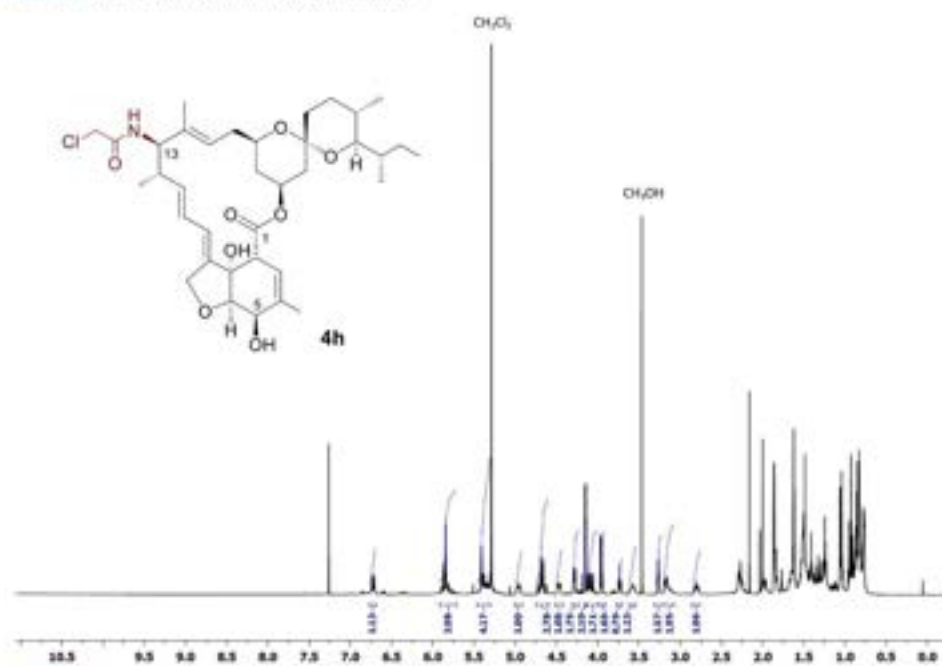


Figure S16. The ^1H NMR spectrum of **4h** in chloroform- d .

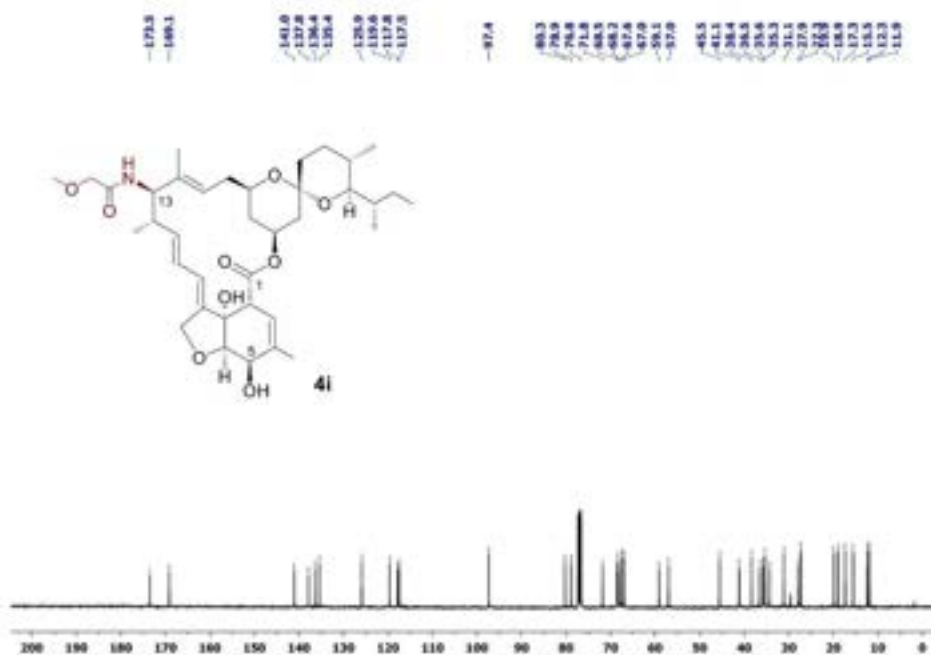


Figure S17. The ^{13}C NMR spectrum of 4i in chloroform- d .

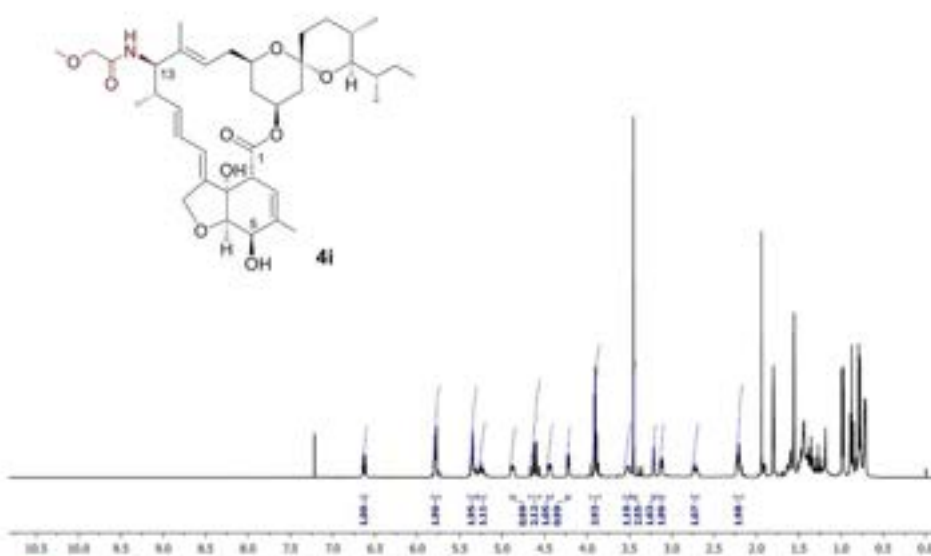


Figure S18. The ^1H NMR spectrum of 4i in chloroform- d .

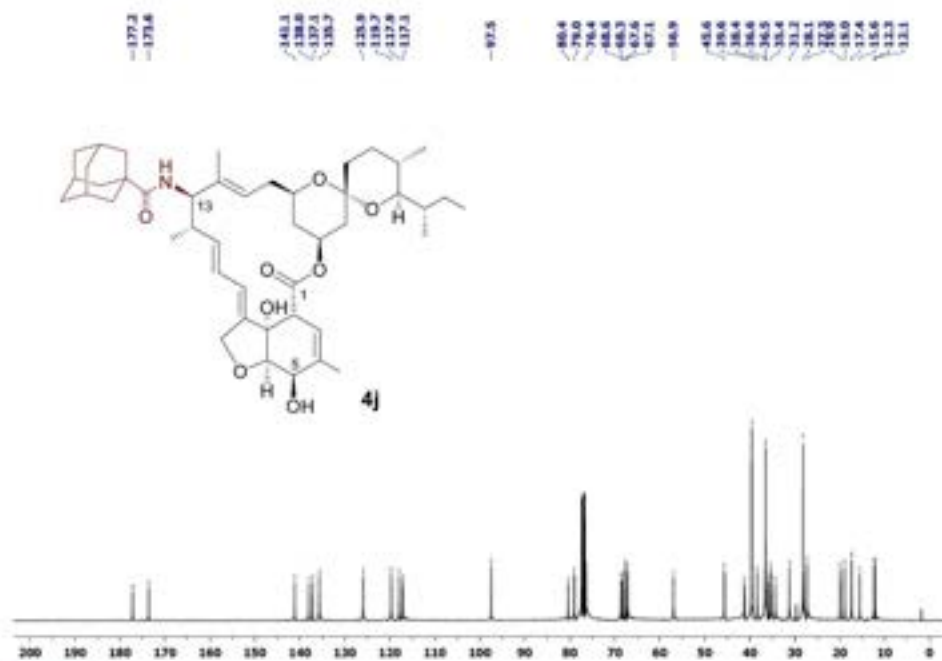


Figure S19. The ^{13}C NMR spectrum of 4j in chloroform-d.

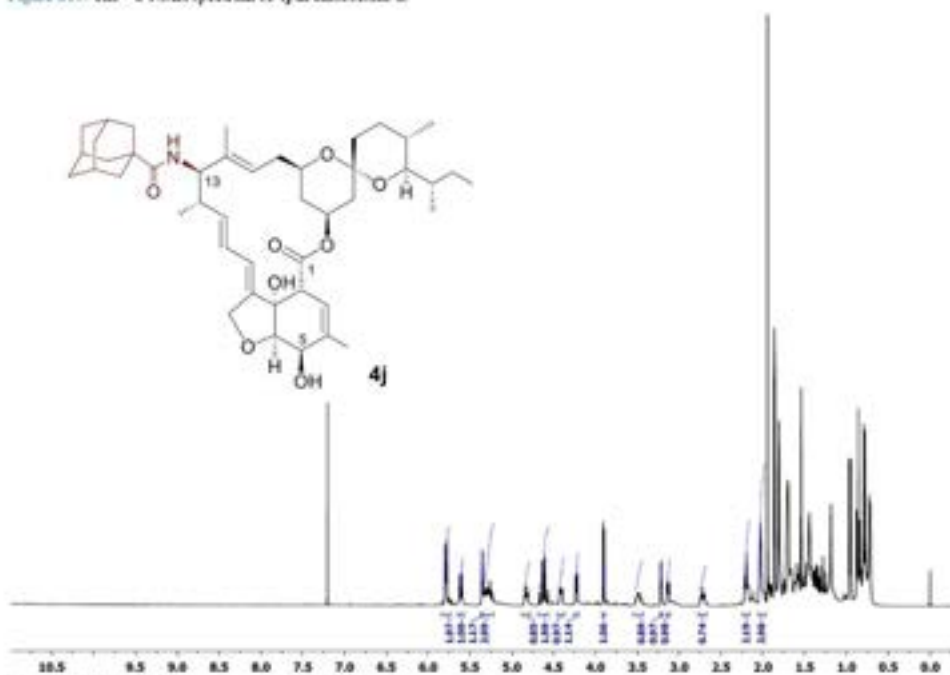


Figure S20. The ^1H NMR spectrum of 4j in chloroform-d.

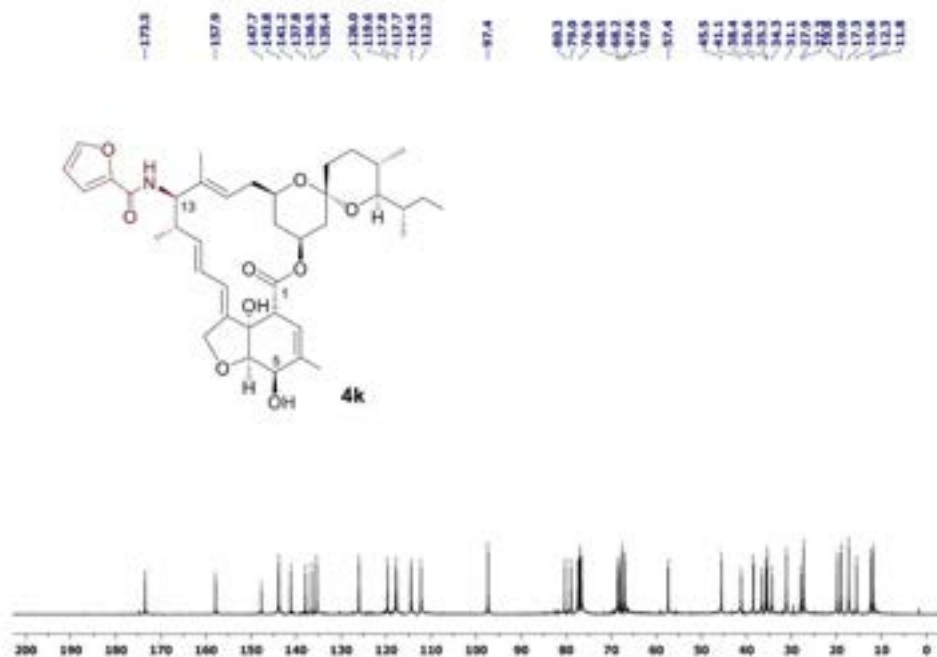


Figure S21. The ^{13}C NMR spectrum of 4k in chloroform-d.

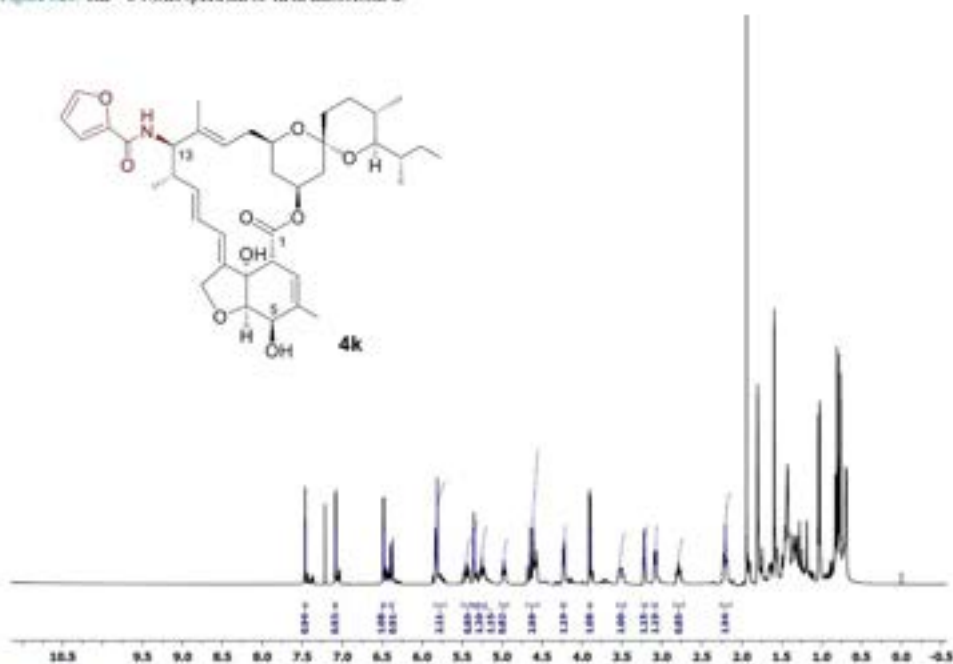


Figure S22. The ^1H NMR spectrum of 4k in chloroform-d.

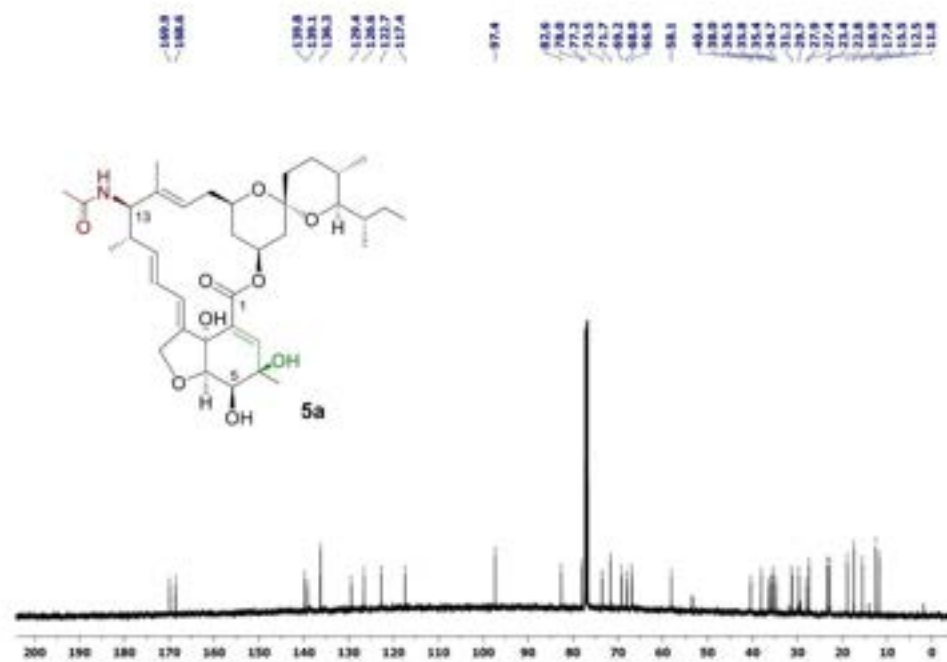


Figure S23. The ^{13}C NMR spectrum of **5a** in chloroform- d .

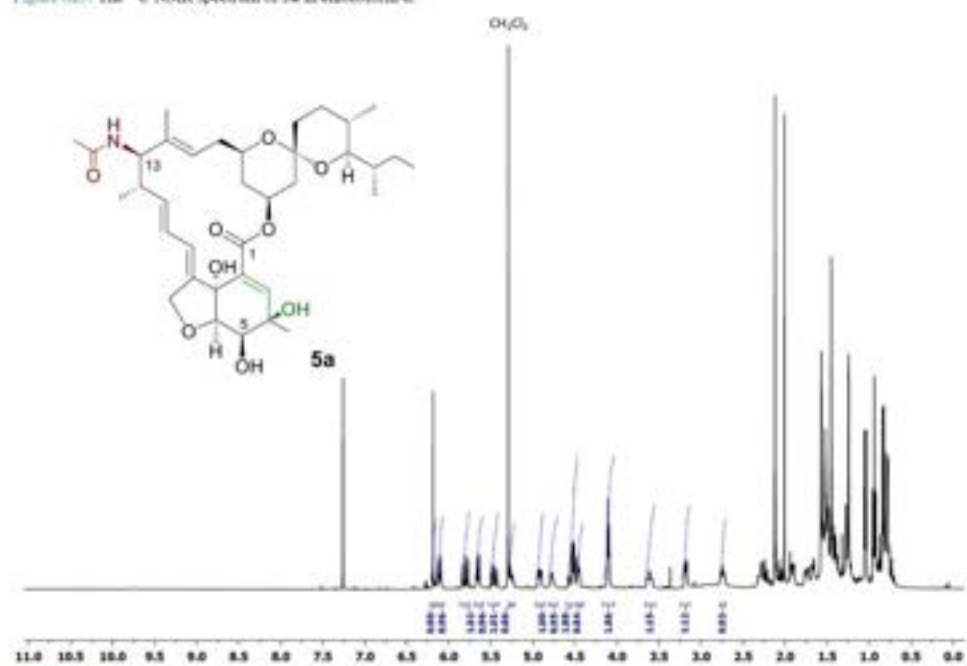
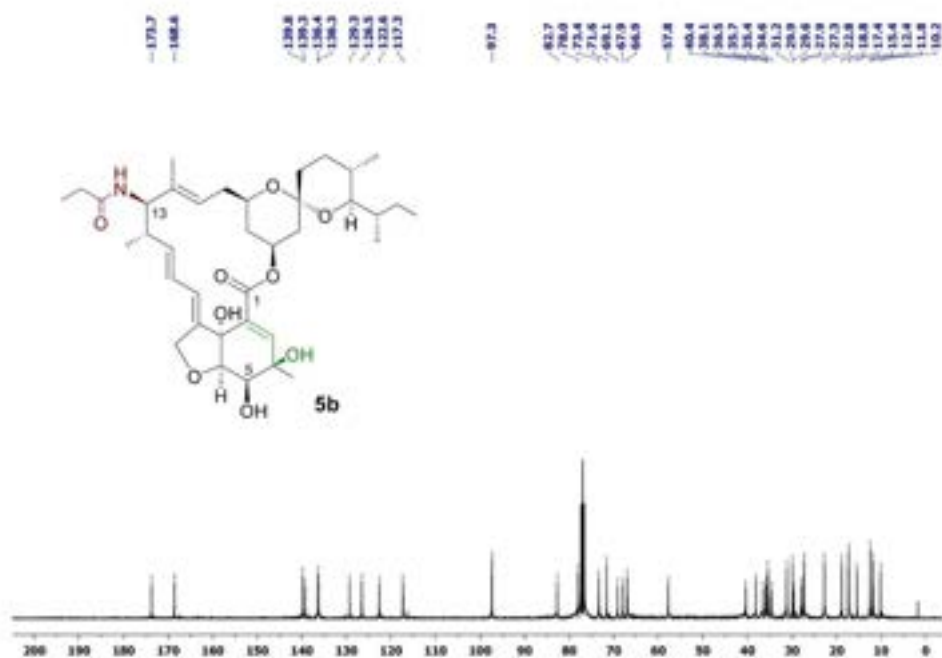
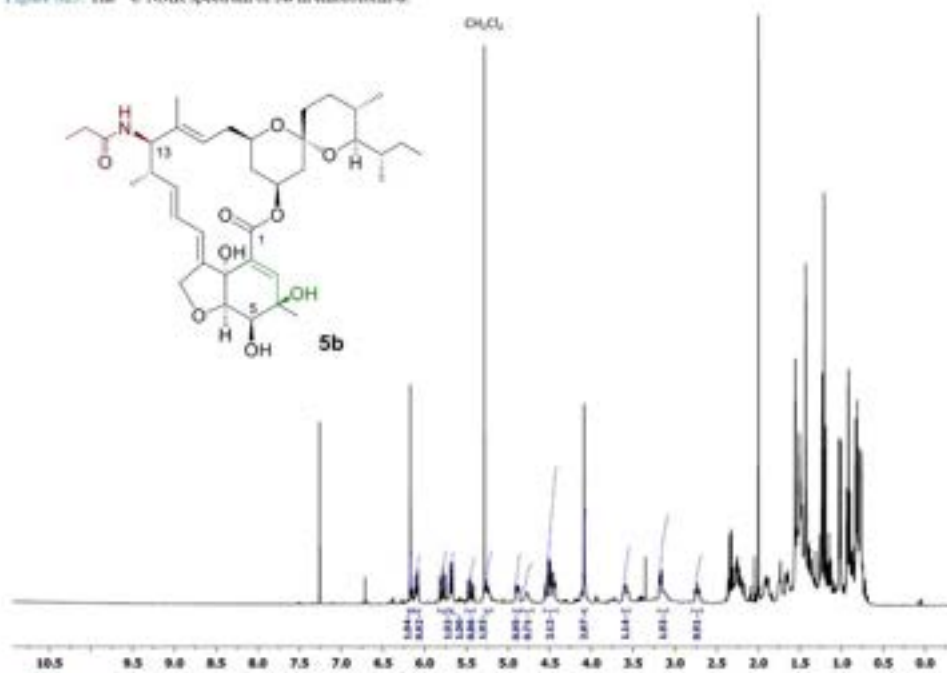


Figure S24. The ^1H NMR spectrum of **5a** in chloroform- d .

Figure S25. The ^{13}C NMR spectrum of **5b** in chloroform- d .Figure S26. The ^1H NMR spectrum of **5b** in chloroform- d .

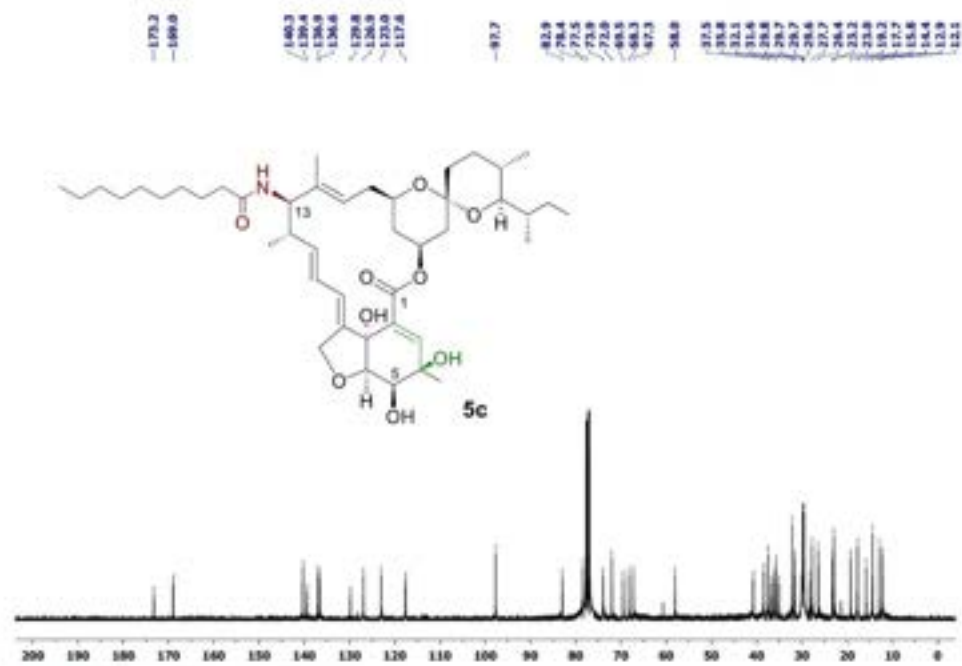


Figure S27. The ^{13}C NMR spectrum of **5c** in chloroform- d .

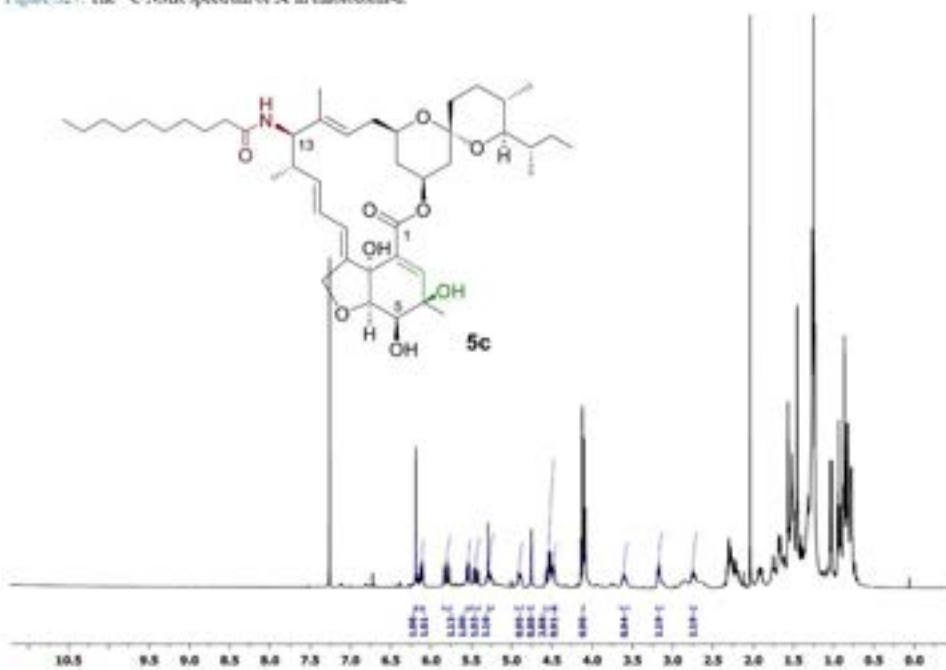


Figure S28. The ^1H NMR spectrum of **5c** in chloroform- d .

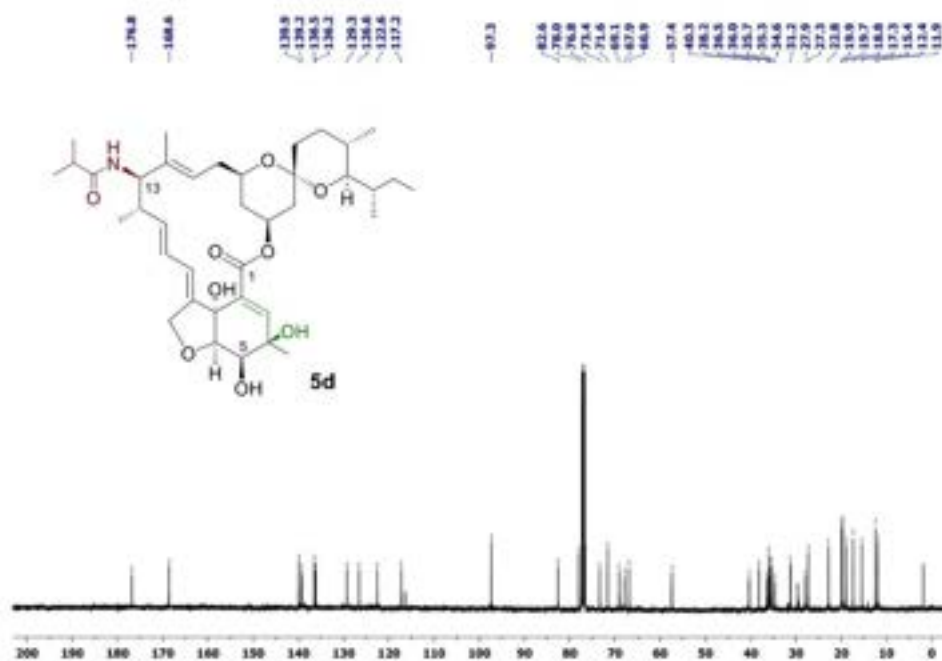


Figure S29. The ^{13}C NMR spectrum of **5d** in chloroform- d .

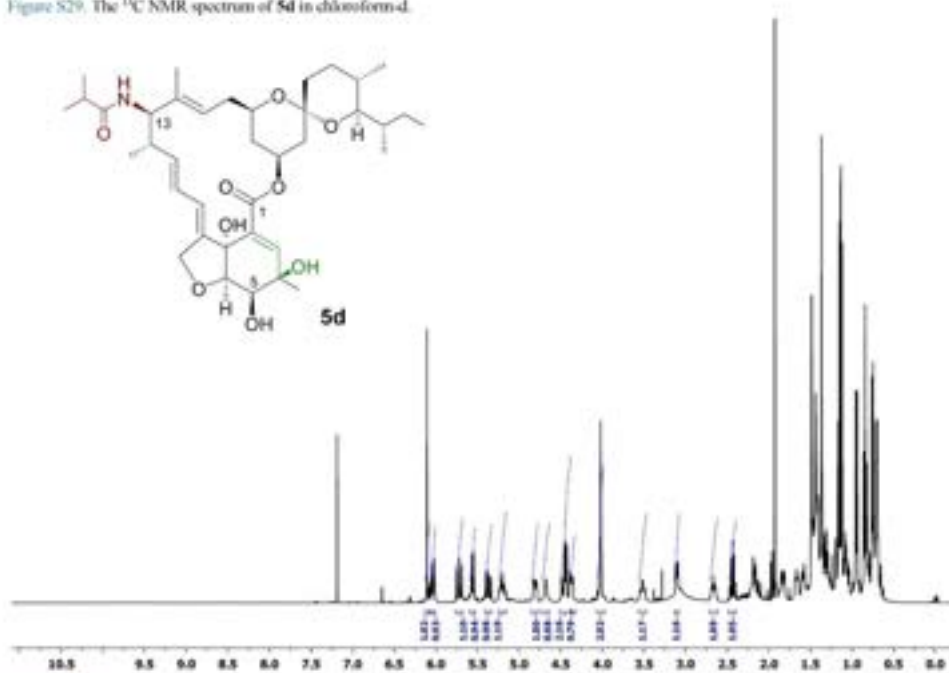


Figure S30. The ^1H NMR spectrum of **5d** in chloroform- d .

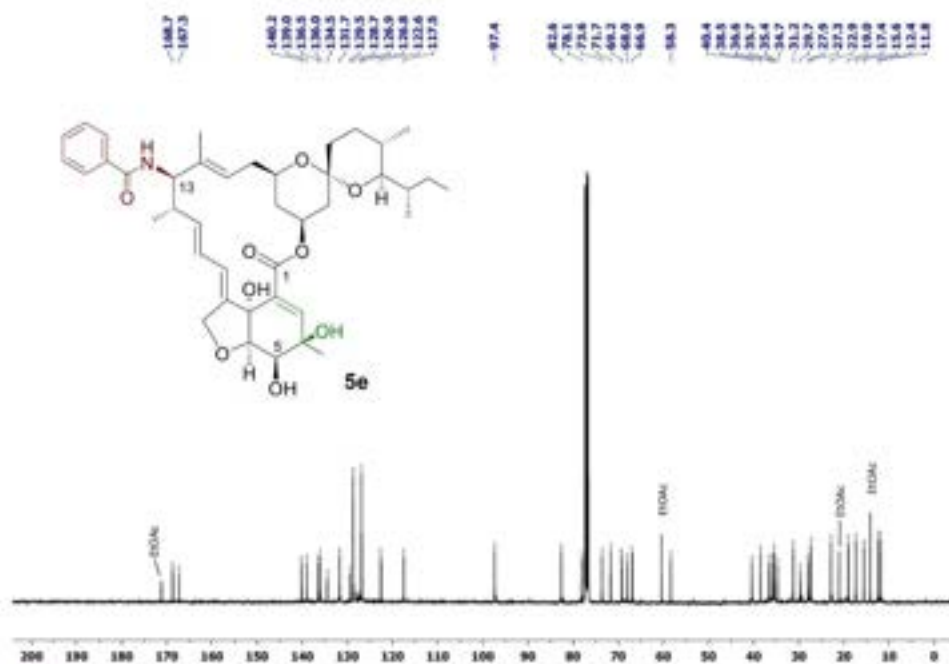


Figure S31. The ^{13}C NMR spectrum of **5e** in chloroform- d .

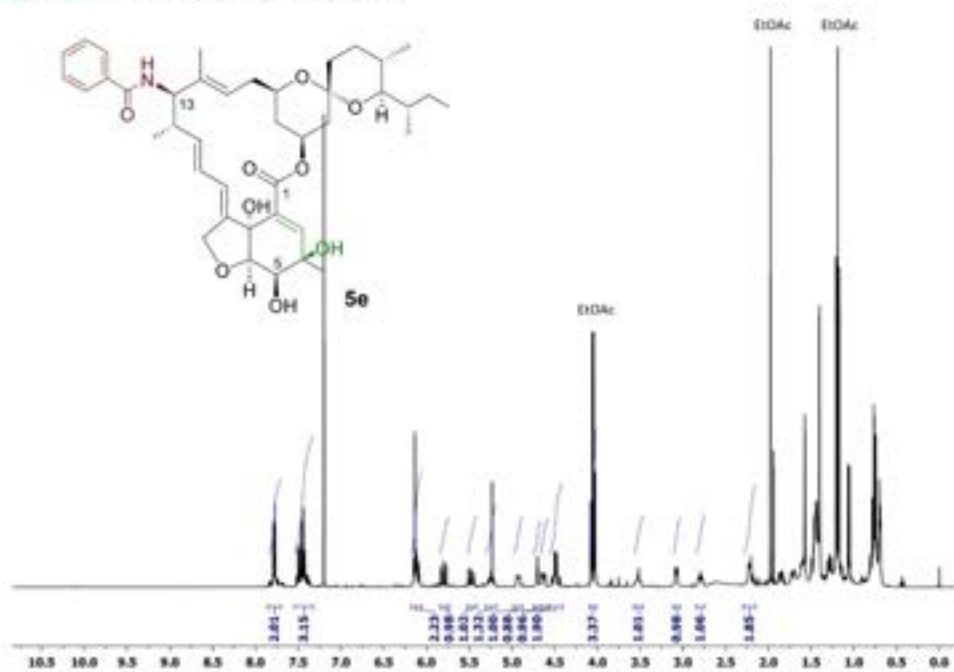
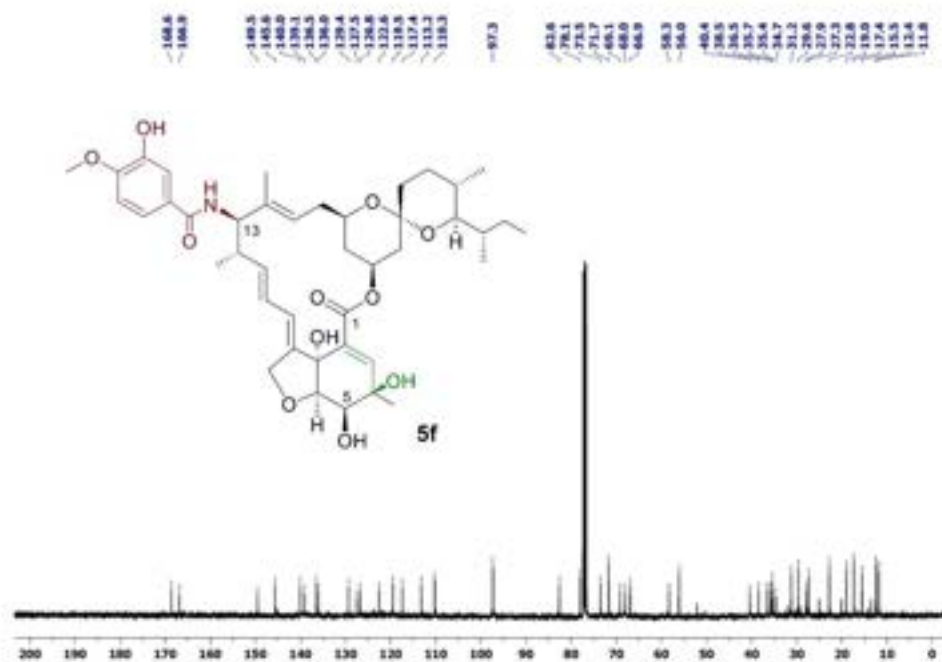
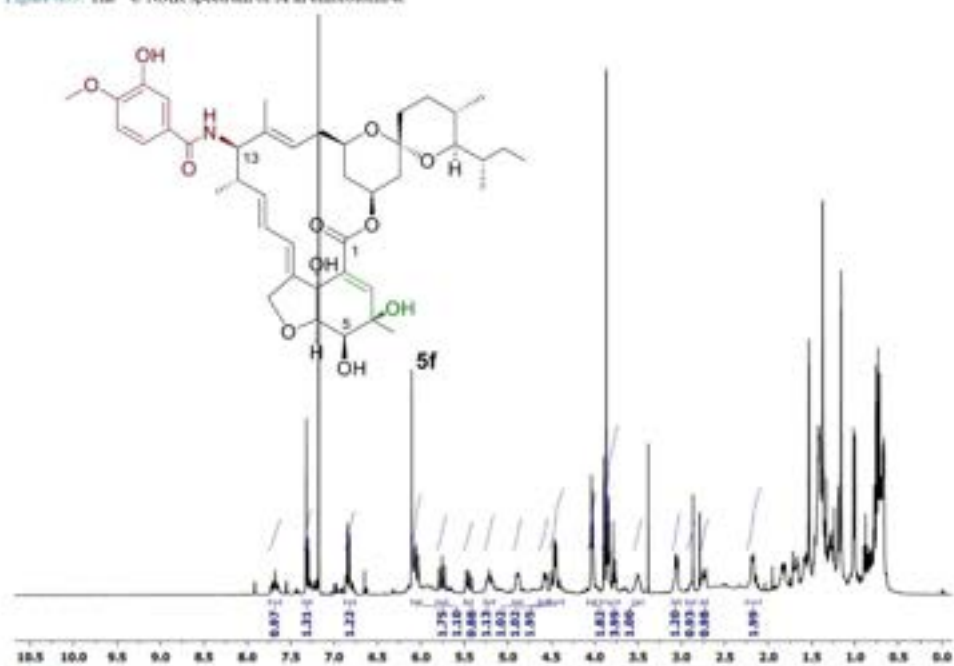


Figure S32. The ^1H NMR spectrum of **5e** in chloroform- d .

Figure S33. The ^{13}C NMR spectrum of **5f** in chloroform- d .Figure S34. The ^1H NMR spectrum of **5f** in chloroform- d .

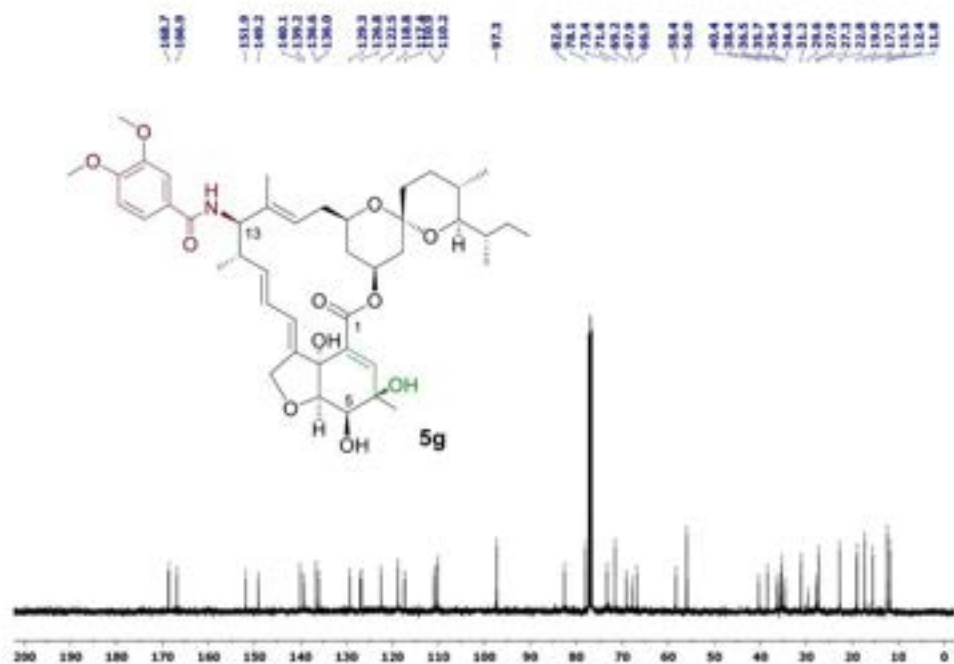


Figure S35. The ^{13}C NMR spectrum of **5g** in chloroform- d .

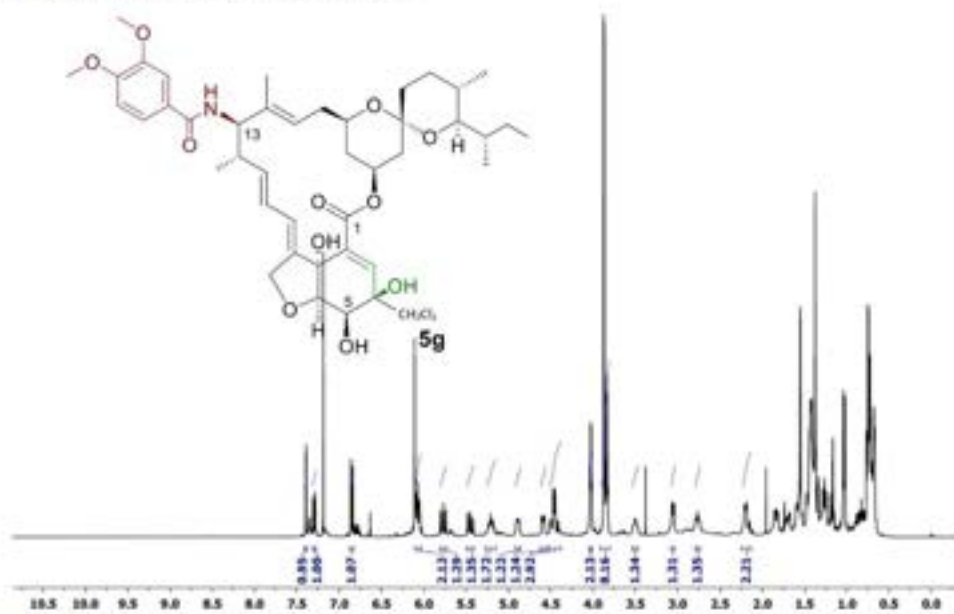
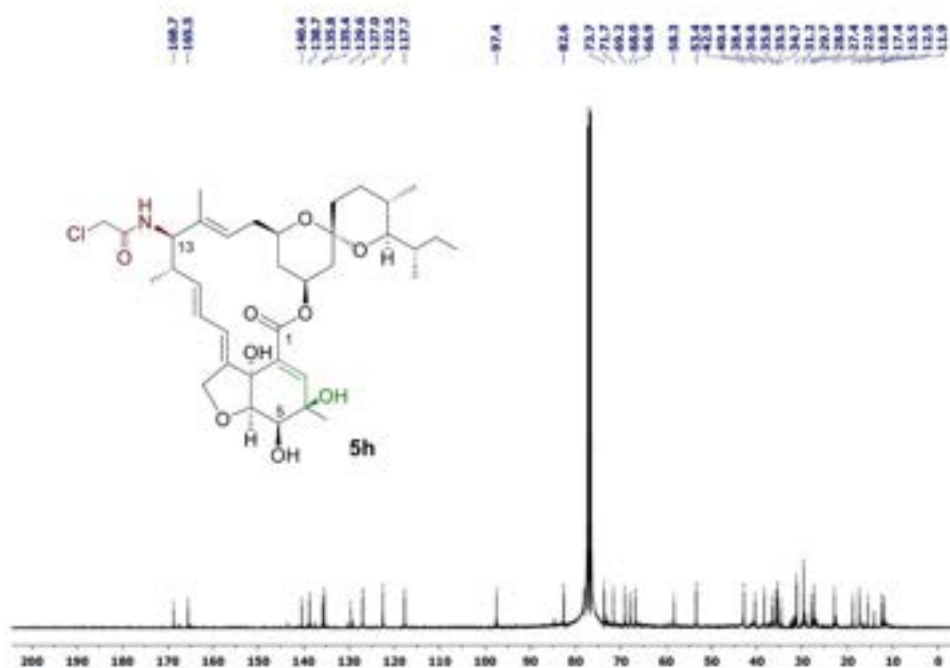
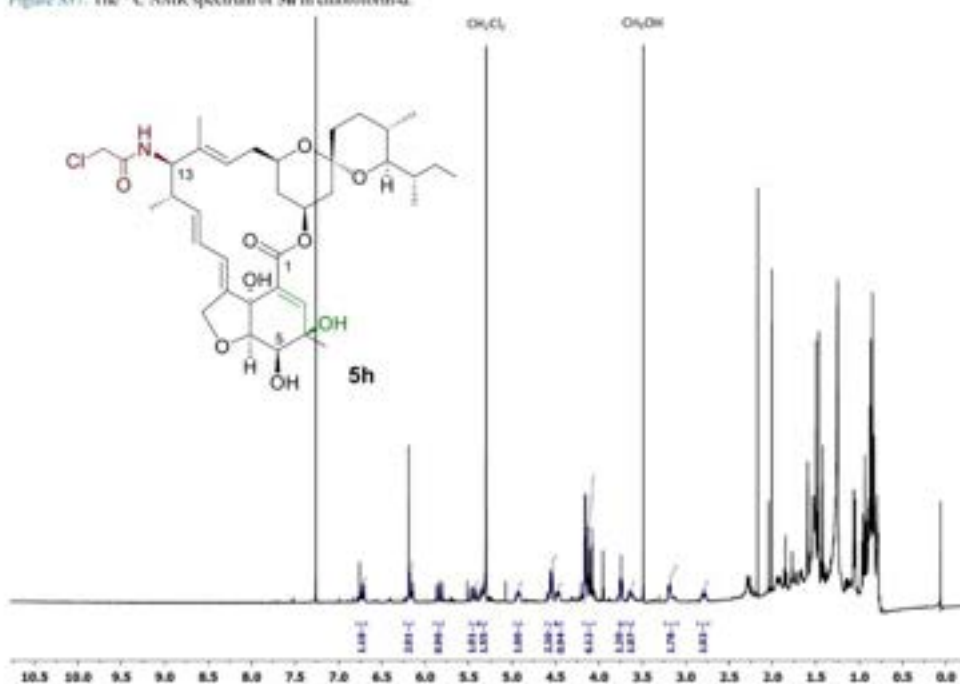


Figure S36. The ^1H NMR spectrum of **5g** in chloroform- d .

Figure S37. The ^{13}C NMR spectrum of **5h** in chloroform- d .Figure S38. The ^1H NMR spectrum of **5h** in chloroform- d .

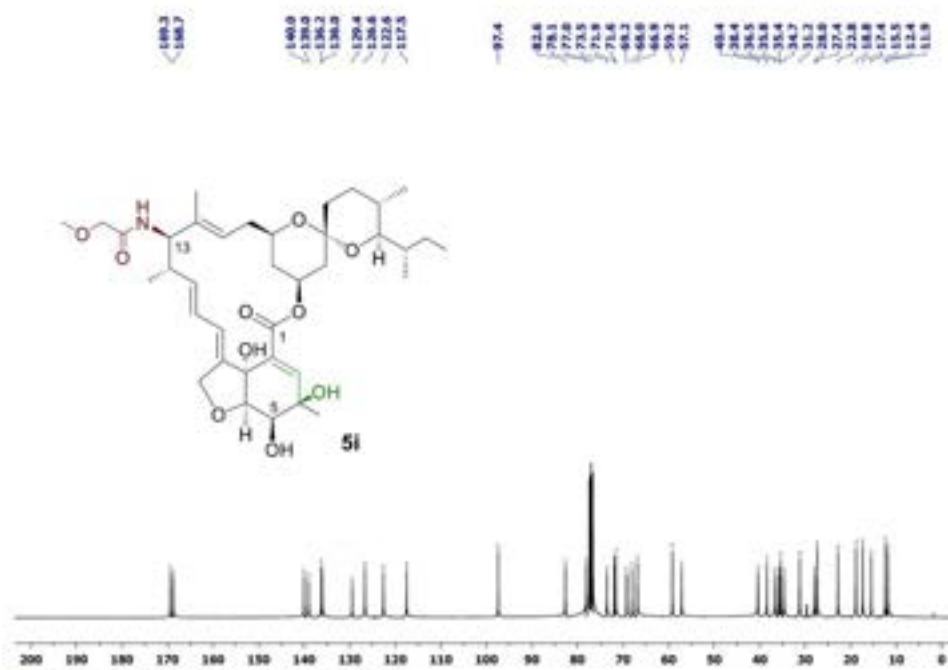


Figure S39. The ^{13}C NMR spectrum of **5i** in chloroform- d .

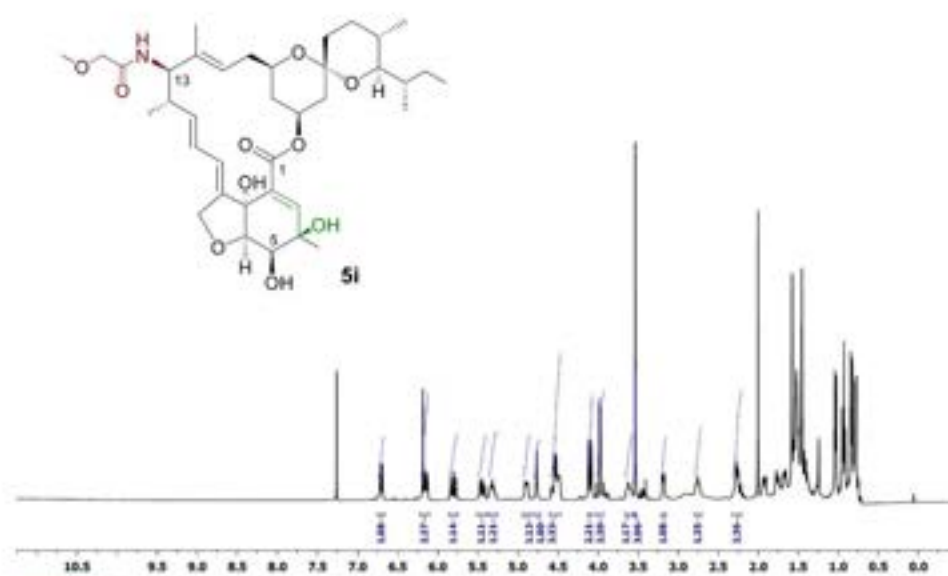


Figure S40. The ^1H NMR spectrum of **5i** in chloroform- d .

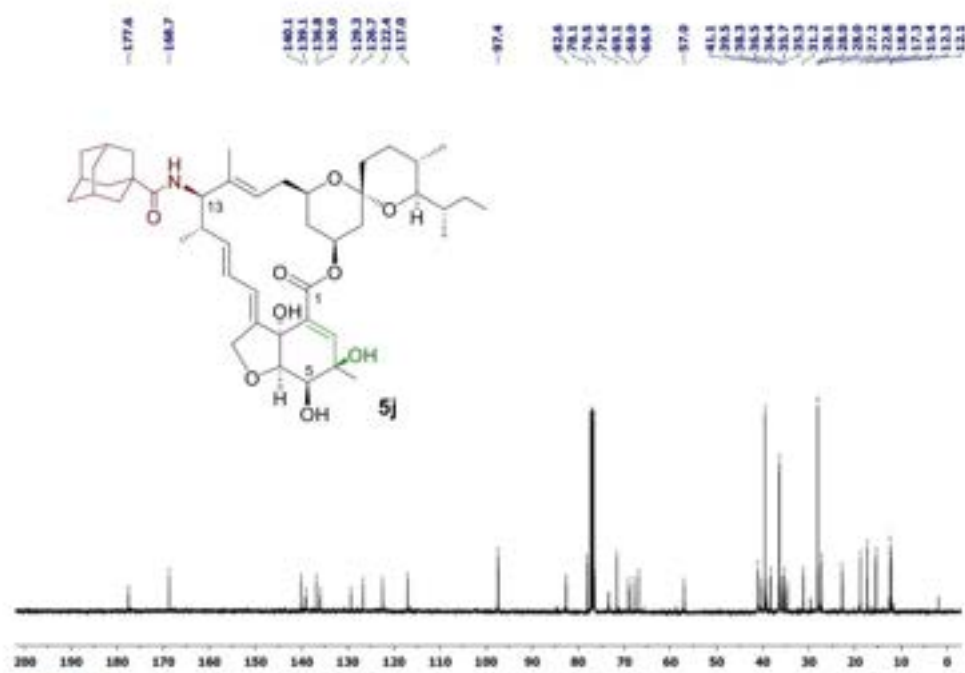


Figure S41. The ^{13}C NMR spectrum of **5j** in chloroform- d .

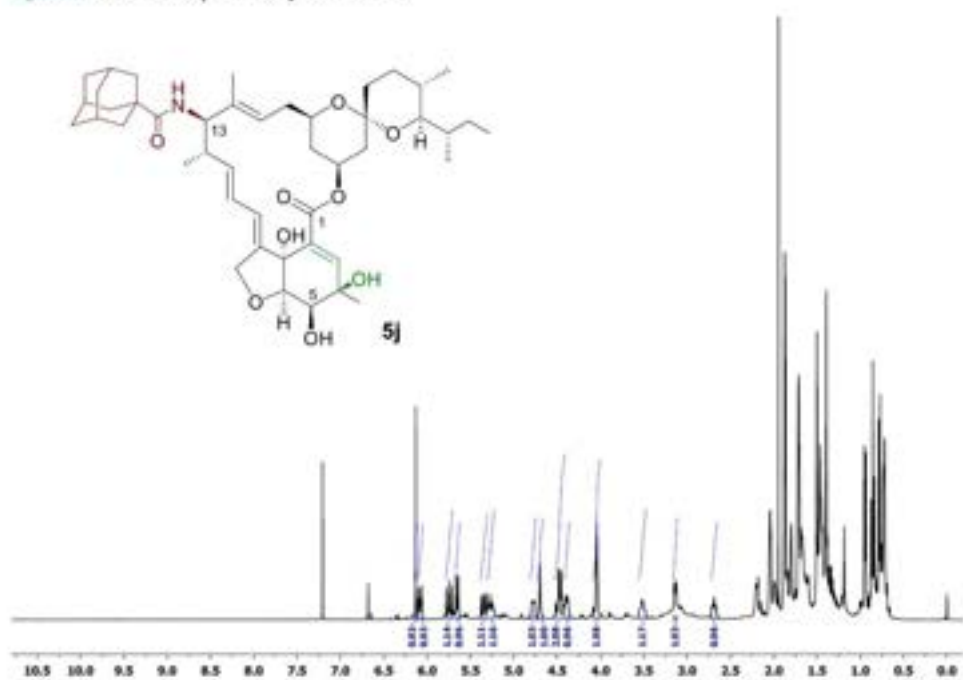


Figure S42. The ^1H NMR spectrum of **5j** in chloroform- d .

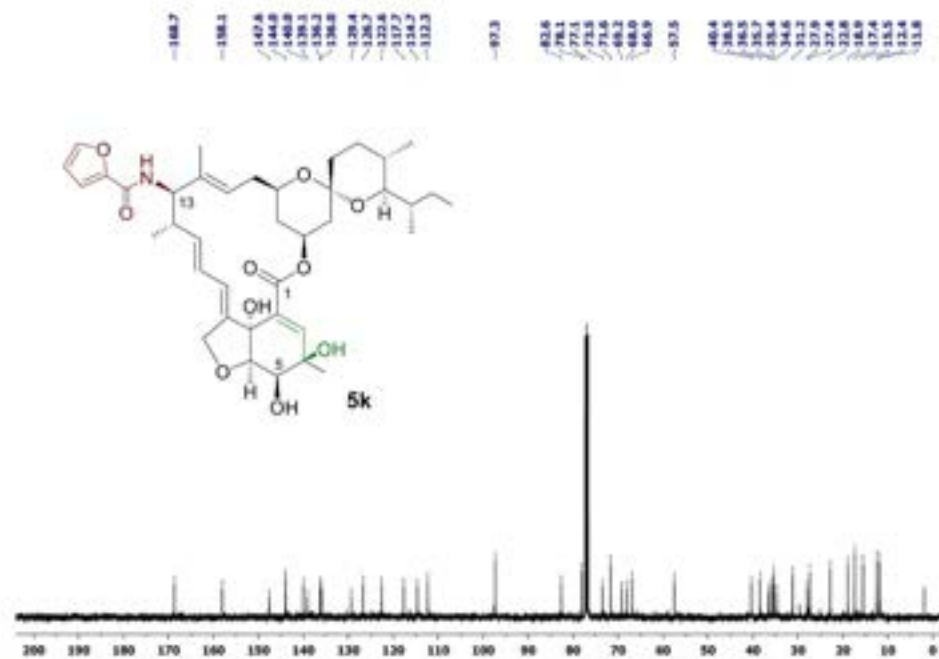


Figure S43. The ^{13}C NMR spectrum of **5k** in chloroform- d .

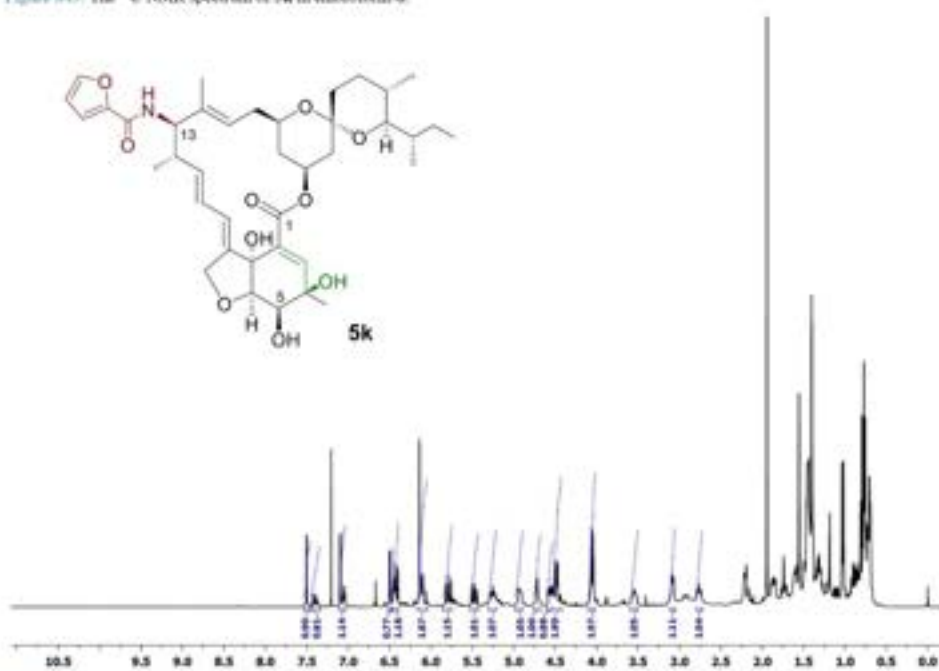


Figure S44. The ^1H NMR spectrum of **5k** in chloroform- d .

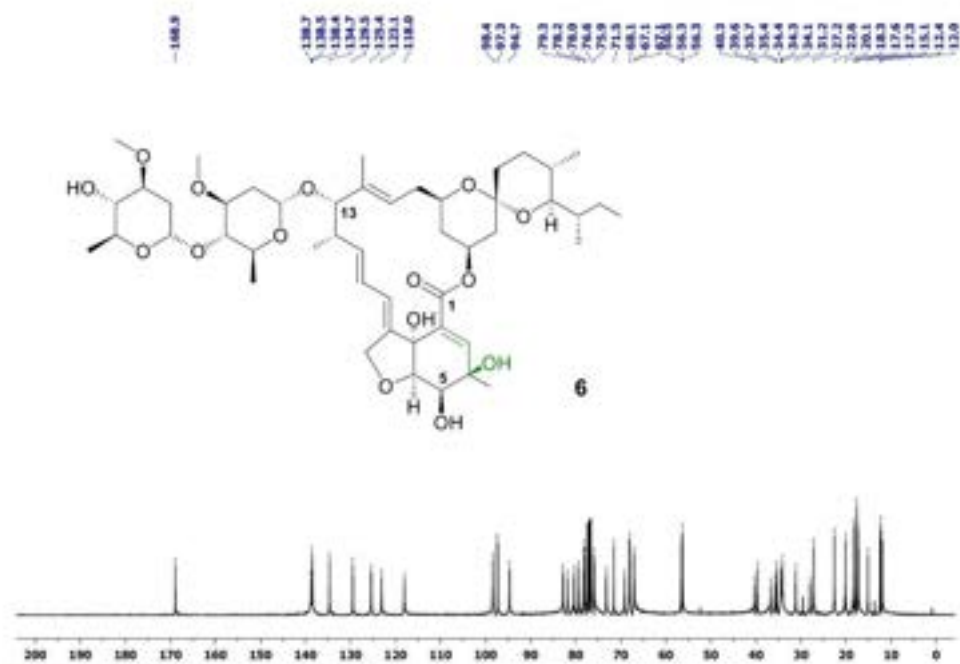


Figure S43. The ^{13}C NMR spectrum of **6** in chloroform- d .

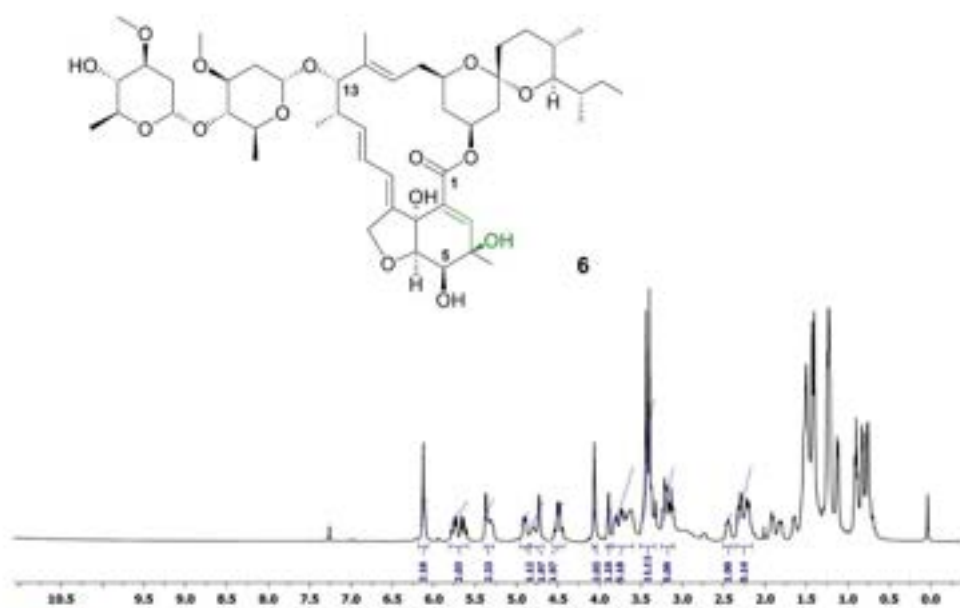


Figure S46. The ^1H NMR spectrum of **6** in chloroform- d .

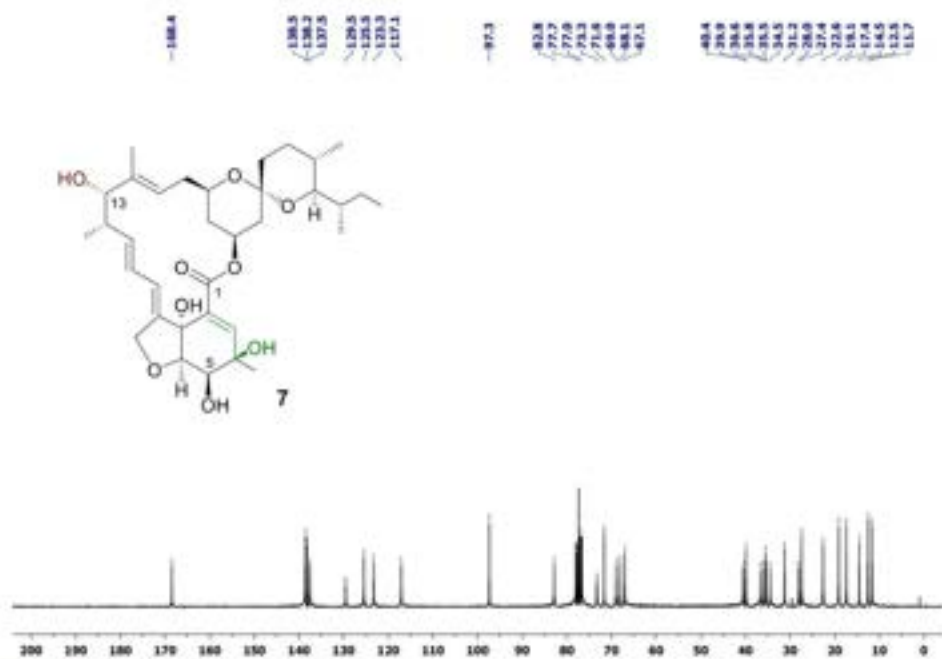


Figure S47. The ^{13}C NMR spectrum of 7 in chloroform- d .

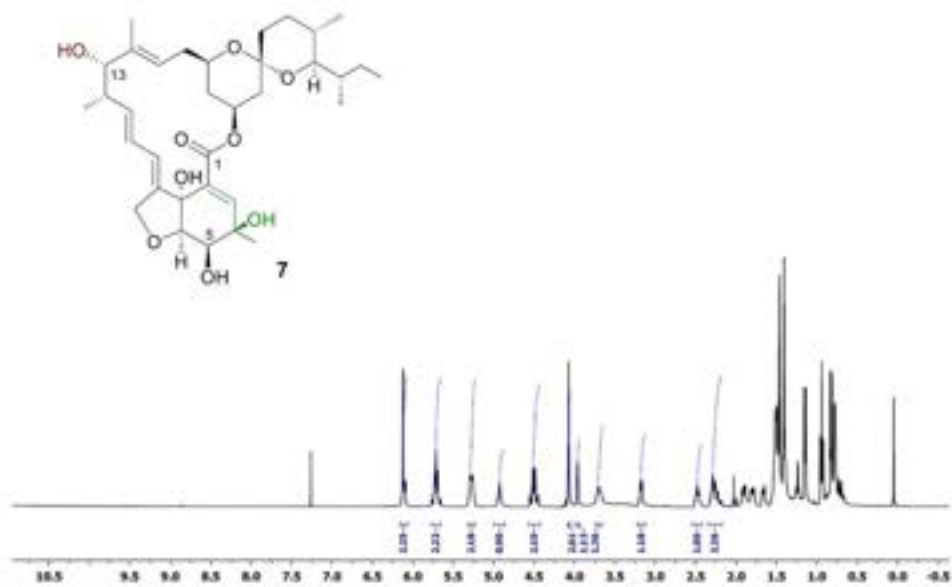
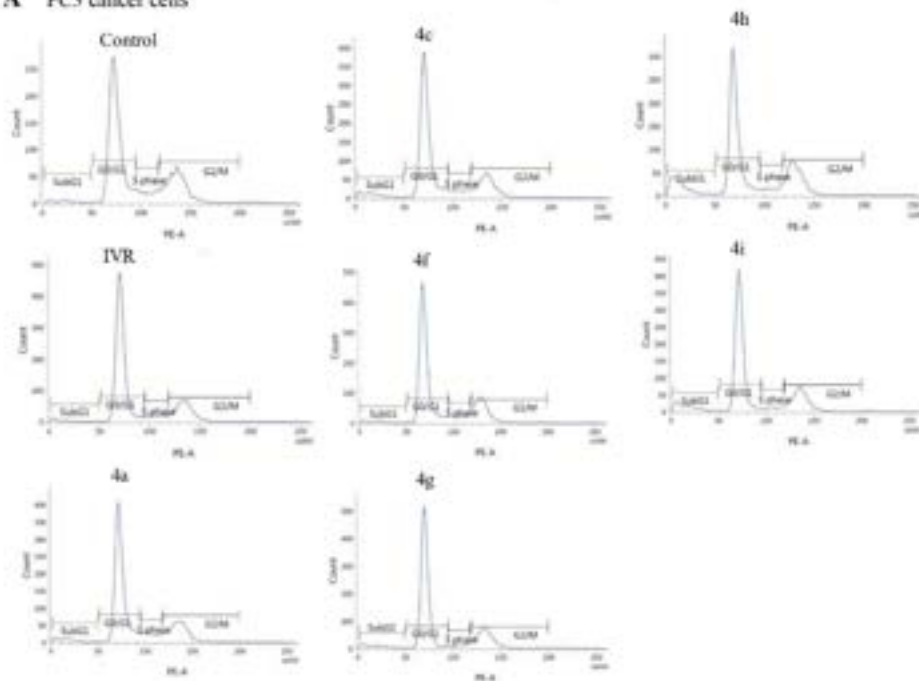


Figure S48. The ^1H NMR spectrum of 7 in chloroform- d .

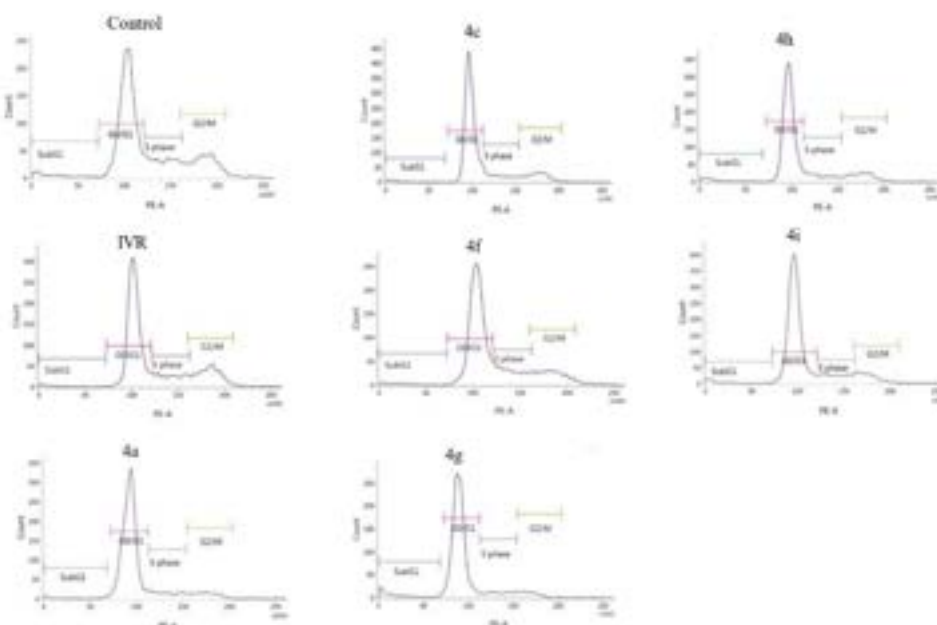
Anticancer activity studies

Ivermectin and its unrearranged derivatives

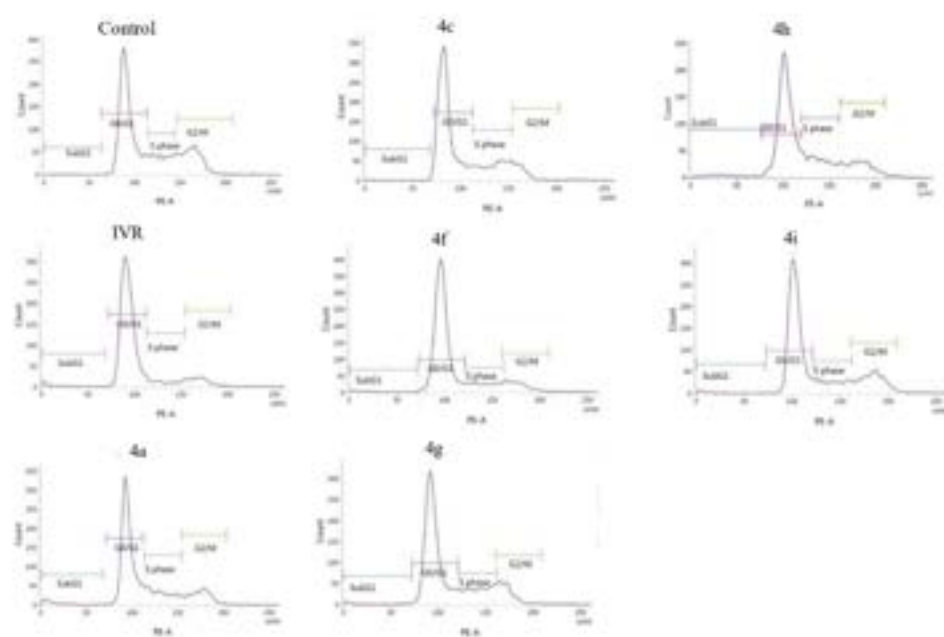
A PC3 cancer cells



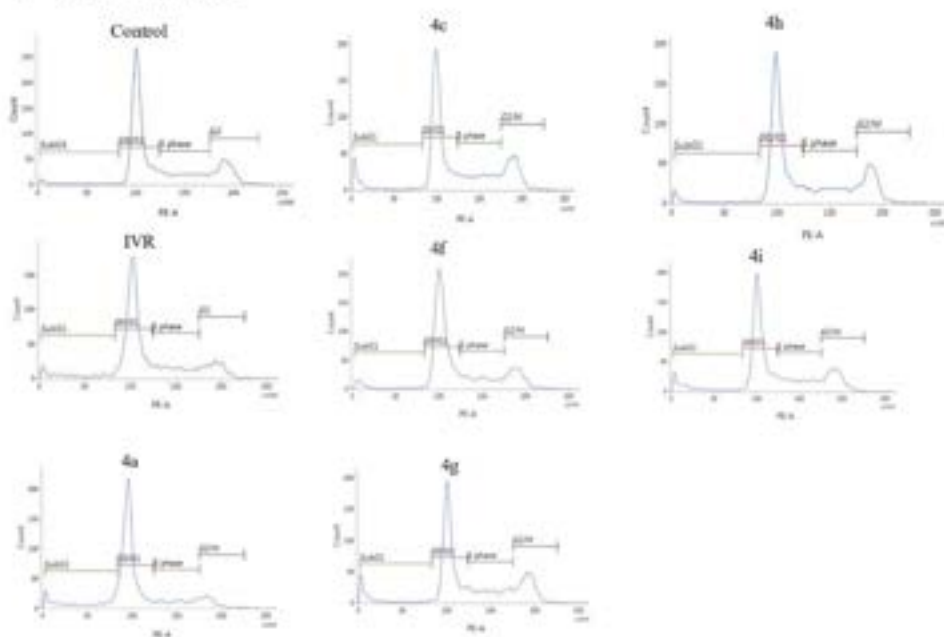
B MDA-MB-231 cancer cells



C A549 cancer cells

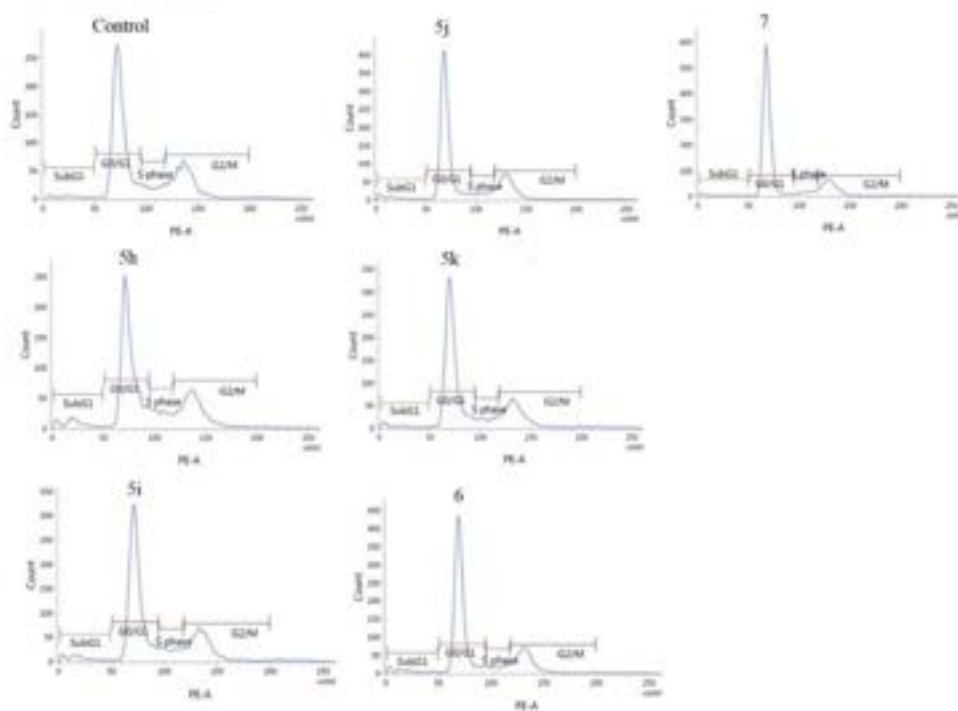


D HCT-116 cancer cells

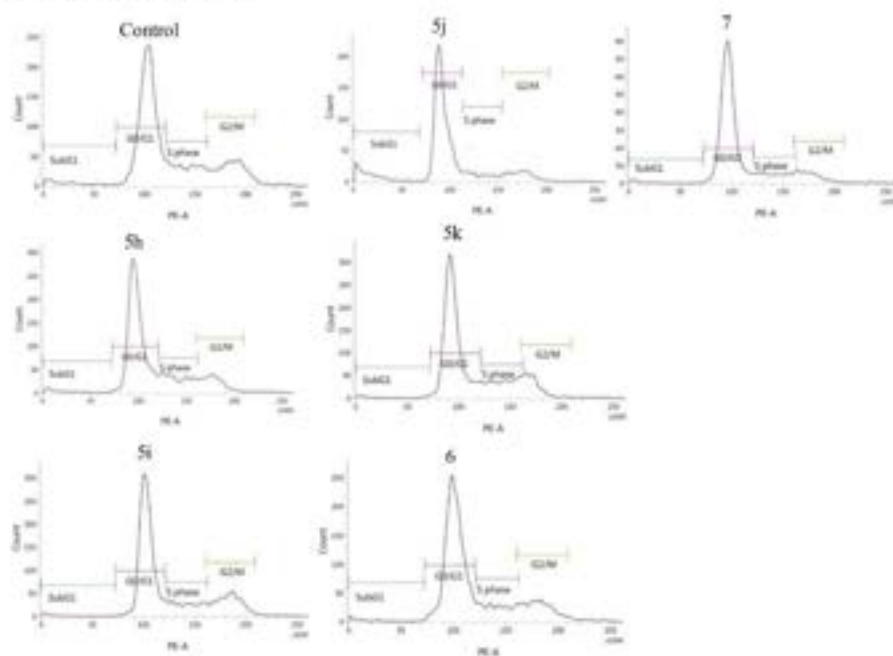


Rearranged derivatives of ivermectin

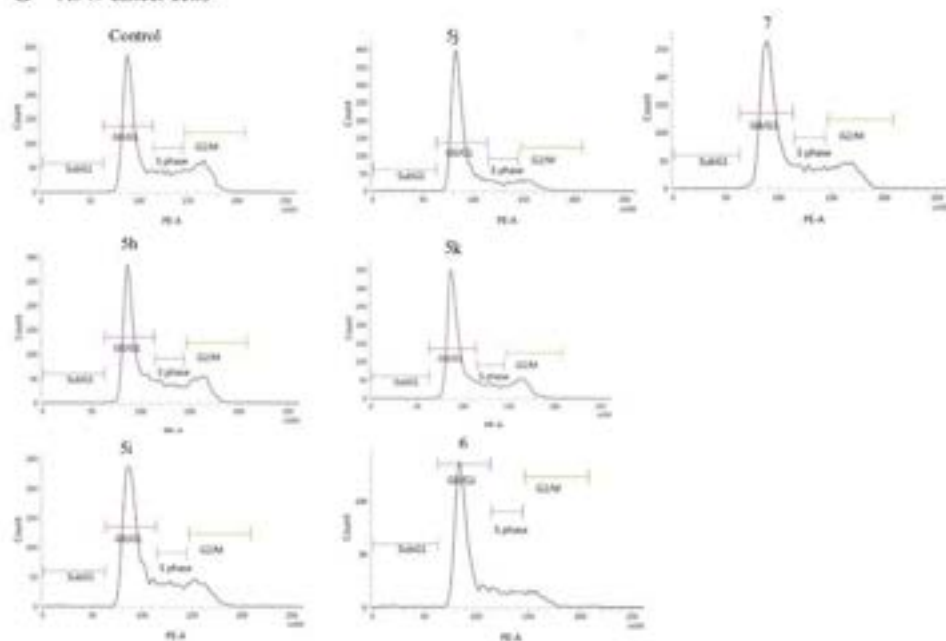
E PC3 cancer cells



F MDA-MB-231 cancer cells



G A549 cancer cells



H HCT-116 cancer cells

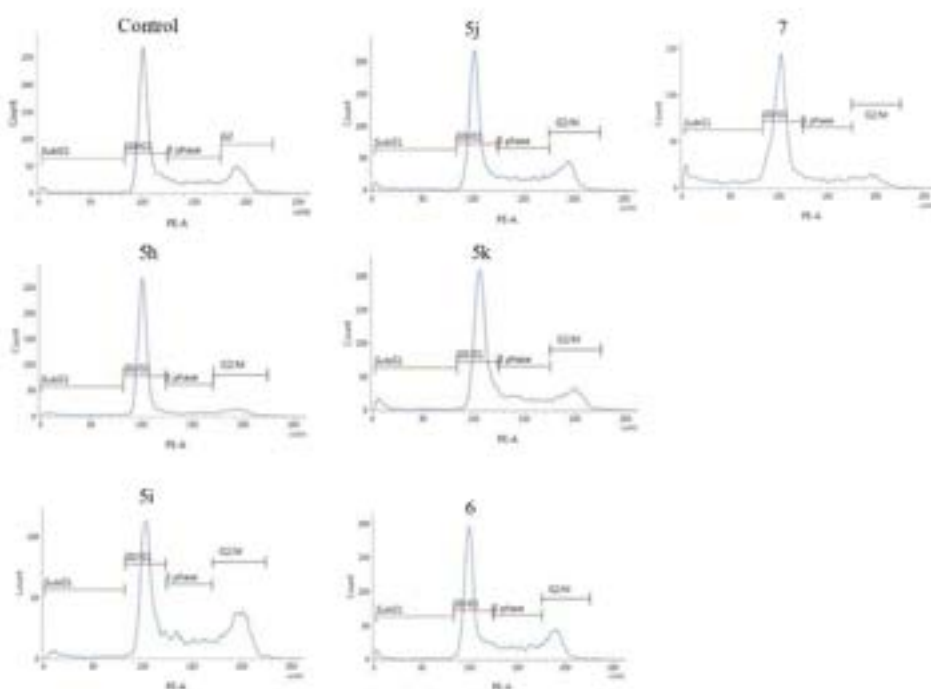
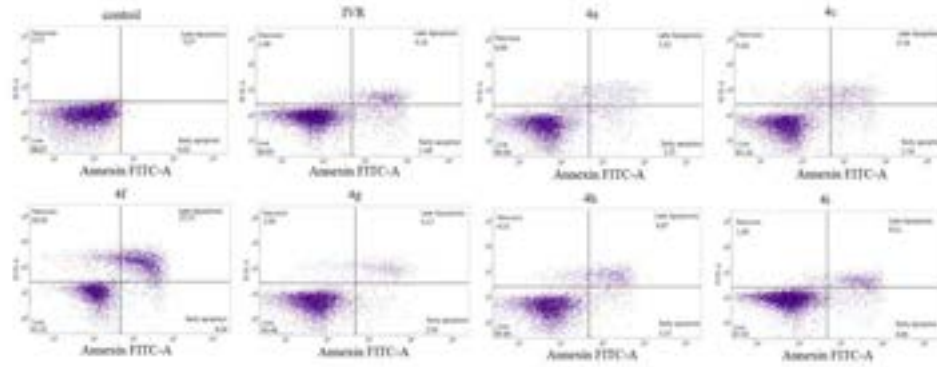


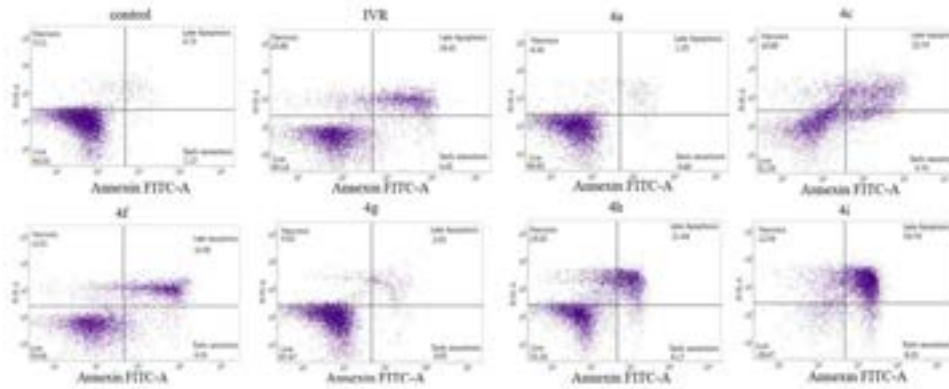
Figure S49. Flow cytometry analysis of cell cycle parameters after incubation of PC3, MDA-MB-231, A549 and HCT-116 cancer cells with (A–D) ivermectin (IVR) and its unrearranged derivatives **4a**, **4c** and **4f–4i**, or (E–H) the corresponding rearranged derivatives **5h–5k**, **6** and **7** at their IC_{50} concentration for 24 h.

Ivermectin and its unrearranged derivatives

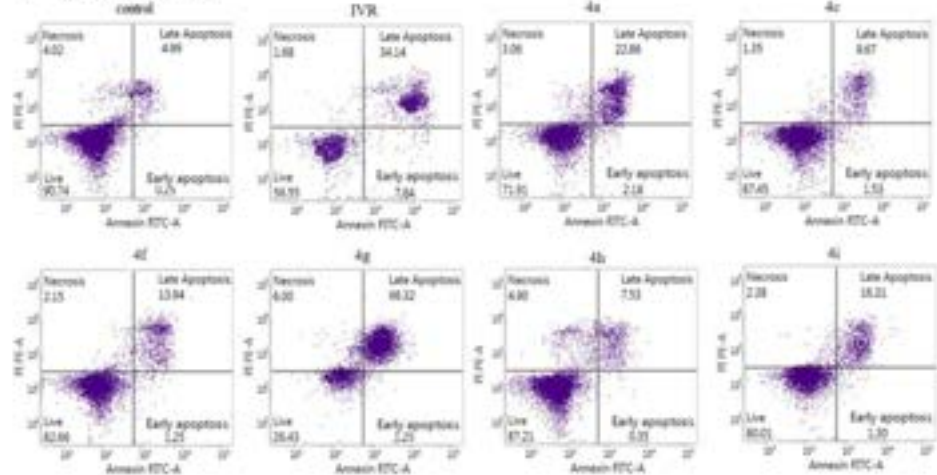
A PC3 cancer cells



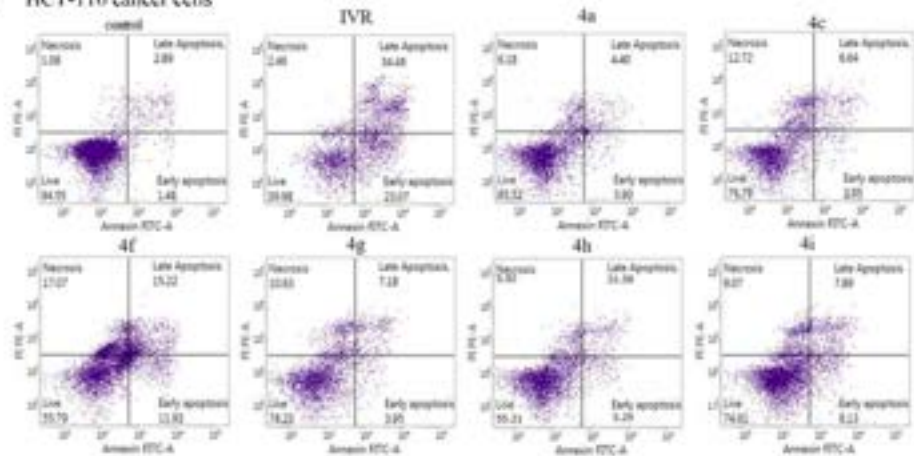
B MDA-MB-231 cancer cells



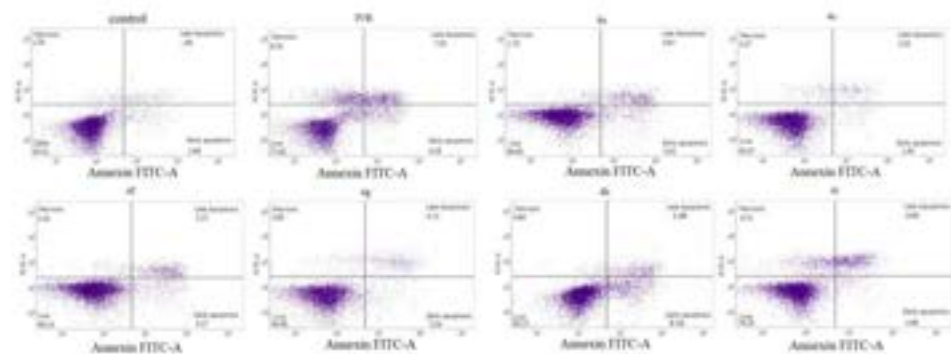
C A549 cancer cells



D HCT-116 cancer cells

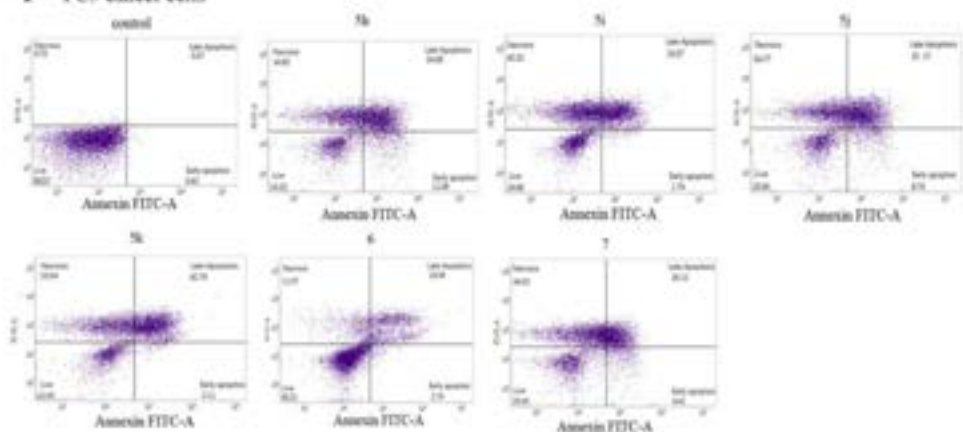


E HaCaT cells

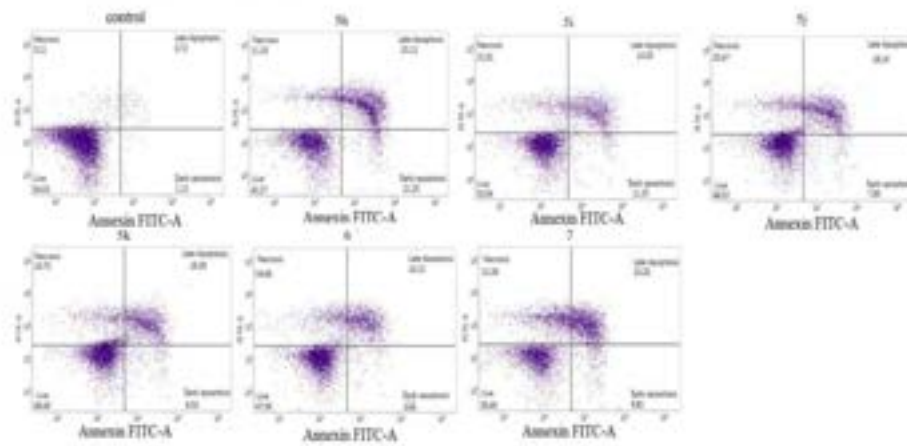


Rearranged derivatives of ivermectin

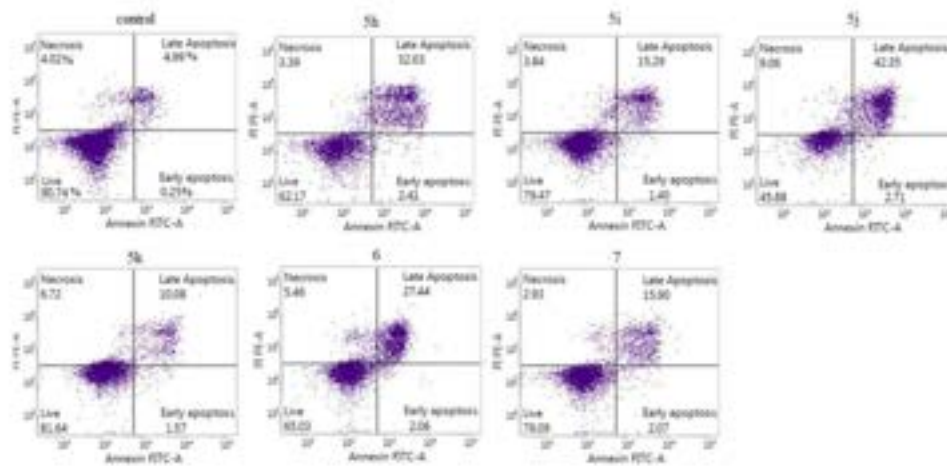
F PC3 cancer cells



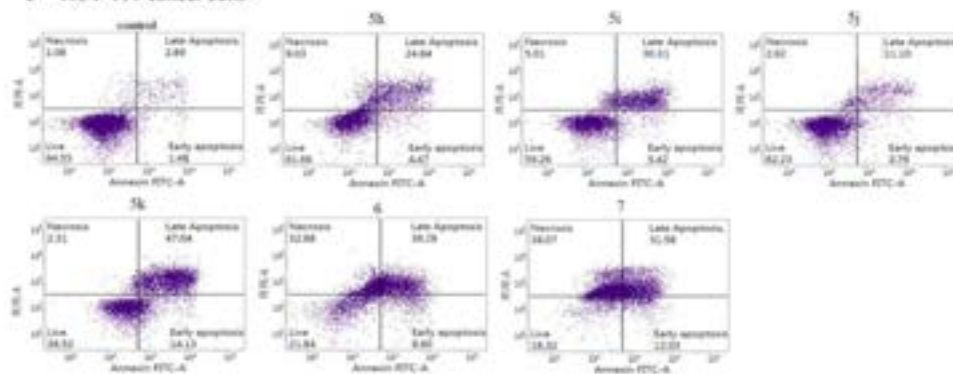
G MDA-MB-231 cancer cells



H A549 cancer cells



I HCT-116 cancer cells



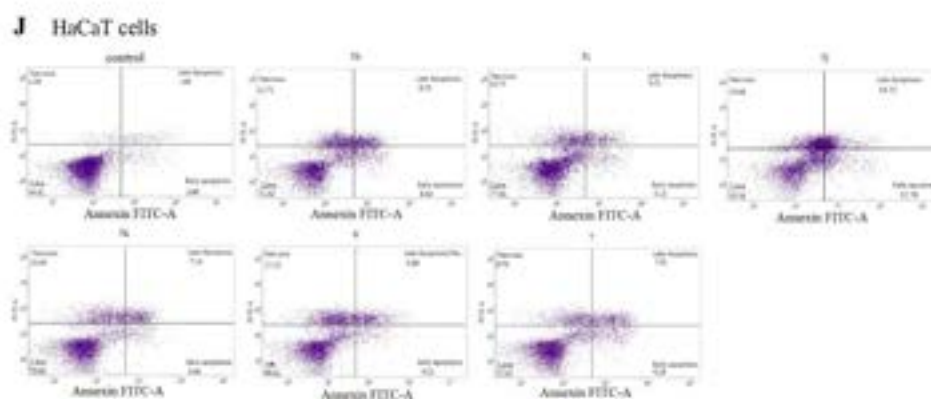


Figure S50. Representative results (%) as dot plots from apoptosis analysis of PC3, MDA-MB-231, A549 and HCT-116 cancer cells, as well as HaCaT cells treated with (A–E) ivermectin (IVR) and its unrecarranged derivatives **4a**, **4c** and **4f–4i**, or (F–J) the corresponding rearranged derivatives **5b–5k**, **6** and **7**, determined by flow cytometry, using the Annexin V-FITC/PI staining bioassay. Q1, necrotic cells; Q2, late apoptotic cells; Q3, viable cells; Q4, early apoptotic cells.

11.4. Publikacja IV

Michał Sulik, Dagmara Otto-Ślusarczyk, Dietmar Steverding,
Marta Struga, Adam Huczyński

Antiproliferative and trypanocidal activity of ivermectin bioconjugates

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Opis wkładu doktoranta w powstanie publikacji: Jestem kierownikiem projektu Diamentowy Grant w ramach którego powstał niniejszy artykuł. Jestem w pełni odpowiedzialny za syntezę oraz oczyszczanie wszystkich biokoniugatów iwermektyny opisanych w pracy. Odpowiadałem za koordynowanie badań w tym kontakt z innymi naukowcami. Przeprowadziłem też analizę otrzymanych wyników, w tym tych z badań biologicznych. Odpowiadałem za napisanie manuskryptu.

Antiproliferative and Trypanocidal Activity of Ivermectin Bioconjugates

Michał Sulik, Dagmara Otto-Ślusarczyk, Dietmar Steverding, Marta Struga, and Adam Huczyński*

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Supporting Information



		Conjugate		Conjugation partners	
		compound 16		ivermectin	artesunate
Parasite cells	T. b. brucei	GI ₅₀ = 6.25 μM	GI ₅₀ = 2.5 μM	GI ₅₀ = 5.0 μM	
Cancer cells	A549	IC ₅₀ = 3.0 μM	IC ₅₀ = 7.2 μM	IC ₅₀ = 24.0 μM	
Normal cells	HeLaT	IC ₅₀ > 100 μM	IC ₅₀ = 12.8 μM	IC ₅₀ = 5.7 μM	

ABSTRACT: Ivermectin (IVR), whose discovery has been Nobel-Prize-honored, is a 16-membered macrocyclic lactone used in medicine as an extremely effective and safe antiparasitic drug. In recent years, interest in this compound has grown due to its potential effectiveness in killing various types of cancer cells. However, research on the anticancer activity of IVR derivatives is limited. Additionally, the growing problem of drug resistance raises concerns about the effectiveness of this drug in the treatment of parasitic diseases. Therefore, in this work, we provide a detailed description of the synthesis of ten new IVR bioconjugates with compounds exhibiting high anticancer and/or antimicrobial activity. We also assess the effectiveness of these hybrids in killing *Trypanosoma brucei* a protozoan parasite that causes African trypanosomiasis, as well as their anticancer activity toward various cancer cell lines. Many of the newly synthesized conjugates exhibited higher biological activity than their respective parent compounds as well as increased selectivity indices. The IVR conjugate with artesunate (compound 16) appears particularly interesting, as it proved not only to be several times more active than the parent compounds but also showed no toxicity toward a reference cell line, indicating its potential as a therapeutic agent.

1. INTRODUCTION

Cancer is a global health issue that continues to be one of the leading causes of death in modern civilization. According to the World Health Organization (WHO), in 2020, approximately 10 million people died from cancer, while more than 19 million new cancer cases were diagnosed worldwide.^{1,2} Despite significant progress in modern therapeutic methods and numerous clinical studies currently conducted across the world, the search for new candidates for effective and safe anticancer drugs remains one of the most important challenges of modern science and medicine.^{1,2} Among the anticancer drugs currently in use, many classes of chemical compounds can be distinguished, each characterized by a different mechanism of biological activity. These include DNA-alkylating agents, mitosis inhibitors, and antimetabolites.³ Many of them—in addition to their high anticancer activity—are also highly toxic, which is often associated with the occurrence of serious side effects.³ One method of mitigating the unfavorable toxicity of anticancer drugs is their bioconjugation with other highly selective chemical compounds.^{4,5} Moreover, if these compounds also exhibit anticancer activity, the resulting hybrids may prove to be extremely effective in the fight against cancer, as multiple cytotoxic mechanisms will act on the cell simultaneously.

Ivermectin (IVR, 1, Figure 1) is a 16-membered lactone isolated in 1967 from a *Streptomyces avermitilis* strain.⁶ This

compound exhibits a broad spectrum of antiparasitic activity and is used in the treatment of various parasitoses, such as onchocerciasis, lymphatic filariasis, and scabies.^{7,8} It is used as a mixture of two homologues: B1a, which has an ethyl group at position C26, and B1b, which has a methyl group at this position.^{6,9} The mixture consists of at least 80% B1a and no more than 20% of B1b.^{6,9} Due to its potent antiparasitic activity and safety profile, IVR is often referred to as a “wonder drug”.^{6,8,10} Its discovery was honored with the Nobel Prize in Physiology or Medicine in 2015.¹¹ Recent studies have shown that, besides its great potential in combating different parasites, this drug exhibits high anticancer activity against various types of cancer cell lines, including glioma, leukemia, pancreatic cancer, and colon cancer.¹² The anticancer mechanism of action of IVR is much more complex and involves many different biochemical processes, including the inhibition of the synthesis of multidrug resistance (MDR) proteins or the increase in reactive oxygen species (ROS) levels.^{12,13} However, the data regarding the anticancer activity of IVR derivatives

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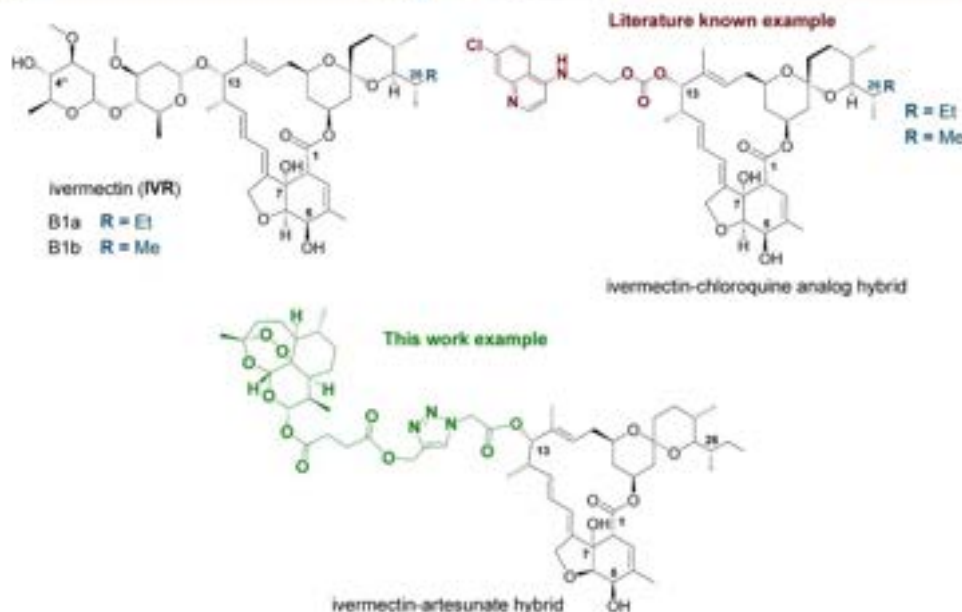


Figure 1. Chemical structure of ivermectin (IVR), its C13-hybrid with a chloroquine analog, and the C13-hybrid of IVR and artesunate studied in this work.

remain limited to only one publication, demonstrating that chemical modification at position C13 can lead to analogs with increased antiproliferative activity and/or improved selectivity.¹⁴ In addition to these findings, IVR is a safe compound with virtually no side effects and, therefore, is an extremely interesting candidate for bioconjugation.

The process of bioconjugation involves the linking of two or more bioactive molecules through covalent bonding.^{4,5} It is one of the most often used strategies in developing new hybrids with higher efficacy compared with that of the parent compounds. Bioconjugates can be created by combining two or more biomolecules in a single molecule or by linking biomolecules with synthetic small molecules.^{4,5} This technique was used for the chemical modification of IVR by synthesizing dual-acting hybrids with different conjugation partners, such as aminoquinolines (Figure 1) or ferrocene derivatives.^{15,16} These compounds showed high *in vitro* activity against the blood-stage and liver-stage of *Plasmodium* parasites, confirming their potential in combating malaria.^{15,16} Linkers with diverse structures and stabilities, such as carbonates, urethanes, or the 1,2,3-triazole ring formed through copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC) between two conjugation partners, were employed in the synthesis of the respective hybrids.^{15,16}

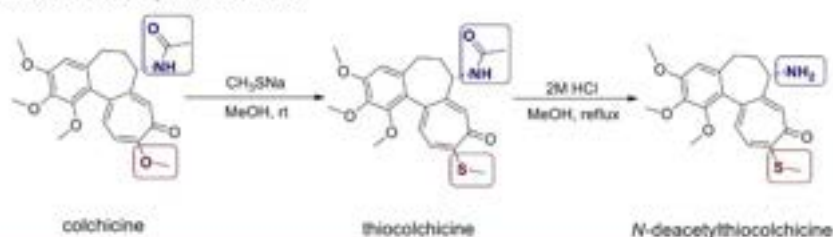
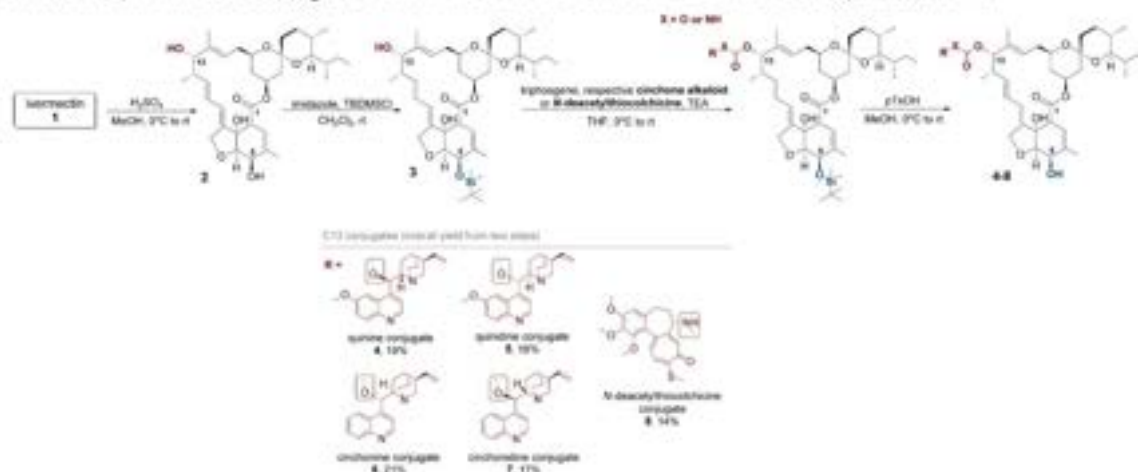
Inspired by the results regarding the anticancer properties of C13 derivatives of IVR and the antimalarial properties of hybrids of this drug, we decided to combine both research directions. Herein, we present synthetic access to a series of ten IVR bioconjugates with other biologically active components, combined using appropriate linkers (Figure 1). Compounds exhibiting diverse biological activities were selected as conjugation partners. These included cinchona bark alkaloids (quinine, quinidine, cinchonine, and cinchonidine), which have been used for many years as antimalarial and antiarrhythmic drugs, but their effectiveness in overcoming

drug resistance in cancer cells has also been documented.^{17,18} In addition to these compounds, we also chose two nucleoside analogs (flouxuridine and azidothymidine), which are used as anticancer drugs but are characterized by high toxicity.^{19,20} Furthermore, we also included in the study *N*-deacetylthiocolchicine, betulinic acid, artesunate, and metronidazole, known for their potent biological activities (antimicrobial and/or anticancer).^{21–23}

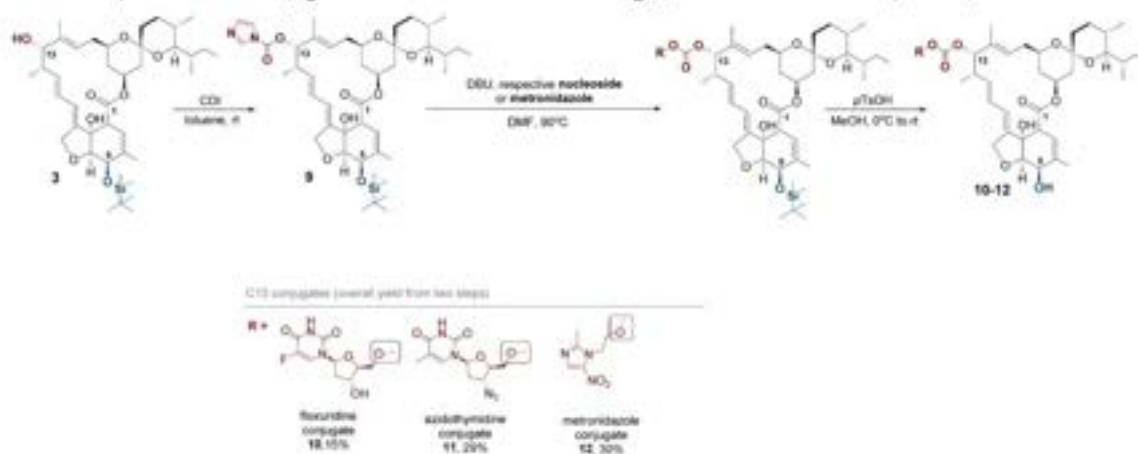
All newly synthesized conjugates were evaluated *in vitro* for their cytotoxic activity toward different cell lines. However, it should be mentioned that the primary use of IVR is as an extremely effective drug to treat various parasitic diseases.⁹ Moreover, many of the conjugation partners named above are also highly effective against parasites. Thus, the resulting hybrids may exhibit potent antiparasitic activities, as well. To test the antiparasitic properties of the bioconjugates, we selected *Trypanosoma brucei*, the causative agent of African trypanosomiasis, a parasite belonging to the group of organisms causing neglected tropical diseases.^{24–28} It should also be noted that C13 derivatives of IVR display promising activity against *T. brucei*.²⁹ Thanks to preventive and new therapeutic interventions introduced by the World Health Organization (WHO), the number of human African trypanosomiasis (sleeping sickness) cases has been significantly reduced in recent years, while animal African trypanosomiasis (nagana disease) remains a problem in sub-Saharan Africa.^{26,30} However, due to the limited number of drugs and the emergence of drug-resistant parasites, the search for new bioactive compounds to combat this disease is necessary.³¹ Therefore, all newly synthesized hybrids were also tested for their *in vitro* activity against bloodstream forms of *T. b. brucei*.

2. RESULTS AND DISCUSSION

2.1. Analogs Design and Synthesis. A characteristic feature of IVR is the presence of a disaccharide moiety at the

Scheme 1. Synthesis of *N*-DeacetylthiocolchicineScheme 2. Synthesis of Bioconjugates of IVR with Cinchona Bark Alkaloids and *N*-Deacetylthiocolchicine

Scheme 3. Synthesis of Bioconjugates of IVR with Nucleoside Analogs (Floxuridine and Azidothymidine) and Metronidazole

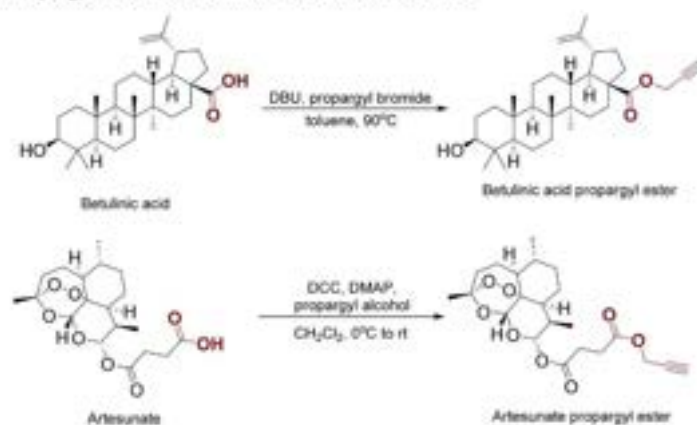


C13 position. It has been shown that its presence is not necessary for the antiparasitic activity of the drug, but the aglycone exhibits generally poorer biological properties than the unmodified IVR.^{32,33} An interesting direction for research is, therefore, the replacement of the sugar moiety with other chemical groups. Thus, the synthesis of C13-hybrids of IVR with compounds showing anticancer and/or antimicrobial activity was conducted. Linkers with diverse chemical properties were used, including carbonates, urethane, and 1,2,3-

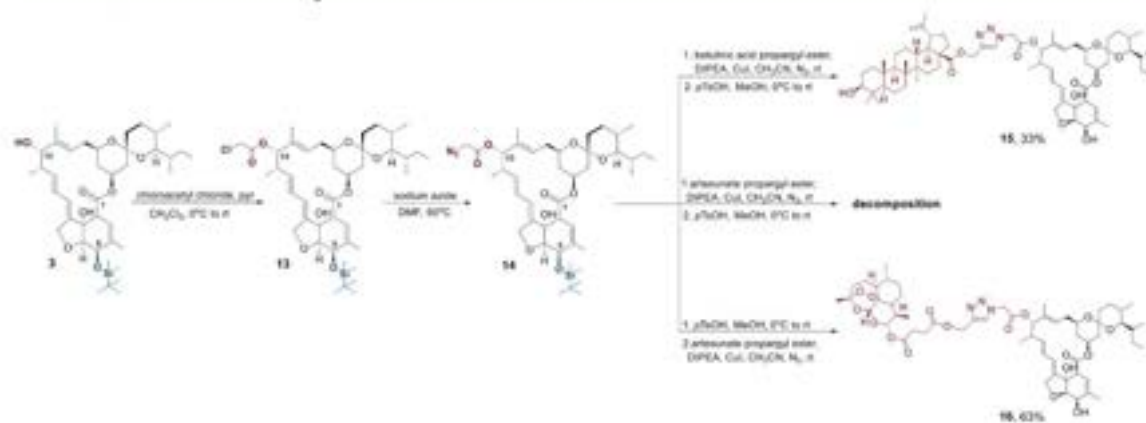
triazole rings. The reactions involved conjugation partners in their unmodified form or after structural modifications. In general, three methods of bioconjugate synthesis were employed.

The first method involved the use of triphosgene, enabling the conjugation of IVR with cinchona bark alkaloids and *N*-deacetylthiocolchicine. The cinchona bark alkaloids were used in their native form, while *N*-deacetylthiocolchicine had to be synthesized. It has been shown that the substitution of

Scheme 4. Synthesis of Propargyl Esters of Betulinic Acid and Artesunate



Scheme 5. Synthesis of Bioconjugates of IVR with Betulinic Acid and Artesunate



colchicine's C10-methoxy group with a thiomethyl group increases the molecular stability of the resulting thiocolchicine.²¹ As further reaction required the presence of a free amine group, a deacetylation of thiocolchicine was carried out, resulting in *N*-deacetylthiocolchicine, which was then used for subsequent bioconjugation (Scheme 1).²¹

The bioconjugation reaction was carried out between the appropriate partners and the C5-protected aglycone of IVR (compound 3, Scheme 2). Compound 3 was obtained through a two-step reaction, which included the solvolysis of the sugar moiety in the presence of sulfuric acid and the protection of the C5-hydroxyl group using *tert*-butyldimethylsilyl chloride (Scheme 2). The reaction between the C13-hydroxyl group of compound 3 and the hydroxyl group of cinchona bark alkaloids, or the amine group of *N*-deacetylthiocolchicine, took place in the presence of triphosgene, which enabled the synthesis of either a carbonate or urethane linker. Deprotection of position C5 using *p*-toluenesulfonic acid allowed the formation of hybrids 4–8 with overall yields from two steps (bioconjugation and deprotection) ranging from 14 to 21% (Scheme 2).

The second method allowed the conjugation of the IVR skeleton with nucleoside analogs (floxuridine and azidothymidine) and metronidazole. Similarly to the first method, the

linker was a carbonate group; however, the use of triphosgene proved unsuitable for these compounds, as no formation of the expected product was observed. Therefore, we decided to use carbonyldiimidazole (CDI) as a conjugative agent, which was proposed by Singh et al. in 2022 for the synthesis of IVR conjugates with aminoquinolines (Scheme 3).¹⁶ For this purpose, C5-protected compound 3 was subjected to a reaction with CDI, which allowed the synthesis of precursor 9. This reagent was then added to a mixture of nucleoside analogue or metronidazole with DBU. The reaction was conducted at 90 °C, and the isolation and subsequent deprotection of the C5-hydroxyl group resulted in the desired hybrids 10–12, with yields ranging from 15 to 30% (Scheme 3).

The third method allowed the conjugation of IVR with betulinic acid and artesunate through the use of the CuAAC reaction under Meldal conditions (copper(I) iodide as the source of Cu(I) ions).²⁴ To facilitate this, it was necessary to prepare the azide and propargyl partners to enable the click reaction. Propargyl ester of betulinic acid was obtained by reacting betulinic acid with propargyl bromide in the presence of DBU. However, these conditions were unsuitable for the synthesis of the propargyl ester of artesunate, as they led to the decomposition of the starting material. Consequently, to

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Table 1. GI₅₀ Values and Ratios of IVR Conjugates and Conjugation Partners for *T. brucei* and HL-60 Cells

compound	<i>T. brucei</i>		selectivity	compound	<i>T. brucei</i>		selectivity
	GI ₅₀ (μM) ^a	GI ₅₀ (μM) ^a			GI ₅₀ (μM) ^a	GI ₅₀ (μM) ^a	
1 (IVR)	2.9 ± 0.1	30.0 ± 1.1	10.3				
bioconjugates				conjugation partner			
4	2.1 ± 0.3	21.7 ± 11.4	10.3	quinine	1.1 ± 0.3	>100	>90.9
5	1.3 ± 0.4	22.9 ± 11.2	17.6	quinidine	0.32 ± 0.04	>100	>312.5
6	1.8 ± 0.4	4.0 ± 1.0	2.2	cinchonine	1.7 ± 0.6	>100	>58.8
7	2.3 ± 0.3	47.2 ± 3.7	20.3	cinchonidine	2.1 ± 0.6	>100	>47.6
8	1.4 ± 0.5	10.1 ± 4.9	7.2	<i>N</i> -deacetylthiocholine	17.4 ± 4.3	>100	>5.7
10	2.4 ± 0.5	19.1 ± 9.1	8.0	flouxuridine	2.2 ± 0.4	>100	>45.5
11	2.0 ± 0.8	11.1 ± 4.1	5.6	azidothymidine	19.1 ± 8.1	>100	>5.2
12	2.3 ± 0.3	9.2 ± 3.4	4.0	metronidazole	>100	>100	>1.0
15	21.9 ± 2.1	>100	>4.6	betulinic acid	15.4 ± 4.4	42.5 ± 2.5	2.8
16	0.39 ± 0.2	18.0 ± 6.0	46.2	artesunate	3.6 ± 0.2	30.8 ± 9.0	8.6
IVR + artesunate	9.0 ± 3.5						
suramin ^b	0.039 ± 0.004	>100	>2564				
ethidium bromide ^c	0.046 ± 0.008	9.3 ± 2.6	202				

^aData shown are mean values ± SD of three independent experiments. ^bGI₅₀ ratio = GI₅₀(HL-60)/GI₅₀ (*T. brucei*). ^cReference controls.

obtain the ester, propargyl alcohol was used in the presence of DCC and DMAP (Scheme 4).

To prepare the C13-azide precursor of IVR, the procedure described by Singh et al. was employed.¹⁵ It involved the synthesis of a chloroacetyl ester 13 formed by the reaction between compound 3 and chloroacetyl chloride (Scheme 5). This ester was then subjected to a nucleophilic substitution reaction with sodium azide (Scheme 5). The reaction did not proceed quantitatively, and the resulting azide 14 was difficult to separate chromatographically. Therefore, the click reaction was carried out using a mixture of azide 14 and substrate 13.

The obtained precursors (azide 14 and the respective ester) were dissolved in acetonitrile, and then DIPEA and copper(I) iodide were added. The reactions were carried out in an inert gas atmosphere to avoid oxidation of copper(I) iodide. Deprotection of position C5 was then carried out using *p*-toluenesulfonic acid (Scheme 5). This method proved suitable for the synthesis of the IVR/betulinic acid bioconjugate (compound 15, yield 33%), but it led to the decomposition of artesunate. Therefore, to obtain compound 16, it was necessary to reverse the synthetic procedure (Scheme 5). Azide 14 was first subjected to deprotection with *p*-toluenesulfonic acid and then used in a click reaction with the propargyl ester of artesunate. This approach allowed the synthesis of compound 16 with a click reaction yield of 63% (Scheme 5).

Purity and structure of the synthesized IVR bioconjugates were determined using spectroscopic (¹H NMR, ¹³C NMR) and spectrometric (ESI-MS) methods. In the ¹³C NMR spectra of IVR bioconjugates, the signals of the highest analytical significance were assigned to the newly introduced carbonyl group of the carbonate, urethane, or ester moiety at position C13. Depending on the type of substituent, the signal from the ester group appeared in the range of 176.3–165.3 ppm. The signals from the carbonate group were detected in the range of 153.9–154.6 ppm, while the signal from the urethane group of compound 8 appeared at 158.4 ppm. The signals from the lactone group at position C1 were observed in the range of 173.4–168.3 ppm. In the ¹H NMR spectra of the click derivatives (compounds 15 and 16), as well as metronidazole bioconjugate 12, a characteristic, intensive singlet originating from the hydrogen atom of the triazole/

imidazole ring emerged in the narrow range of 7.88–7.78 ppm. The ESI-MS analysis confirmed the formation of the desired products, with [M + Na]⁺ or [M + H]⁺ as the main peak (intensity = 100%). The NMR and ESI-MS spectra of all novel IVR derivatives are included in the Supporting Information (Figures S1–S23).

2.2. Biological Activity. **2.2.1. Trypanocidal Activity.** The trypanocidal and cytotoxic activities of the newly synthesized IVR bioconjugates and their conjugation partners were determined for bloodstream forms of *T. brucei* and human myeloid HL-60 cells in vitro using the resazurin cell viability assay as previously described.²⁹ For most bioconjugates (4–12), the trypanocidal activity was found to be only slightly better than that of IVR, with 50% growth inhibition (GI₅₀) values ranging between 1.3–2.4 μM (Table 1). In the case of bioconjugates 4, 5, 6, 7, and 10, the observed antitrypanosomal activity was between that of IVR and the corresponding conjugation partner. As *N*-deacetylthiocholine, azidothymidine, and metronidazole displayed trypanocidal activities with GI₅₀ values >10 μM (Table 1), it may be suggested that these conjugation partners can enhance the trypanocidal activity of IVR. The antitrypanosomal activity of the bioconjugate 15 (GI₅₀ = 21.9 μM) was lower than that of IVR and its conjugation partner (Table 1), suggesting that the combination of IVR and betulinic acid reduces their individual effectiveness when linked together. An encouraging result was obtained for bioconjugate 16 (GI₅₀ = 0.39 μM) as its antitrypanosomal activity was 7 times and 9 times stronger than those of IVR and artesunate, respectively (Table 1), indicating synergistic action of the two partners. Since the trypanocidal activity of an equimolar mixture of IVR and artesunate was 23 times lower than that of 16 (Table 1), it can be concluded that IVR and artesunate act synergistically only if linked together. The cytotoxic activity of the bioconjugates was found to be lower compared with their trypanocidal activity (Table 1). Bioconjugates 6 and 15 displayed the strongest and lowest cytotoxic activity with GI₅₀ values of 4.0 and >100 μM, respectively (Table 1). Except for 7 and 15, all other bioconjugates exhibited lower GI₅₀ values towards HL-60 cell line than IVR (Table 1). All conjugation partners displayed low (betulinic acid and artesunate) or no cytotoxic activity (Table 1).

Table 2. Antiproliferative Activity (IC_{50} , μM) of IVR, its Conjugates, and Conjugation Partners^a

compound	cancer cells								normal cells
	PC3		MDA-MB-231		A549		HCT-116		HaCaT
	IC_{50}	SI ^b	IC_{50}	SI ^b	IC_{50}	SI ^b	IC_{50}	SI ^b	IC_{50}
1 (IVR)	33.9 $\pm$ 6.3	0.4	8.7 $\pm$ 3.2	1.4	7.2 $\pm$ 1.4	1.8	9.9 $\pm$ 1.1	1.3	12.6 $\pm$ 4.3
4	13.1 $\pm$ 3.5	2.0	16.3 $\pm$ 4.9	1.6	56.3 $\pm$ 5.1	0.5	16.7 $\pm$ 2.3	1.6	26.8 $\pm$ 4.9
5	12.4 $\pm$ 3.1	1.2	10.1 $\pm$ 1.4	1.5	42.3 $\pm$ 0.5	0.4	13.8 $\pm$ 3.5	1.1	14.9 $\pm$ 3.6
6	4.1 $\pm$ 1.7	3.6	13.8 $\pm$ 1.6	1.1	35.3 $\pm$ 6.5	0.4	12.2 $\pm$ 2.8	1.2	14.6 $\pm$ 1.6
7	7.2 $\pm$ 3.8	7.0	22.4 $\pm$ 1.2	2.2	13.6 $\pm$ 1.1	3.7	20.1 $\pm$ 3.7	2.5	50.3 $\pm$ 4.9
8	85.1 $\pm$ 9.8	>1.2	6.6 $\pm$ 0.6	>15.2	12.4 $\pm$ 2.6	>8.1	7.5 $\pm$ 1.6	>13.3	>100
10	7.8 $\pm$ 2.9	2.0	13.2 $\pm$ 1.1	1.2	15.5 $\pm$ 3.2	1.0	14.3 $\pm$ 1.9	1.1	15.4 $\pm$ 1.1
11	8.1 $\pm$ 3.2	3.2	12.5 $\pm$ 1.5	2.1	8.2 $\pm$ 1.9	3.2	7.7 $\pm$ 3.1	3.4	26.1 $\pm$ 3.3
12	5.9 $\pm$ 1.7	4.5	13.6 $\pm$ 3.7	2.0	13.2 $\pm$ 3.4	2.0	9.1 $\pm$ 0.6	2.9	26.7 $\pm$ 2.6
15	93.9 $\pm$ 6.4	>1.1	>100	>1.0	>100	>1.0	70.1 $\pm$ 6.4	>1.4	>100
16	20.8 $\pm$ 7.2	>4.8	13.0 $\pm$ 4.1	>7.7	3.8 $\pm$ 1.3	>26.3	4.5 $\pm$ 0.6	>22.2	>100
quinine	56.3 $\pm$ 7.2	>1.8	54.4 $\pm$ 7.0	>1.8	56.4 $\pm$ 3.2	>1.8	32.8 $\pm$ 3.8	>1.0	>100
quinidine	86.1 $\pm$ 1.2	>1.2	76.2 $\pm$ 4.6	>1.3	55.8 $\pm$ 5.3	>1.8	67.4 $\pm$ 8.9	>1.5	>100
cinchonine	74.3 $\pm$ 5.4	>1.3	65.3 $\pm$ 2.8	>1.5	>100	>1.0	78.2 $\pm$ 5.8	>1.3	>100
cinchonidine	86.7 $\pm$ 3.9	>1.2	>100	>1.0	>100	>1.0	87.1 $\pm$ 12.1	>1.1	>100
N-deacetylthiocolchicine	56.1 $\pm$ 2.5	1.6	84.2 $\pm$ 7.1	1.0	>100	<0.9	70.3 $\pm$ 5.9	1.2	87.4 $\pm$ 10.4
floxuridine	15.7 $\pm$ 3.3	1.5	22.2 $\pm$ 3.4	1.1	26.4 $\pm$ 3.8	0.9	11.7 $\pm$ 4.2	2.1	24.3 $\pm$ 7.3
azidothymidine	64.3 $\pm$ 3.7	>1.6	52.2 $\pm$ 4.1	>1.9	72.1 $\pm$ 11.6	>1.4	60.2 $\pm$ 9.7	>1.7	>100
metronidazole	57.0 $\pm$ 6.1	>1.8	14.9 $\pm$ 3.9	>6.7	>100	>1.0	>100	>1.0	>100
betulinic acid	66.6 $\pm$ 2.3	>1.5	46.5 $\pm$ 4.6	>2.2	98.1 $\pm$ 3.1	>1.0	80.1 $\pm$ 6.1	>1.2	>100
artesunate	48.5 $\pm$ 5.7	0.1	12.6 $\pm$ 3.9	0.5	24.0 $\pm$ 6.9	0.2	37.8 $\pm$ 4.2	0.2	5.7 $\pm$ 0.1
IVR + artesunate	1.8 $\pm$ 0.5	8.9	22.5 $\pm$ 2.6	0.7	2.4 $\pm$ 0.7	6.7	1.7 $\pm$ 0.2	9.5	16.1 $\pm$ 2.9
doxorubicin ^c	0.6 $\pm$ 0.02	0.5	1.83 $\pm$ 0.1	0.2	0.63 $\pm$ 0.2	0.5	0.6 $\pm$ 0.02	0.5	0.29 $\pm$ 0.1

^aData are expressed as mean $\pm$ SD; IC_{50} (μM), the concentration of the compound that corresponds to a 50% growth inhibition of the cell line (compared to the control) after culturing the cells for 72 h with the individual compound; PC3, human metastatic prostate cancer cell line; MDA-MB-231, human triple-negative breast cancer cell line; A549, human lung cancer cell line; HCT-116, human primary colon cancer cell line; HaCaT, human immortalized keratinocyte cell line. ^bSI (selectivity index) was calculated using the formula: $SI = IC_{50}$ for normal cell line (HaCaT)/ IC_{50} for respective cancer cell line (PC3, MDA-MB-231, A549, or HCT-116). Briefly, an SI > 1.0 indicates that the compounds exhibit greater potency against cancer cells than toxicity toward nontumor cells. ^cReference compound commonly used in cancer treatment.

The selectivity index (ratio of cytotoxic to trypanocidal activity) of all bioconjugates was found to be in the range of moderate values (<100) (Table 1). Only compounds 5, 7, and 16 had a better selectivity index than IVR, with 16 having the best one due to its increased trypanocidal activity (Table 1). On the other hand, the cinchona bark alkaloids (quinine, quinidine, cinchonine, and cinchonidine) had a better selectivity index than bioconjugate 16, which can be attributed to their noncytotoxicity ($GI_{50} > 100 \mu M$, Table 1). Encouraging, however, is the finding that 16 is nontoxic against normal cells (HaCaT, Table 2). Using the cytotoxicity of normal cells (HaCaT) as the reference value, the selectivity index for 16 would then be >256. As the commercial drugs suramin and ethidium bromide (homidium bromide) used for the treatment of human and animal African trypanosomiasis, respectively, display 10 times higher trypanocidal activity, their selectivity indices are much higher (>200) (Table 1).

2.2.2. Antiproliferative Activity. The antiproliferative activities of IVR, its newly synthesized bioconjugates, and its precursors were evaluated against four human cancer cell lines: PC3 (metastatic prostate cancer), MDA-MB-231 (triple-negative breast adenocarcinoma), A549 (lung cancer), and HCT-116 (primary colon cancer) using the MTT colorimetric assay (Table 2). Additionally, to assess the selectivity of these compounds toward cancer cells, we included an immortalized keratinocyte cell line from adult human skin (HaCaT) in the antiproliferative activity tests. Doxorubicin served as the reference anticancer drug. First, it is important to highlight

the antiproliferative activity exhibited by IVR. This drug demonstrates the highest activity among all compounds used for bioconjugation across all tested cell lines, with the exception of floxuridine, which displays a lower IC_{50} value than IVR for the PC3 cell line (Table 2). However, it should be noted that IVR exhibits the highest cytotoxicity toward the HaCaT cell line among all compounds used for bioconjugation, except artesunate. Despite that, IVR selectively targets cancer cells from the MDA-MB-231, A549, and HCT-116 lines more effectively than the reference HaCaT cell line (Table 2). Consequently, the selectivity indices of IVR are comparable to those of the other conjugation partners. Second, the bioconjugates had, in most cases, lower biological activity than IVR. The IVR bioconjugates with cinchona bark alkaloids (compounds 4–8) generally showed reduced antiproliferative activity compared to unmodified IVR (Table 2). However, an interesting result was observed for the PC3 cell line, which was particularly resistant to the effects of IVR and cinchona bark alkaloids. The obtained bioconjugates are effective against the PC3 cancer cell line, with 6 showing an IC_{50} value of 4.1 μM , making it one of the most active compounds in the entire series of bioconjugates (Table 2).

Furthermore, most IVR bioconjugates with cinchona bark alkaloids showed satisfactory selectivity index values, with compound 7 reaching a value of 7.0 (Table 2). Although N-deacetylthiocolchicine exhibited low bioactivity across all tested cancer cell lines, the corresponding bioconjugate (compound 8) had IC_{50} values comparable to those of

unmodified IVR. In addition, compound **8** showed high selectivity, as it was completely nontoxic to the reference HaCaT cell line (SI > 15.2 for the MDA-MB-231 cell line). IVR bioconjugates with the nucleoside analogs and metronidazole (compounds **10–12**) exhibited the highest antiproliferative activity against the PC3 cancer cell line (IC_{50} = 5.9–8.1 μ M), comparable to that of cinchona bark alkaloids, which indicates that the replacement of the sugar moiety at position C13 of IVR with small-molecular conjugation partners enhanced their activity against the PC3 cell line (Table 2).

Bioconjugates with partners characterized by high molecular weight and greater steric hindrance, such as *N*-deacetylthiocolchicine or betulinic acid, seemed to be ineffective against cancer cells of the PC3 line. Moreover, the IVR bioconjugate with betulinic acid (compound **15**) proved to be the least active compound from the entire series (Table 2). This is likely due to its low bioavailability, as this hybrid showed no activity against either cancer cell lines or the reference cell line. A particularly promising compound was the IVR bioconjugate with artesunate (compound **16**). This hybrid proved to be not only the most active bioconjugate from the entire series (IC_{50} = 3.8 μ M against the A549 cell line and IC_{50} = 4.5 μ M against the HCT-116 cell line), but it also showed no toxicity against the HaCaT cell line (Table 2). This indicates that compound **16** is highly selective (SI > 26.3 against the A549 cell line) and could be a lead compound for further drug development.

Inspired by the high potential of compound **16**, we evaluated the antiproliferative activity of an equimolar mixture of IVR and artesunate (Table 2). As shown, the results are ambiguous. In the case of the PC3 cell line, the mixture of the two compounds exhibited significantly greater activity compared to that of compound **16**, IVR, and artesunate tested individually (Table 2). For the A549 and HCT-116 cell lines, the mixture and the bioconjugate **16** demonstrated comparable levels of activity, slightly in favor of the mixture. In contrast, for the MDA-MB-231 cell line, compound **16** showed superior antiproliferative activity relative to that of the drug combination. Despite these inconsistencies, it is important to note the differences in cytotoxicity with respect to the HaCaT reference cell line. Bioconjugation of the two agents effectively mitigated the negative effects of both drugs on noncancerous cells, underscoring the beneficial outcomes of the bioconjugation strategy.

Finally, although doxorubicin showed strong antiproliferative activity against all tested cancer cell lines, it is important to emphasize its high cytotoxicity toward HaCaT cells (Table 2). In all cases, the SI values of doxorubicin were lower than 1.0, indicating that the compound preferentially targets healthy cells over cancerous ones (Table 2). In contrast, almost all bioconjugates displayed SI values greater than 1.0, underscoring their high anticancer potential (Table 2).

3. CONCLUSIONS

A series of new IVR bioconjugates with partners exhibiting high antitumor or antimicrobial activity have been synthesized. These partners included four cinchona bark alkaloids, nucleoside analogs, metronidazole, *N*-deacetylthiocolchicine, betulinic acid, and artesunate. The hybrids were obtained using three different synthetic procedures, allowing the introduction of various linkers such as carbonate, urethane, or a 1,2,3-triazole ring. All obtained bioconjugates, along with their respective conjugation partners, were evaluated in vitro for their activity against the protozoan parasite *T. b. brucei*. For

most bioconjugates, the trypanocidal activity was slightly better than that of IVR, with GI_{50} values ranging between 1.3–2.4 μ M. For the bioconjugates **4**, **5**, **6**, **7**, and **10**, the antitrypanosomal activity was between that of IVR and the corresponding conjugation partner. In addition to the antitrypanosomal studies, in vitro experiments were also conducted against various cancer cell lines. It has been shown that the conjugation of IVR with cinchona bark alkaloids, nucleosides, or metronidazole is an effective method to enhance the antiproliferative activity of IVR against the PC3 cancer cell line, which was most resistant to the action of IVR and the conjugation partners. On the other hand, compound **8** exhibited antiproliferative activity similar to that of IVR, but its lack of toxicity against the HaCaT reference cell line suggests that it is a highly selective anticancer agent. In both antiparasitic and anticancer activity assays, compound **16** (IVR-artesunate bioconjugate) showed the highest activity. This hybrid was not only several times more active than its components but also demonstrated low toxicity against the reference cell line. Moreover, trypanocidal studies of the IVR-artesunate equimolar mixture revealed that the synergistic effect of the combination of these two drugs was observed only after conjugation. A mixture of the two compounds acts antagonistically, showing lower activity than IVR or artesunate alone. Analogous antiproliferative studies gave more variable results; however, compound **16** proved to be significantly less cytotoxic against noncancerous cells than the combination of IVR and artesunate. These observations confirm that conjugation of IVR is a promising approach to develop new bioactive compounds with potential applications in anticancer and antiparasitic therapies.

4. EXPERIMENTAL SECTION

4.1. General Procedure. Ivermectin (IVR, form B1a) was purchased from Trimen Chemicals S.A. All other reagents were commercially available and purchased from two sources, Merck or Trimen Chemicals S.A., and were used without further purification. A detailed description of the general procedures, measurement parameters, and equipment can be found in the Supporting Information.

4.2. Synthesis of Ivermectin Aglycone (Compound 2). IVR (4.95 g, 5.66 mmol, 1.0 equiv) was dissolved in methanol (79.2 mL) and the solution was cooled in an ice bath. Concentrated sulfuric acid (0.8 mL) was then added dropwise to the reaction mixture. The solution changed color to greenish, and after a few hours, it turned yellow. After 20 h, the reaction mixture was concentrated by using a rotary evaporator, diluted with CH_2Cl_2 , and extracted with an aqueous sodium carbonate solution (0.1 M). The organic layers were then concentrated under reduced pressure. The product was purified on silica gel using the CombiFlash system (0% → 30% EtOAc/ CH_2Cl_2), yielding pure aglycone **2** as a clear oil. After two evaporation steps with *n*-pentane to remove any remaining solvent, the oily product was fully converted to a white, amorphous solid (2.79 g, 84% yield). The spectroscopic data were in agreement with previously published data.¹⁵

4.3. Protection of the C5 Hydroxyl Group of Ivermectin Aglycone (Compound 3). To a stirred solution of **2** (2.00 g, 3.41 mmol, 1.0 equiv) in anhydrous CH_2Cl_2 (40 mL), imidazole (2.32 g, 34.07 mmol, 10.0 equiv) was added. Once the imidazole had dissolved, *tert*-butyldimethylsilyl chloride (1.13 g, 7.50 mmol, 2.2 equiv) was added in one portion. The reaction mixture was stirred at room temperature

for 24 h. After this period, the reaction mixture was concentrated to dryness using a rotary evaporator. The product was then purified on silica gel using the CombiFlash system (0% → 10% EtOAc/CHCl₃), yielding pure product 3 as a clear oil. After two evaporation steps with *n*-pentane to remove residual solvent, the oily product was fully converted to a yellow amorphous solid (2.17 g, 91% yield). The spectroscopic data were in agreement with previously published data.¹⁵

4.4. Synthesis of *N*-Deacetylthiocolchicine. To a mixture of colchicine (500 mg, 1.25 mmol) in MeOH (5 mL) was added a sodium methanethiolate solution (21% in H₂O, 0.83 mL, 2.5 mmol). The mixture was stirred at room temperature, and the reaction progress was monitored by TLC. After 24 h, the reaction mixture was quenched by adding water (150 mL). The mixture was then extracted four times with CH₂Cl₂, and the combined organic layers were dried over MgSO₄, filtered, and evaporated under reduced pressure. The residue was purified by the CombiFlash system (EtOAc/MeOH, increasing concentration gradient) to yield thiocolchicine as an amorphous yellow solid (78% yield). Next, a solution of thiocolchicine (500 mg, 1.18 mmol) in MeOH (3 mL) was treated with a 2 M HCl solution (5 mL). The mixture was stirred at 90 °C for 72 h, and the reaction progress was monitored by TLC. Afterward, the solvent was evaporated under reduced pressure. The residue was purified by CombiFlash system (EtOAc/MeOH, increasing concentration gradient) to yield *N*-deacetylthiocolchicine (82% yield). The spectroscopic data were in agreement with previously published data.²¹

4.5. General Procedure for the Preparation of Ivermectin Conjugates with *N*-Deacetylthiocolchicine and Cinchona Bark Alkaloids (Compounds 4–8). Precursor 3 (400 mg, 0.57 mmol, 1.0 equiv) was dissolved in anhydrous THF (20 mL). The solution was then cooled in an ice bath, and triethylamine (288 mg, 2.85 mmol, 5.0 equiv). Next, triphosgene (85 mg, 0.29 mmol, 0.5 equiv) was added to the reaction mixture in one portion, and stirring was continued for 1 h. The formation of a white precipitate was observed. Then, the appropriate conjugation partner (*N*-deacetylthiocolchicine or cinchona bark alkaloid, 0.86 mmol, 1.5 equiv) was added to the reaction mixture, and stirring was continued for 24 h. Thereafter, the reaction mixture was concentrated to dryness using a rotary evaporator, diluted with CH₂Cl₂, and extracted with brine. The collected organic layers were subsequently concentrated *in vacuo*. Purification on silica gel using the CombiFlash system (0% → 100% EtOAc/CHCl₃) gave the respective C5-protected bioconjugates as clear oils.

To remove the C5-protecting group, the individual hybrids were dissolved in methanol (10 mL) and *para*-toluenesulfonic acid hydrate (80 mg, 0.46 mmol, 1.3 equiv) was added. After 2 h, the organic solvent was evaporated, diluted with CH₂Cl₂, and extracted with an aqueous solution of sodium carbonate (0.1 M). Next, the collected organic layers were subsequently concentrated *in vacuo*. Purification on silica gel using the CombiFlash system (0% → 100% EtOAc/CHCl₃) gave the pure products 4–8 as clear oils. After twice evaporation to dryness with *n*-pentane, the oily products were completely converted to white or yellow amorphous solids.

4.5.1. Ivermectin–Quinine Conjugate 4. 86 mg, 19% yield (over two steps). Isolated as a white, amorphous solid, a single spot by TLC. UV-active and strains green with PMA; ¹H NMR (401 MHz, CDCl₃) δ 8.74 (d, *J* = 4.5 Hz, 1H), 8.02 (d, *J* = 9.9

Hz, 1H), 7.40 (d, *J* = 4.6 Hz, 1H), 7.38–7.34 (m, 2H), 6.30 (d, *J* = 5.2 Hz, 1H), 5.88–5.83 (m, 1H), 5.83–5.76 (m, 1H), 5.76–5.72 (m, 1H), 5.68 (dd, *J* = 14.7, 9.3 Hz, 1H), 5.41 (s, 1H), 5.37–5.27 (m, 1H), 5.13 (dd, *J* = 10.6, 4.9 Hz, 1H), 4.98 (dd, *J* = 13.7, 8.3 Hz, 2H), 4.87 (s, 1H), 4.66 (qd, *J* = 14.5, 2.1 Hz, 2H), 4.30–4.26 (m, 1H), 4.25–4.21 (m, 1H), 3.97–3.92 (m, 4H), 3.70–3.62 (m, 1H), 3.33 (q, *J* = 7.9 Hz, 1H), 3.26 (dd, *J* = 4.4, 2.2 Hz, 1H), 3.17 (t, *J* = 11.2 Hz, 2H), 3.04 (dd, *J* = 13.7, 10.2 Hz, 1H), 2.73–2.52 (m, 4H), 2.35–0.71 (m, 38H) ppm; ¹³C NMR (101 MHz, CDCl₃) δ 173.4, 158.0, 154.2, 147.3, 144.7, 140.8, 137.8, 136.0, 134.3, 131.8, 126.7, 125.5, 121.9, 119.9, 118.2, 118.0, 117.8, 114.6, 101.1, 97.4, 83.1, 80.3, 79.2, 77.1, 68.4, 68.3, 67.6, 67.0, 59.3, 56.7, 55.6, 45.6, 42.6, 41.3, 39.7, 39.0, 36.8, 35.8, 35.5, 34.2, 34.1, 31.2, 29.2, 28.0, 27.6, 27.4, 23.8, 22.3, 19.9, 18.5, 17.4, 14.5, 14.0, 12.5, 11.8 ppm; ESI-MS *m/z*: [M + H]⁺ Calcd for C₅₁H₇₇N₂O₁₁⁺ 938; Found 937; [M + Na]⁺ Calcd for C₅₁H₇₇N₂NaO₁₁⁺ 960; Found 959.

4.5.2. Ivermectin–Quinidine Conjugate 5. 102 mg, 16% yield (over two steps). Isolated as a white amorphous solid, a single spot by TLC. UV-active and strains green with PMA; ¹H NMR (401 MHz, CDCl₃) δ 8.69 (d, *J* = 4.6 Hz, 1H), 8.00 (d, *J* = 9.2 Hz, 1H), 7.38–7.30 (m, 3H), 6.48 (d, *J* = 5.7 Hz, 1H), 6.15–6.04 (m, 1H), 5.84 (dt, *J* = 10.7, 2.1 Hz, 1H), 5.81–5.72 (m, 1H), 5.66 (dd, *J* = 14.5, 9.8 Hz, 1H), 5.40 (s, 1H), 5.34–5.24 (m, 1H), 5.14 (s, 1H), 5.12–5.09 (m, 1H), 4.98 (dd, *J* = 10.1, 5.2 Hz, 1H), 4.93 (s, 1H), 4.65 (qd, *J* = 14.5, 2.1 Hz, 2H), 4.28 (d, *J* = 5.8 Hz, 1H), 4.25 (s, 1H), 3.95 (d, *J* = 6.1 Hz, 1H), 3.92 (s, 3H), 3.63–3.54 (m, 1H), 3.29–3.21 (m, 2H), 3.15 (d, *J* = 7.3 Hz, 1H), 2.94 (d, *J* = 9.0 Hz, 2H), 2.90–2.83 (m, 1H), 2.75 (dt, *J* = 13.3, 8.7 Hz, 1H), 2.67–2.57 (m, 2H), 2.28 (dd, *J* = 16.5, 8.5 Hz, 1H), 2.21–2.08 (m, 3H), 2.06–0.66 (m, 34H) ppm; ¹³C NMR (101 MHz, CDCl₃) δ 173.4, 158.1, 154.2, 147.1, 144.6, 143.3, 140.8, 140.1, 137.8, 135.9, 133.9, 131.7, 127.0, 125.5, 122.1, 119.9, 118.0, 118.0, 117.9, 117.5, 115.0, 100.8, 97.4, 83.2, 80.3, 79.2, 76.9, 68.5, 68.3, 67.6, 66.9, 59.0, 55.5, 49.9, 49.2, 45.6, 41.2, 39.8, 39.1, 36.7, 35.7, 35.4, 34.0, 31.2, 28.0, 27.9, 27.4, 26.3, 22.5, 19.8, 18.6, 17.4, 14.4, 12.5, 11.8 ppm; ESI-MS *m/z*: [M + H]⁺ Calcd for C₅₁H₇₇N₂O₁₁⁺ 938; Found 938.

4.5.3. Ivermectin–Cinchonine Conjugate 6. 109 mg, 21% yield (over two steps). Isolated as a white amorphous solid, a single spot by TLC. UV-active and strains green with PMA; ¹H NMR (400 MHz, CDCl₃) δ 8.84 (d, *J* = 4.5 Hz, 1H), 8.15–8.10 (m, 2H), 7.74–7.67 (m, 1H), 7.63–7.56 (m, 1H), 7.41 (d, *J* = 4.5 Hz, 1H), 6.47 (d, *J* = 6.1 Hz, 1H), 6.14–6.03 (m, 1H), 5.84–5.70 (m, 2H), 5.58 (dd, *J* = 13.7, 10.2 Hz, 1H), 5.40 (s, 1H), 5.35–5.25 (m, 1H), 5.13 (s, 1H), 5.10 (d, *J* = 4.4 Hz, 1H), 4.99 (dd, *J* = 9.6, 5.6 Hz, 1H), 4.92 (s, 1H), 4.64 (qd, *J* = 14.6, 1.9 Hz, 2H), 4.28 (s, 2H), 4.09 (dd, *J* = 14.3, 7.1 Hz, 1H), 3.97–3.90 (m, 1H), 3.61 (dd, *J* = 15.2, 9.7 Hz, 1H), 3.29–3.20 (m, 2H), 3.17 (d, *J* = 7.3 Hz, 1H), 2.99–2.87 (m, 2H), 2.87–2.75 (m, 1H), 2.75–2.54 (m, 3H), 2.26 (dd, *J* = 15.6, 7.5 Hz, 1H), 2.19–2.06 (m, 2H), 2.04–0.61 (m, 34H) ppm; ¹³C NMR (101 MHz, CDCl₃) δ 173.4, 154.0, 149.6, 148.3, 145.1, 140.7, 140.0, 137.7, 135.9, 133.9, 130.3, 129.3, 127.0, 125.8, 125.4, 122.9, 119.9, 117.9, 117.8, 117.5, 115.0, 97.4, 83.1, 80.2, 79.1, 68.4, 68.2, 67.6, 66.9, 60.3, 59.5, 49.8, 49.1, 45.6, 41.2, 39.8, 39.0, 36.6, 35.7, 35.4, 34.0, 31.2, 27.9, 27.8, 27.4, 26.3, 22.8, 19.8, 18.6, 17.4, 14.4, 14.1, 12.5, 11.8 ppm; ESI-MS *m/z*: [M + H]⁺ Calcd for C₅₄H₇₇N₂O₁₀⁺ 908; Found 908.

4.5.4. Ivermectin–Cinchonidine Conjugate 7. 88 mg, 17% yield (over two steps). Isolated as a white amorphous solid, a single spot by TLC. UV-active and strains green with PMA; ^1H NMR (400 MHz, CDCl_3) δ 8.89 (d, J = 4.5 Hz, 1H), 8.18 (d, J = 8.3 Hz, 1H), 8.13 (dd, J = 8.5, 0.9 Hz, 1H), 7.71 (ddd, J = 8.3, 6.9, 1.2 Hz, 1H), 7.59 (ddd, J = 8.3, 6.9, 1.2 Hz, 1H), 7.44 (d, J = 4.5 Hz, 1H), 6.37 (d, J = 6.5 Hz, 1H), 5.89–5.63 (m, 5H), 5.41 (s, 1H), 5.36–5.27 (m, 1H), 5.16–5.10 (m, 1H), 5.02–4.94 (m, 2H), 4.86 (s, 1H), 4.65 (qd, J = 14.5, 2.3 Hz, 2H), 4.28 (s, 2H), 4.10 (dd, J = 14.3, 7.1 Hz, 1H), 3.95 (d, J = 6.1 Hz, 1H), 3.71–3.62 (m, 1H), 3.34 (dd, J = 15.5, 7.3 Hz, 1H), 3.26 (dd, J = 4.5, 2.2 Hz, 1H), 3.19 (d, J = 7.6 Hz, 1H), 3.12 (s, 1H), 3.02 (dd, J = 13.8, 10.1 Hz, 1H), 2.70–2.53 (m, 4H), 2.33–2.22 (m, 3H), 2.08–0.69 (m, 33H) ppm; ^{13}C NMR (101 MHz, CDCl_3) δ 173.4, 154.1, 149.8, 148.4, 144.9, 141.4, 140.7, 137.7, 136.0, 134.3, 130.3, 129.3, 126.9, 125.7, 125.4, 123.0, 119.9, 118.1, 118.0, 117.7, 114.6, 97.4, 83.0, 80.2, 79.2, 77.9, 68.4, 68.2, 67.6, 67.0, 60.3, 59.7, 56.6, 45.6, 42.5, 41.2, 39.6, 39.0, 36.7, 35.7, 35.4, 34.2, 31.2, 28.0, 27.6, 27.4, 23.9, 19.8, 18.4, 17.4, 14.5, 14.1, 12.5, 11.8 ppm; ESI-MS m/z : $[\text{M} + \text{H}]^+$ Calcd for $\text{C}_{54}\text{H}_{71}\text{N}_5\text{O}_{16}$ 908; Found 908.

4.5.5. Ivermectin–N-Deacetylthiocolchicine Conjugate 8. 79 mg, 14% yield (over two steps). Isolated as a yellow, amorphous solid, a single spot by TLC. UV-active and strains green with PMA; ^1H NMR (400 MHz, CDCl_3) δ 7.65 (s, 1H), 7.19 (dd, J = 38.4, 10.5 Hz, 2H), 7.05 (s, 1H), 6.54 (s, 1H), 5.68–5.57 (m, 1H), 5.47–5.35 (m, 2H), 5.27–5.17 (m, 1H), 5.03 (d, J = 9.7 Hz, 1H), 4.78–4.50 (m, 3H), 4.28 (d, J = 5.4 Hz, 1H), 3.97 (d, J = 6.1 Hz, 1H), 3.89 (d, J = 3.8 Hz, 3H), 3.75–3.60 (m, 2H), 3.50 (s, 2H), 3.28–3.20 (m, 1H), 2.62–0.69 (m, 48H) ppm; ^{13}C NMR (101 MHz, CDCl_3) δ 182.2, 173.0, 158.5, 155.0, 153.5, 151.0, 151.0, 141.4, 139.5, 138.0, 137.7, 137.3, 135.2, 134.7, 134.3, 129.1, 126.9, 125.6, 124.6, 120.4, 118.2, 117.3, 107.4, 97.4, 80.5, 79.3, 79.2, 76.0, 68.7, 68.2, 67.6, 67.1, 61.4, 61.3, 56.1, 53.4, 45.5, 41.3, 38.9, 37.5, 36.5, 35.7, 35.3, 34.0, 31.1, 30.0, 29.6, 28.1, 27.4, 19.9, 19.2, 17.4, 15.1, 14.4, 12.5, 12.4 ppm; ESI-MS m/z : $[\text{M} + \text{H}]^+$ Calcd for $\text{C}_{55}\text{H}_{77}\text{NO}_{15}\text{S}^+$ 986; Found 987; $[\text{M} + \text{Na}]^+$ Calcd for $\text{C}_{55}\text{H}_{77}\text{NNaO}_{15}\text{S}^+$ 1008; Found 1009.

4.6. Synthesis of IVR Aglycon-1H-imidazole-1-carboxylate (Compound 9). To a suspension of CDI (148 mg, 0.91 mmol, 2 equiv) in dry toluene (10 mL) was added dropwise a solution of compound 3 (320 mg, 0.46 mmol, 1 equiv) in dry toluene (5 mL). The resulting mixture was stirred at room temperature for 24 h. Thereafter, the reaction mixture was concentrated to dryness by using a rotary evaporator, dissolved in CH_2Cl_2 , and extracted with brine. The organic layers were then concentrated under reduced pressure. The product was purified on silica gel using the CombiFlash system (0% $\rightarrow$ 10% EtOAc/ CHCl_3), yielding pure product 9 as an oil. After two extractions with *n*-pentane to remove any remaining solvent, the oily product was fully converted to a yellow amorphous solid (215 mg, 59% yield). The spectroscopic data were in agreement with previously published data.¹⁰

4.7. General Procedure for the Preparation of Ivermectin Conjugates with Nucleosides and Metronidazole (Compounds 10–12). The appropriate conjugation partner (nucleoside analog or metronidazole, 1.5 equiv) was dissolved in DMF (20 mL), and after being heated to 90 $^\circ\text{C}$, DBU (1.5 equiv) was added. After 30 min, a solution of 9 (1.0 g, 1.26 mmol, 1 equiv) in DMF (20 mL) was added dropwise to the reaction mixture, and stirring was continued for 24 h. Then, the reaction mixture was concentrated to dryness by

using a rotary evaporator, diluted with CH_2Cl_2 , and extracted with brine. The collected organic layers were subsequently concentrated in *vacuo*. Purification on silica gel using the CombiFlash system (0% $\rightarrow$ 100% EtOAc/ CHCl_3) gave the respective C5-protected bioconjugates as clear oils.

To remove the C5-protecting group, the individual hybrids were dissolved in methanol (10 mL), and para-toluenesulfonic acid hydrate (80 mg, 0.46 mmol, 1.3 equiv) was added. After 2 h, the organic solvent was evaporated, diluted with CH_2Cl_2 , and extracted with an aqueous solution of sodium carbonate (0.1 M). Next, the collected organic layers were subsequently concentrated in *vacuo*. Purification on silica gel using the CombiFlash system (0% $\rightarrow$ 100% EtOAc/ CHCl_3) gave the pure products 10–12 as clear oils. After twice the evaporation to dryness with *n*-pentane, the oily products were completely converted into white amorphous solids.

4.7.1. Ivermectin–Floxadine Conjugate 10. 162 mg, 15% yield (over two steps). Isolated as a white amorphous solid, a single spot by TLC. UV-active and strains green with PMA; ^1H NMR (400 MHz, CDCl_3) δ 9.83 (s, 1H), 7.73 (d, J = 6.1 Hz, 1H), 6.27 (t, J = 6.0 Hz, 1H), 6.18–6.10 (m, 2H), 5.70 (ddd, J = 24.3, 14.9, 10.1 Hz, 2H), 5.30 (td, J = 10.6, 5.5 Hz, 1H), 5.09 (d, J = 9.8 Hz, 1H), 4.98 (s, 1H), 4.62–4.33 (m, 5H), 4.21 (d, J = 3.3 Hz, 1H), 4.05 (d, J = 1.6 Hz, 1H), 3.73–3.64 (m, 1H), 3.59 (d, J = 9.1 Hz, 1H), 3.17 (d, J = 8.1 Hz, 1H), 2.66–2.58 (m, 2H), 2.55–2.46 (m, 2H), 2.37–0.57 (m, 35H) ppm; ^{13}C NMR (101 MHz, CDCl_3) δ 168.3, 156.9, 156.6, 154.6, 148.9, 141.9, 140.0, 139.5, 138.6, 135.7, 133.5, 129.6, 126.5, 122.4, 118.1, 97.3, 85.8, 84.5, 83.6, 83.0, 78.5, 77.2, 72.1, 71.0, 68.7, 68.1, 66.9, 40.5, 39.1, 36.7, 35.8, 35.5, 34.4, 33.1, 31.2, 29.7, 28.0, 27.2, 18.7, 17.4, 16.8, 14.5, 12.5, 11.7 ppm; ESI-MS m/z : $[\text{M} + \text{Na}]^+$ Calcd for $\text{C}_{44}\text{H}_{50}\text{FN}_2\text{NaO}_{14}^+$ 881; Found 881.

4.7.2. Ivermectin–Azidothymidine Conjugate 11. 321 mg, 29% yield (over two steps). Isolated as a white amorphous solid, a single spot by TLC. UV-active and strains green with PMA; ^1H NMR (400 MHz, CDCl_3) δ 9.55 (s, 1H), 7.27 (d, J = 0.8 Hz, 1H), 6.15–6.05 (m, 2H), 5.67 (ddd, J = 24.7, 14.8, 10.6 Hz, 3H), 5.29–5.20 (m, 1H), 5.05 (d, J = 8.2 Hz, 1H), 4.96 (s, 1H), 4.84 (s, 1H), 4.53 (dd, J = 30.0, 14.4 Hz, 2H), 4.39 (ddd, J = 15.4, 12.0, 4.2 Hz, 2H), 4.22 (dd, J = 12.3, 5.2 Hz, 1H), 4.10–4.01 (m, 2H), 3.68–3.59 (m, 1H), 3.58–3.52 (m, 1H), 3.13 (d, J = 7.7 Hz, 1H), 2.79 (d, J = 6.5 Hz, 1H), 2.64–2.55 (m, 1H), 2.53–2.41 (m, 2H), 2.41–2.30 (m, 1H), 2.30–2.15 (m, 2H), 2.04–0.55 (m, 33H) ppm; ^{13}C NMR (101 MHz, CDCl_3) δ 168.3, 163.6, 154.3, 150.2, 140.1, 138.8, 135.4, 133.6, 129.5, 126.5, 122.2, 117.8, 111.3, 97.2, 85.6, 83.6, 83.0, 81.6, 78.3, 77.1, 71.9, 68.5, 68.0, 66.8, 66.7, 60.4, 40.3, 38.9, 37.4, 36.6, 35.7, 35.4, 34.4, 33.1, 31.1, 29.6, 27.8, 27.2, 18.6, 17.3, 16.8, 14.4, 12.6, 12.4, 11.6 ppm; ESI-MS m/z : $[\text{M} + \text{Na}]^+$ Calcd for $\text{C}_{45}\text{H}_{51}\text{N}_5\text{NaO}_{15}^+$ 902; Found 902.

4.7.3. Ivermectin–Metronidazole Conjugate 12. 296 mg, 30% yield (over two steps). Isolated as a white amorphous solid, a single spot by TLC. UV-active and strains green with PMA; ^1H NMR (401 MHz, CDCl_3) δ 7.88 (s, 1H), 6.07 (dd, J = 7.2, 2.2 Hz, 2H), 5.61 (ddd, J = 24.8, 15.0, 10.3 Hz, 2H), 5.25–5.16 (m, 1H), 4.91 (d, J = 10.8 Hz, 1H), 4.81 (s, 1H), 4.79 (d, J = 15.5 Hz, 1H), 4.59–4.49 (m, 4H), 4.45 (dd, J = 12.2, 2.0 Hz, 2H), 4.37 (ddd, J = 11.3, 10.5, 5.4 Hz, 2H), 3.95 (d, J = 2.1 Hz, 1H), 3.64–3.56 (m, 1H), 3.50 (dd, J = 9.4, 2.0 Hz, 1H), 3.38 (s, 1H), 3.11 (d, J = 7.7 Hz, 1H), 2.57–0.51 (m, 35H) ppm; ^{13}C NMR (101 MHz, CDCl_3) δ 168.5, 154.2, 150.8, 140.3, 138.6, 135.6, 133.5, 133.0, 129.6, 126.4, 122.2, 117.9, 97.2, 83.6, 82.9, 78.4, 77.2, 72.0, 68.6, 68.0, 66.8, 65.7,

45.2, 40.4, 38.9, 36.7, 35.8, 35.4, 34.4, 33.1, 31.2, 27.9, 27.3, 18.5, 17.3, 16.8, 14.4, 14.3, 12.4, 11.6 ppm; ESI-MS m/z : $[M + Na]^+$ Calcd for $C_{41}H_{72}N_3NaO_{12}$ 806; Found 806.

4.8. Synthesis of Betulinic Acid Propargyl Ester. A mixture of betulinic acid (2.0 g, 4.39 mmol, 1.0 equiv), DBU (1.0 g, 6.58 mmol, 1.5 equiv), and propargyl bromide (1.57 g, 13.16 mmol, 3.0 equiv) in anhydrous toluene (50 mL) was heated at 90 °C for 24 h. Subsequently, the mixture was concentrated under reduced pressure. Purification on silica gel using the CombiFlash system (0 → 50% EtOAc/*n*-hexane) yielded the pure product of the reaction (62% yield) as a clear oil. The oil was then diluted in *n*-pentane and evaporated to dryness three times to yield an amorphous solid. The spectroscopic data were in agreement with previously published data.³⁴

4.9. Synthesis of Artesunate Propargyl Ester. To a stirred solution of artesunate (1.0 g, 2.60 mmol, 1.0 equiv) in anhydrous CH_2Cl_2 (40 mL) were added DCC (1.07 g, 5.20 mmol, 2.0 equiv) and a catalytic amount of DMAP. After 30 min, propargyl alcohol (730 mg, 13.02 mmol, 5 equiv) was added dropwise, and stirring was continued for 24 h. The formation of a white precipitate of DCU was observed. Afterward, the white precipitate was filtered off, and the reaction mixture was concentrated to dryness using a rotary evaporator. The product was then purified on silica gel using the CombiFlash system (0% → 100% EtOAc/ $CHCl_3$), yielding the pure product of the reaction (47% yield) as a clear oil. The oil was then diluted in *n*-pentane and evaporated to dryness three times to yield an amorphous solid. The spectroscopic data were in agreement with previously published data.³⁷

4.10. Synthesis of Ivermectin C13-Chloroacetyl Ester (Compound 13). Precursor 3 (1.0 g, 1.43 mmol, 1.0 equiv) was dissolved in anhydrous CH_2Cl_2 (30 mL). The solution was then cooled in an ice bath, and pyridine (564 mg, 7.13 mmol, 5.0 equiv) was added. Next, chloroacetyl chloride (322 mg, 2.85 mmol, 2.0 equiv) was added dropwise to the reaction mixture. The solution turned brown, and stirring was continued for 24 h. Thereafter, the reaction mixture was concentrated to dryness using a rotary evaporator, diluted with CH_2Cl_2 , and extracted with an aqueous solution of sulfuric acid (pH = 1) and then with water. The collected organic layers were subsequently concentrated *in vacuo*. Purification on silica gel using the CombiFlash system (0% → 10% EtOAc/ $CHCl_3$) gave the respective product of the reaction (72% yield) as a clear oil. The oil was then diluted in *n*-pentane and evaporated to dryness three times to yield an amorphous solid. The spectroscopic data were in agreement with the previously published data.³⁵

4.11. Synthesis of Ivermectin C13-Azide Precursor (Compound 14). Ivermectin C13-chloroacetyl ester 13 (400 mg, 0.51 mmol, 1.0 equiv) was dissolved in DMF (10 mL). Sodium azide (67 mg, 1.02 mmol, 2.0 equiv) was then added in one portion; the mixture was heated to 60 °C, and stirring was continued for 24 h. Afterward, the reaction mixture was diluted with a large amount of water, and extracted several times with methylene chloride. This step can be hazardous due to the potential for an explosion resulting from the reaction between sodium azide and the chlorinated solvent, so it is essential to follow the sequence of operations precisely. The collected organic layers were subsequently concentrated *in vacuo*. This raw mixture was used directly in the next step.

4.12. Synthesis of Ivermectin-Betulinic Acid Conjugate (Compound 15). Under a nitrogen atmosphere, a solution of 14 (~1.0 equiv) in anhydrous CH_3CN was prepared. Betulinic acid propargyl ester (157 mg, 0.32 mmol, 1.0 equiv) and DIPEA (123 mg, 0.95 mmol, 3.0 equiv) were then added, followed by the addition of catalytic CuI (6.05 mg, 0.03 mmol, 0.1 equiv) in one portion. The reaction mixture was stirred at room temperature for 24 h. After complete consumption of the propargyl partner (monitored by TLC and ESI-MS), the organic solvent was removed by using a rotary evaporator. The oily residue was dissolved in a small amount of CH_2Cl_2 and extracted several times with 10% aqueous EDTA solution. The organic phases were separated and concentrated under reduced pressure. The product was purified by silica gel chromatography using the CombiFlash system (0 → 100% EtOAc/ $CHCl_3$), yielding the pure product of the click reaction as a clear oil. The oil was diluted in *n*-pentane and evaporated to dryness three times to obtain the amorphous solid.

To remove the C5-protecting group, the hybrid was dissolved in methanol (10 mL), and *para*-toluenesulfonic acid hydrate (80 mg, 0.46 mmol, 1.3 equiv) was added. After 2 h, the organic solvent was evaporated, diluted with CH_2Cl_2 , and extracted with an aqueous solution of sodium carbonate (0.1 M). Next, the collected organic layers were subsequently concentrated *in vacuo*. Purification on silica gel using the CombiFlash system (0% → 100% EtOAc/ $CHCl_3$) gave the pure product 15 as a clear oil. After twice evaporation to dryness with *n*-pentane, the oily product was completely converted into a white amorphous solid.

4.12.1. Ivermectin-Betulinic Acid Conjugate 15. 122 mg, 33% yield (over two steps). Isolated as a white amorphous solid, a single spot by TLC. UV-active and stains green with PMA; ^1H NMR (401 MHz, CD_2Cl_2) δ 7.78 (s, 1H), 6.17 (d, J = 2.0 Hz, 1H), 6.12 (dt, J = 10.8, 2.4 Hz, 1H), 5.81 (ddd, J = 20.3, 10.5, 6.7 Hz, 1H), 5.72–5.62 (m, 1H), 5.32 (m, 1H), 5.30–5.25 (m, 3H), 5.22 (dd, J = 9.7, 3.6 Hz, 3H), 5.06–5.00 (m, 1H), 4.75 (s, 1H), 4.72 (d, J = 2.3 Hz, 1H), 4.58 (dd, J = 2.4, 1.4 Hz, 1H), 4.58–4.45 (m, 2H), 3.97 (d, J = 2.2 Hz, 1H), 3.76–3.67 (m, 1H), 3.55–3.47 (m, 1H), 3.24 (dd, J = 9.9, 5.1 Hz, 1H), 3.13 (dd, J = 12.1, 4.0 Hz, 1H), 3.02–2.95 (m, 1H), 2.73–2.62 (m, 1H), 2.53–0.62 (m, 76H) ppm; ^{13}C NMR (101 MHz, CD_2Cl_2) δ 176.3, 169.2, 166.2, 151.3, 144.2, 141.2, 139.3, 136.2, 134.0, 130.4, 127.2, 125.8, 122.7, 118.7, 109.9, 97.9, 83.7, 81.6, 79.2, 79.0, 77.7, 72.7, 69.3, 68.7, 67.5, 57.6, 57.0, 55.8, 51.2, 51.1, 50.0, 47.6, 42.9, 41.3, 41.0, 39.5, 39.3, 39.3, 38.8, 37.7, 37.4, 36.4, 36.1, 35.0, 34.8, 33.9, 32.5, 31.8, 31.1, 30.1, 28.5, 28.3, 28.0, 28.0, 26.1, 21.4, 19.6, 19.2, 18.8, 17.8, 17.2, 16.5, 16.1, 15.8, 15.0, 14.9, 13.0, 12.1 ppm; ESI-MS m/z : $[M + Na]^+$ Calcd for $C_{60}H_{101}N_3NaO_{12}$ 1187; Found 1188.

4.13. Synthesis of Ivermectin-Artesunate Conjugate (Compound 16). To remove the C5-protecting group, azide 14 was dissolved in methanol (10 mL), and *para*-toluenesulfonic acid hydrate (80 mg, 0.46 mmol, 1.3 equiv) was added. After 2 h, the organic solvent was evaporated, diluted with CH_2Cl_2 , and extracted with an aqueous solution of sodium carbonate (0.1 M). Next, the collected organic layers were subsequently concentrated *in vacuo*. This raw mixture was used directly in the next step.

Under a nitrogen atmosphere, a solution of deprotected azide (~1.0 equiv) in anhydrous CH_3CN was prepared. Artesunate propargyl ester (135 mg, 0.32 mmol, 1.0 equiv) and DIPEA (123 mg, 0.95 mmol, 3.0 equiv) were then added,

followed by the addition of catalytic CuI (6.05 mg, 0.03 mmol, 0.1 equiv) in one portion. The reaction mixture was stirred at room temperature for 24 h. After complete consumption of the propargyl partner (monitored by TLC and ESI-MS), the organic solvent was removed using a rotary evaporator. The oily residue was dissolved in a small amount of CH_2Cl_2 and extracted several times with 10% aqueous EDTA solution. The organic phases were separated and concentrated under reduced pressure. The product was purified by silica gel chromatography using the CombiFlash system (0 $\rightarrow$ 100% EtOAc/ CHCl_3), yielding the pure product **16** of the click reaction as a clear oil. The oil was diluted in *n*-pentane and evaporated to dryness three times to obtain the amorphous solid.

4.13.1. Ivermectin–Artesunate Conjugate **16.** 220 mg, 63% yield (yield of one step–click reaction). Isolated as a white amorphous solid, a single spot by TLC. UV-active and strains green with PMA₆. ^1H NMR (401 MHz, CDCl_3) δ 7.80 (s, 1H), 6.18–6.13 (m, 2H), 5.75 (dt, J = 11.0, 8.0 Hz, 2H), 5.62 (dd, J = 15.0, 10.0 Hz, 1H), 5.42 (s, 1H), 5.35–5.26 (m, 5H), 5.21 (s, 1H), 4.94 (d, J = 11.2 Hz, 1H), 4.83 (s, 1H), 4.53 (qd, J = 14.1, 2.2 Hz, 2H), 4.04 (d, J = 2.2 Hz, 1H), 3.74–3.65 (m, 1H), 3.61–3.55 (m, 1H), 3.20 (d, J = 7.8 Hz, 1H), 2.78–0.61 (m, 60H) ppm; ^{13}C NMR (101 MHz, CDCl_3) δ 171.8, 170.9, 168.6, 165.3, 143.2, 140.5, 138.7, 135.5, 133.4, 129.6, 126.5, 125.2, 122.2, 118.1, 104.4, 97.3, 92.1, 91.4, 82.8, 81.0, 80.0, 78.4, 72.1, 68.7, 68.1, 66.8, 57.9, 51.5, 50.5, 45.1, 40.4, 38.8, 37.2, 36.7, 36.1, 35.8, 35.4, 34.4, 34.0, 33.2, 31.7, 31.2, 29.6, 29.1, 28.7, 27.9, 27.4, 25.8, 24.5, 21.9, 20.1, 18.8, 17.4, 16.8, 14.5, 12.6, 11.9, 11.7 ppm; ESI-MS m/z : $[\text{M} + \text{Na}]^+$ Calcd for $\text{C}_{28}\text{H}_{40}\text{N}_2\text{NaO}_{17}$ 1115; Found 1114.

4.14. In Vitro Biological Studies. **4.14.1. Trypanocidal and Cytotoxic Assays.** The 427–221a clone of bloodstream forms of *T. b. brucei*⁵⁰ and human promyelocytic leukemia HL-60 cells⁵⁰ were used for evaluating the trypanocidal and cytotoxic activity of test compounds. Trypanosomes and HL-cells were seeded in 96-well plates at cell densities of 1×10^4 and $5 \times 10^4 \text{ mL}^{-1}$, respectively, in 200 μL of Baltz medium⁵⁰ supplemented with 16.7% bovine serum. Test compounds were assayed at 10-fold dilutions ranging from 100 μM to 1 nM in the presence of 0.9% DMSO. Controls were grown in the culture medium containing only 0.9% DMSO. The assays were cultured in an incubator at 37 $^\circ\text{C}$ in a humidified atmosphere containing 5% CO_2 . After 24 h incubation, 20 μL of 0.5 mM resazurin (prepared in sterile PBS) was added, and the cultures were grown for another 48 h. Then, the proliferation of the cells was determined by measuring the absorbance at 570 nm (test wavelength) and 630 nm (reference wavelength) using a microplate reader. The half-maximal growth inhibition (GI_{50}) values, i.e., the concentration of a test compound that reduces the growth rate of cells by 50% compared to the growth rate of the control cells, were calculated using the method described by Huber and Koella.⁵¹

4.14.2. Human Cell Lines. The human cancer cell lines PC3 (metastatic prostate cancer), MDA-MB-231 (breast cancer), A549 (lung cancer), HCT-116 (colon carcinoma), and the immortalized human keratinocyte line HaCaT were obtained from the repository of the Medical University of Warsaw. PC3 cells were cultured in RPMI (Biowest SAS, France) and HCT-116 cells in MEM (Thermo Scientific, USA), while A549, MDA-MB-231, and HaCaT cells were maintained in DMEM High Glucose (Biowest SAS, France). The culture media were enriched with 10% fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO, USA), 20 mM HEPES (Biowest, Nuaille, France),

and antibiotics (100 U/mL penicillin and 100 $\mu\text{g/mL}$ streptomycin) obtained from Gibco (Grand Island, NY, USA). The cells were maintained in a humidified incubator at 37 $^\circ\text{C}$ with 5% CO_2 until they reached 80–90% confluence, after which they were used for further experiments.

4.14.3. Cytotoxicity. To assess the cytotoxicity, the cells ($0.5 \times 10^4/\text{well}$) were plated in 96-well plates and allowed to adhere for 24 h. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to evaluate the cytotoxic effects of IVR bioconjugates on all tested cell lines. After 24 h incubation, cells were exposed to varying concentrations (1 to 100 μM) of the test compounds for 72 h. Following the treatment, the medium was discarded, and a 0.5 mg mL^{-1} MTT solution was added to each well and incubation was continued for another 4 h. Mitochondrial enzyme activity facilitated the formation of formazan crystals, which were subsequently dissolved in DMSO-isopropanol (1:1, v/v), producing a violet-colored solution. A 100 μL aliquot of this solution was transferred to the well, and absorbance was recorded at 570 nm using a MultiscanGo spectrophotometer (ThermoFisher Scientific, Carlsbad, CA, USA).

Cytotoxicity was quantified by comparing the absorbance of the treated cells to that of untreated controls using the formula: $[\text{A}]/[\text{B}] \times 100$, where [A] represents the absorbance of the treated sample and [B] corresponds to the absorbance of the control sample. A decrease in MTT levels reflects a decrease in cell viability. IC_{50} values, the concentration required to inhibit 50% of cell viability, were calculated using GraphPad Prism 8.0.1 software.

■ ASSOCIATED CONTENT

Data Availability Statement

The data underlying this study are available in the published article and its Supporting Information.

■ Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.omega.5c02998>.

General procedures, as well as NMR and ESI-MS spectra of the reported compounds (PDF)

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ABBREVIATIONS

A549, lung cancer cell line; CDI, 1,1'-carbonyldiimidazole; CuAAC, copper-catalyzed azide-alkyne cycloaddition; DBU, 1,8-diazabicyclo[5.4.0]undec-7-en; DCC, *N,N*'-dicyclohexylcarbodiimide; DIPEA, *N,N*-diisopropylethylamine; DMAP, 4-dimethylaminopyridine; DMF, *N,N*-dimethylformamide; DNA, DNA; ESI-MS, electrospray ionization mass spectrometry; GI50, half-maximal growth inhibition; HaCaT, human keratinocytes; HCT-116, primary colon cancer; HL-60, promyelocytic leukemia cell line; IC50, half-maximal inhibitory concentration; IVR, ivermectin; MDA-MB-231, triple-negative breast adenocarcinoma; MDR, multidrug resistance; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NMR, nuclear magnetic resonance; PC3, metastatic prostate cancer; pTsOH, para-toluenesulfonic acid; pyr, pyridine; ROS, reactive-oxygen species; rt, room temperature; SI, selectivity index; TBDMSCl, *tert*-butyldimethylsilyl chloride; TEA, triethylamine; THF, tetrahydrofuran; WHO, World Health Organization

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Antiproliferative and trypanocidal activity of ivermectin bioconjugates

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Supplementary material

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General procedures

All reagents were purchased from two sources – Merck or Trimen Chemicals S.A., and used without further purification. CDCl_3 and CD_2Cl_2 spectral grade were stored over 3 Å molecular sieves for several days. Reaction mixtures were stirred using Teflon-coated magnetic stir bars and were monitored by thin layer chromatography (TLC) using aluminum-backed plates (Merck 60 F₂₅₄). TLC plates were visualized by UV-light (254 nm), followed by treatment with phosphomolybdic acid (PMA) (5% in absolute ethanol) and gentle heating. Products of the reactions were purified using CombiFlash® Rf+ Lumen Flash Chromatography System (Teledyne Isco) with integrated ELS and UV detectors. All solvents used in flash chromatography were of HPLC grade (Merck) and were used as received. Solvents were removed using a rotary evaporator.

NMR spectra were recorded on a Varian 400 (^1H NMR at 400 MHz and ^{13}C NMR at 101 MHz) magnetic resonance spectrometer. ^1H NMR spectra are reported in chemical shifts downfield from TMS using the respective residual solvent peak as internal standard (CDCl_3 δ 7.26 ppm; CD_2Cl_2 δ 5.32 ppm). ^1H NMR spectra are reported as follows: chemical shift (δ , ppm), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, dd = doublet of doublets, dt = doublet of triplets, td = triplet of doublets, qd = quartet of doublets, ddd = doublet of doublets of doublets, m = multiplet), coupling constant(s) in Hz, and integration. Significant peaks are reported within the overlapping ~ 2.50 – 0.50 ppm region of the ^1H NMR spectra. ^{13}C NMR spectra are reported in chemical shifts downfield from TMS using the respective residual solvent peak as internal standard (CDCl_3 δ 77.16 ppm; CD_2Cl_2 δ 53.84 ppm). Line broadening parameters were 0.5 or 1.0 Hz, while the error of chemical shift value was 0.1 ppm.

The electrospray ionization (ESI) mass spectra were recorded on a Waters/Micromass ZQ mass spectrometer (Waters Alliance) equipped with a Harvard syringe pump. The samples were prepared in dry acetonitrile, and were infused into the ESI source using a Harvard pump at a flow rate of 20 ml/min. The ESI source potentials were: capillary 3 kV, lens 0.5 kV, and extractor 4 V. The standard ESI mass spectra were recorded at the cone voltages of 10 V. The source temperature was 120 °C and the desolvation temperature was 300 °C. Nitrogen was used as the nebulizing and desolvation gas at flow-rates of 100 dm³/h. Mass spectra were acquired in the positive ion detection mode with unit mass resolution at a step of 1 m/z unit. The mass range for ESI experiments was from m/z = 600 to m/z = 1200 or from m/z = 600 to m/z = 1400.

Spectroscopic and spectrometric analysis of newly synthesized compounds

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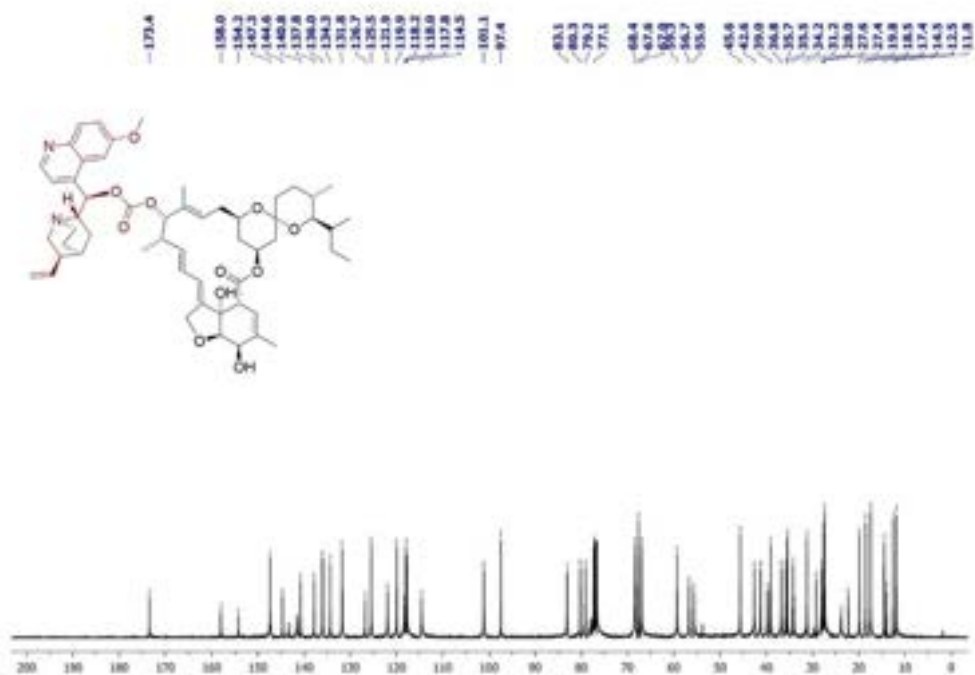


Figure S1. The ^{13}C NMR (101 MHz) spectrum of 4 in chloroform-d.

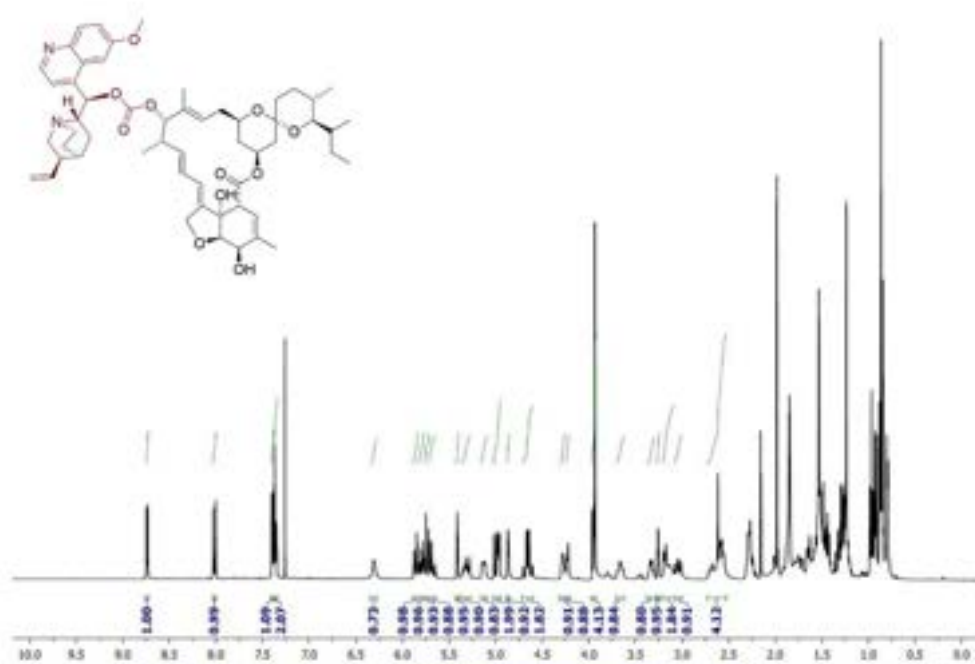
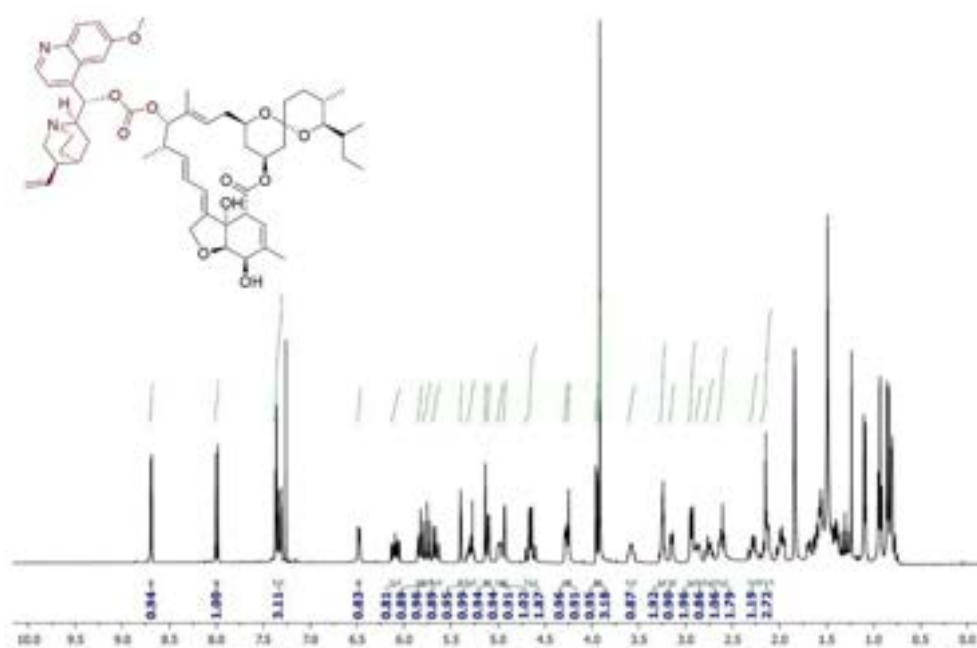
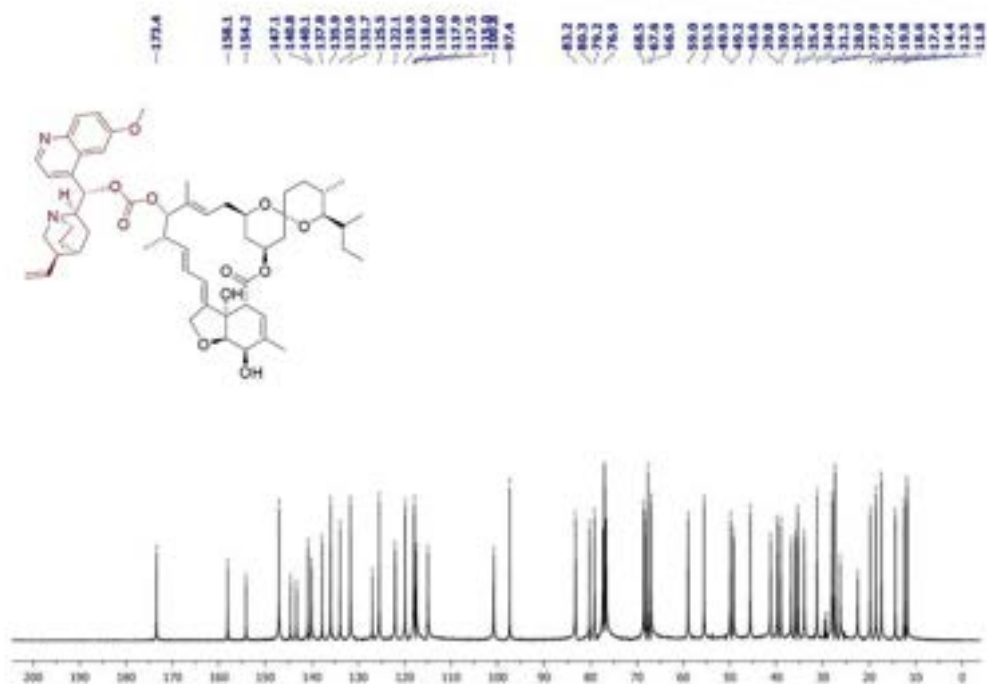
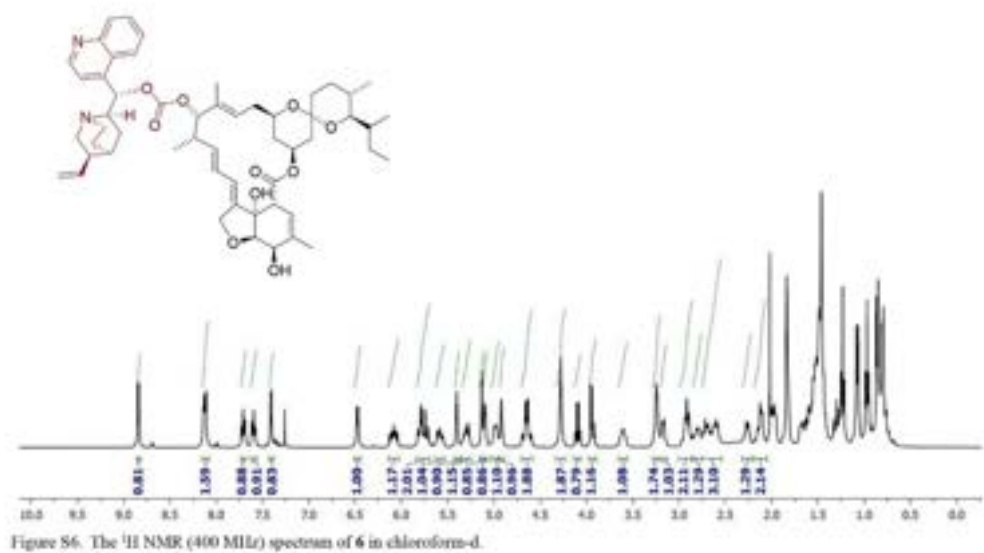
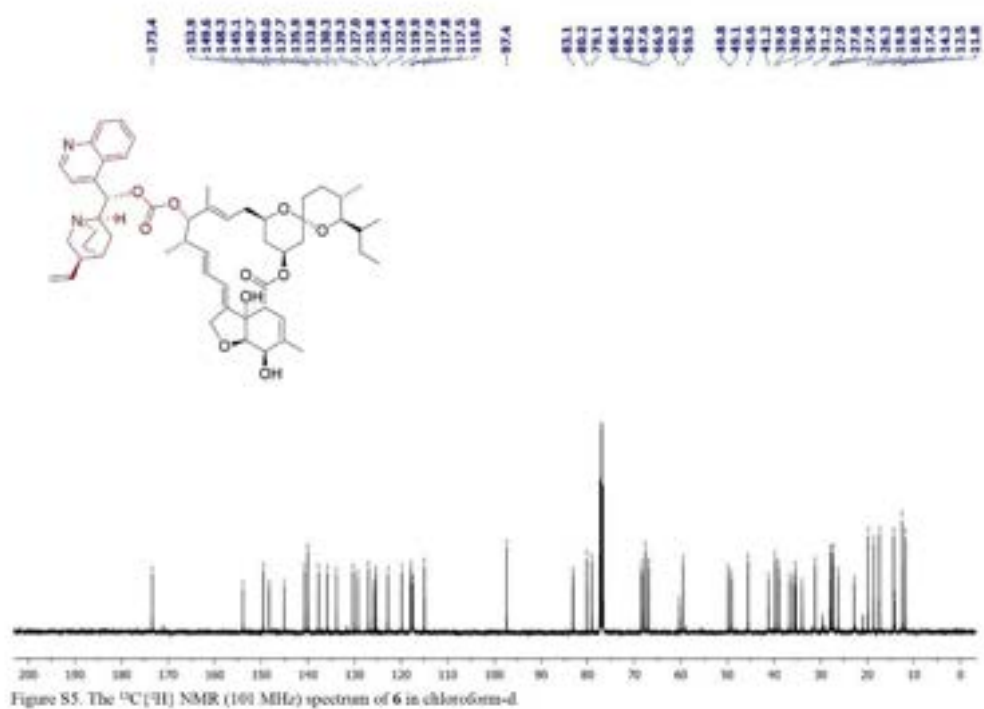
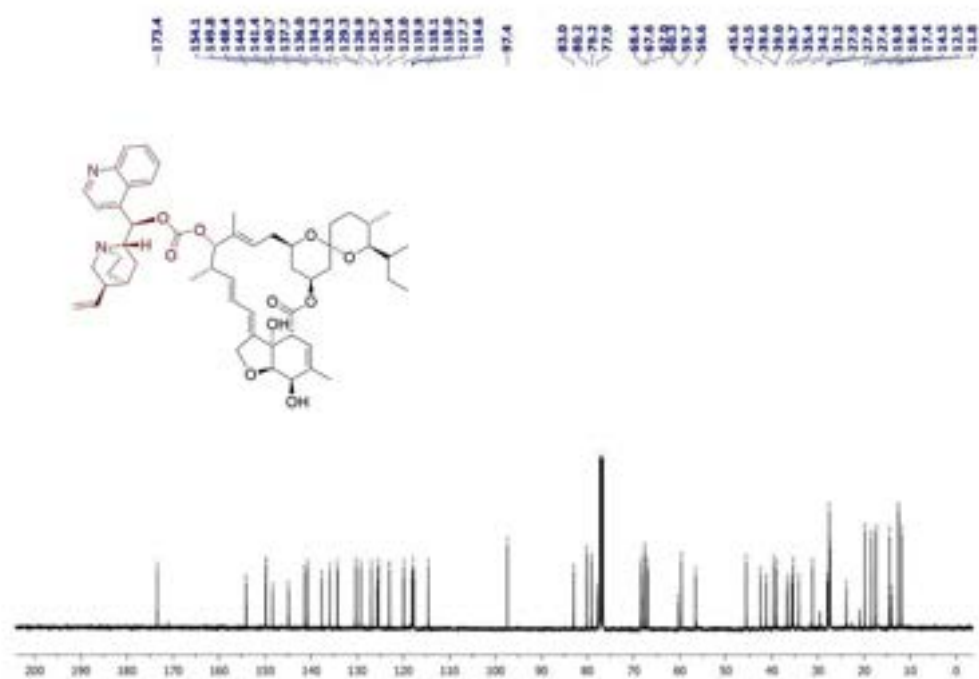
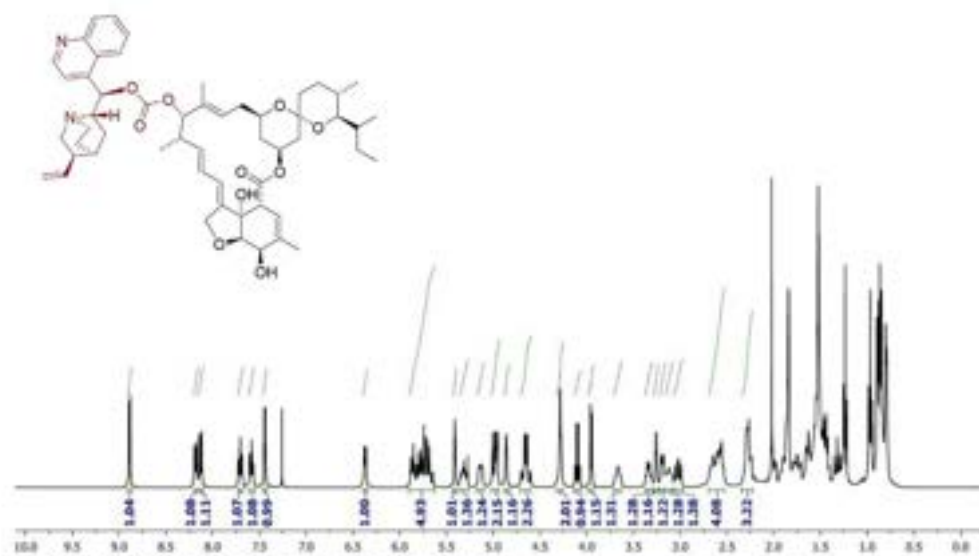


Figure S2. The ^1H NMR (400 MHz) spectrum of 4 in chloroform-d.





Figure S7. The ^{13}C NMR (101 MHz) spectrum of 7 in chloroform- d .Figure S8. The ^1H NMR (400 MHz) spectrum of 7 in chloroform- d .

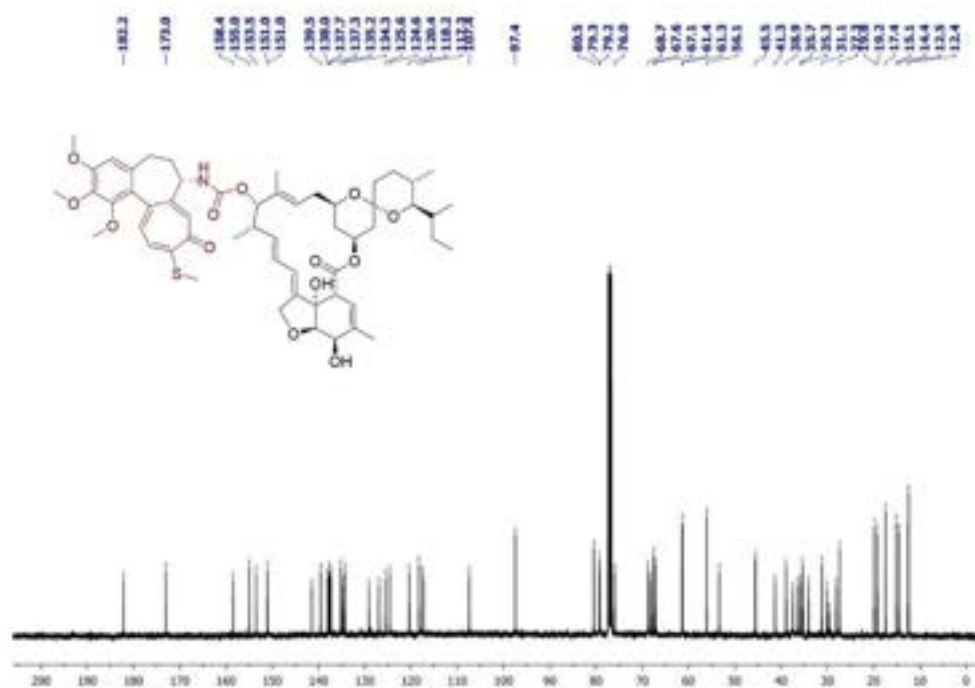


Figure S9. The ^{13}C NMR (101 MHz) spectrum of **8** in chloroform- d .

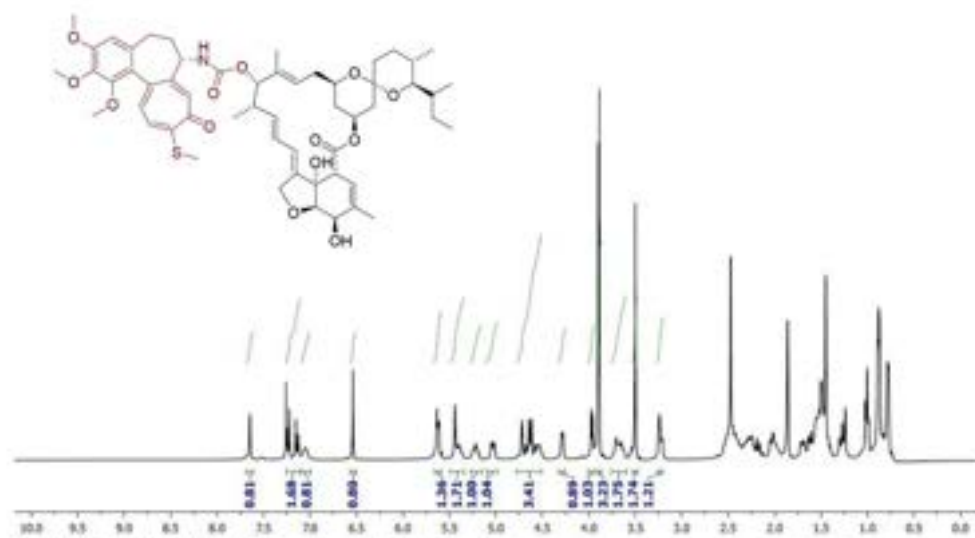


Figure S10. The ^1H NMR (400 MHz) spectrum of **8** in chloroform- d .

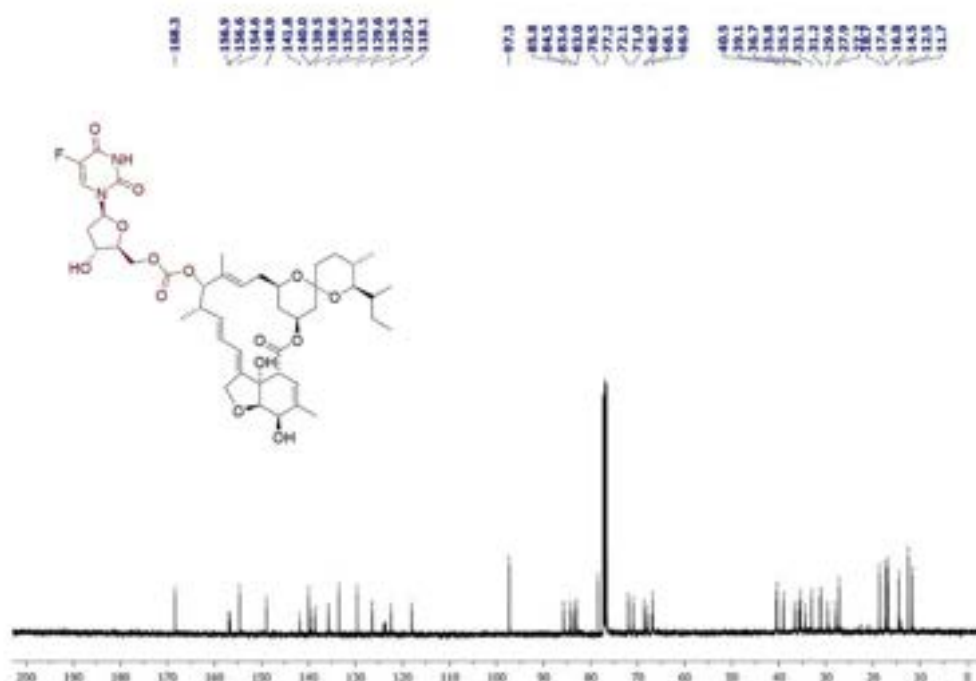


Figure S11. The $^{13}\text{C}\{^1\text{H}\}$ NMR (101 MHz) spectrum of **10** in chloroform- d .

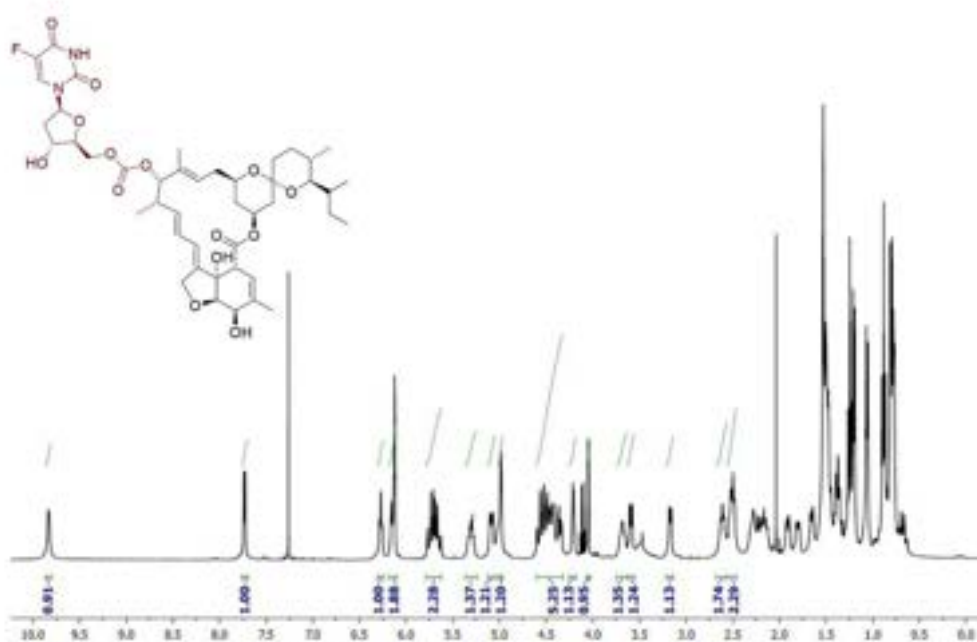


Figure S12. The ^1H NMR (400 MHz) spectrum of **10** in chloroform- d .

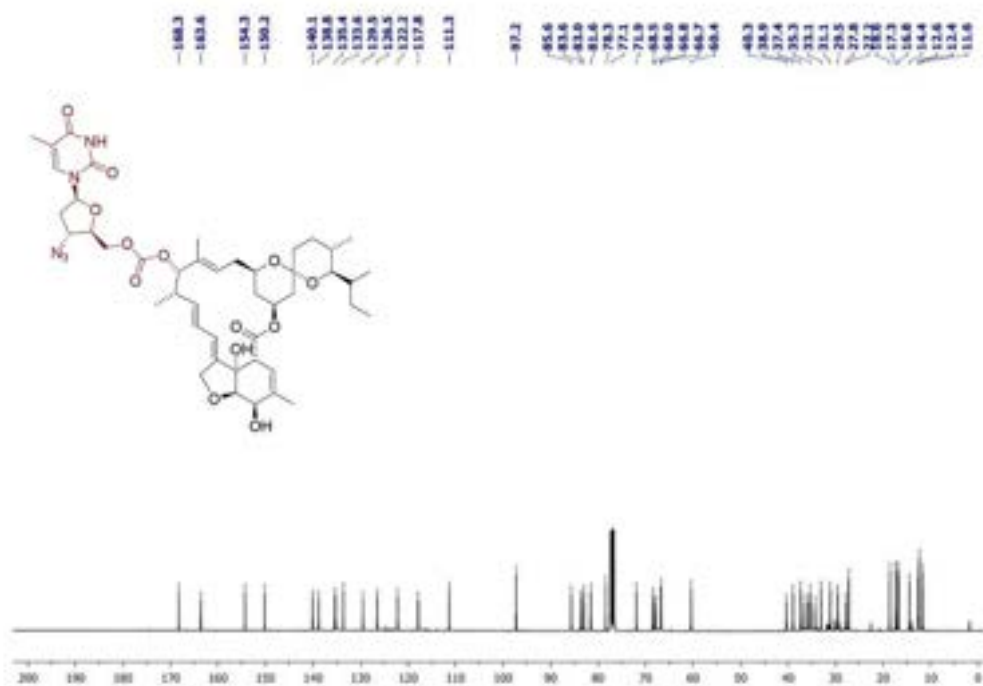


Figure S13. The ^{13}C NMR (101 MHz) spectrum of 11 in chloroform- d .

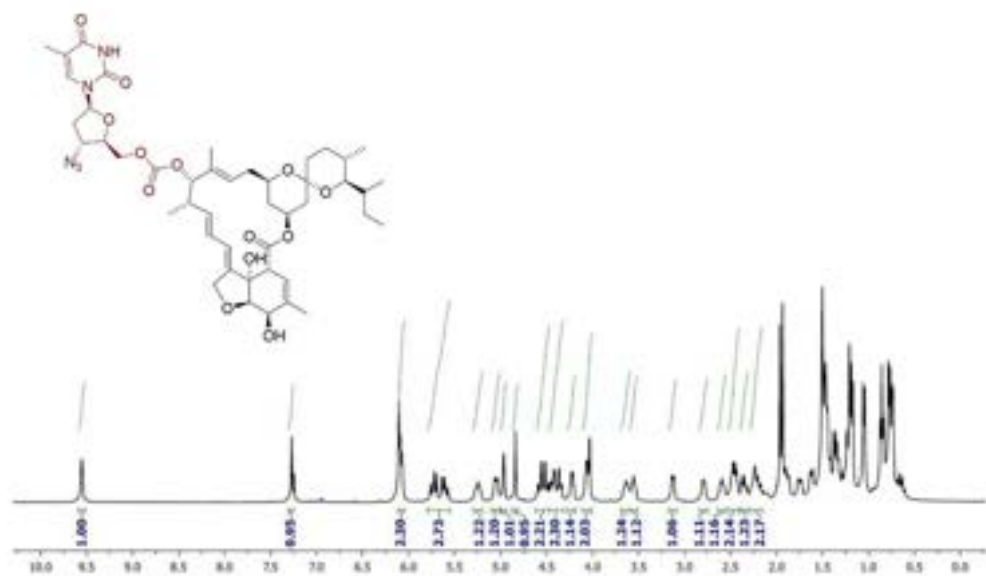
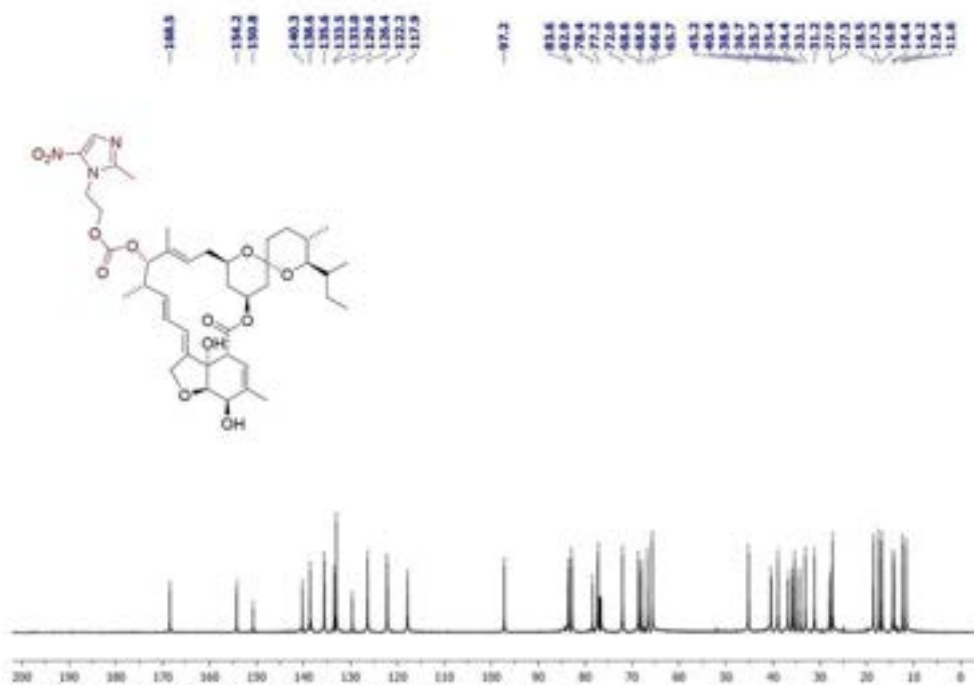
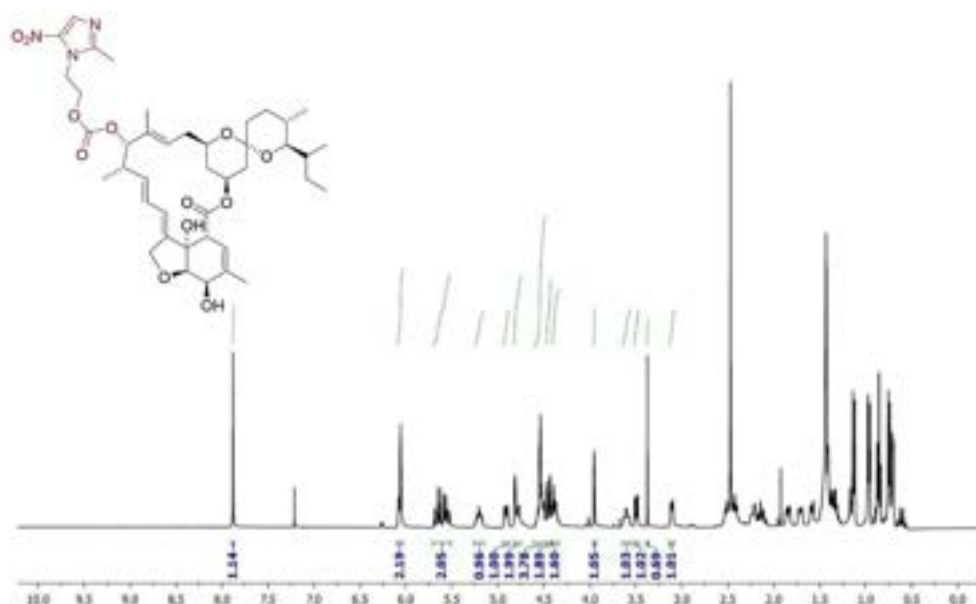


Figure S14. The ^1H NMR (400 MHz) spectrum of 11 in chloroform- d .

Figure S15. The ^{13}C (^1H) NMR (101 MHz) spectrum of **12** in chloroform- d .Figure S16. The ^1H NMR (400 MHz) spectrum of **12** in chloroform- d .

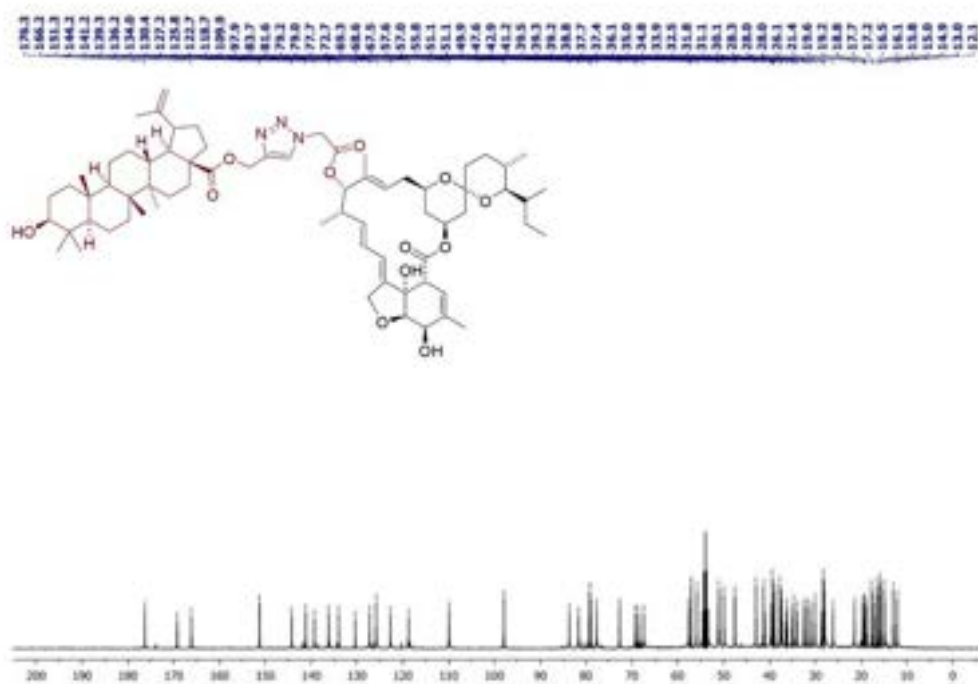


Figure S17. The ^{13}C (^1H) NMR (101 MHz) spectrum of **15** in methylene chloride- d_2 .

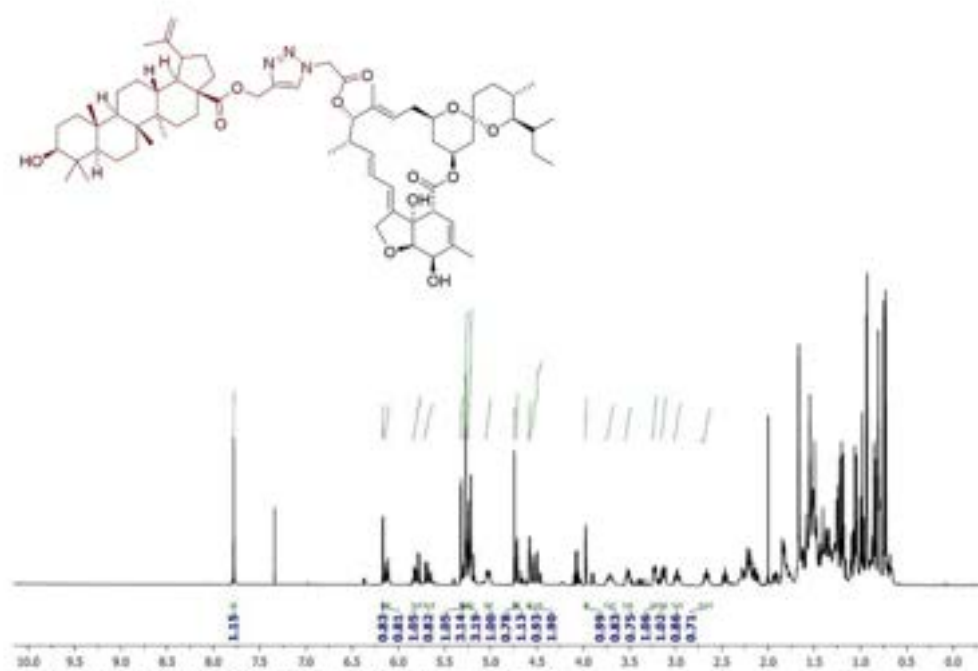
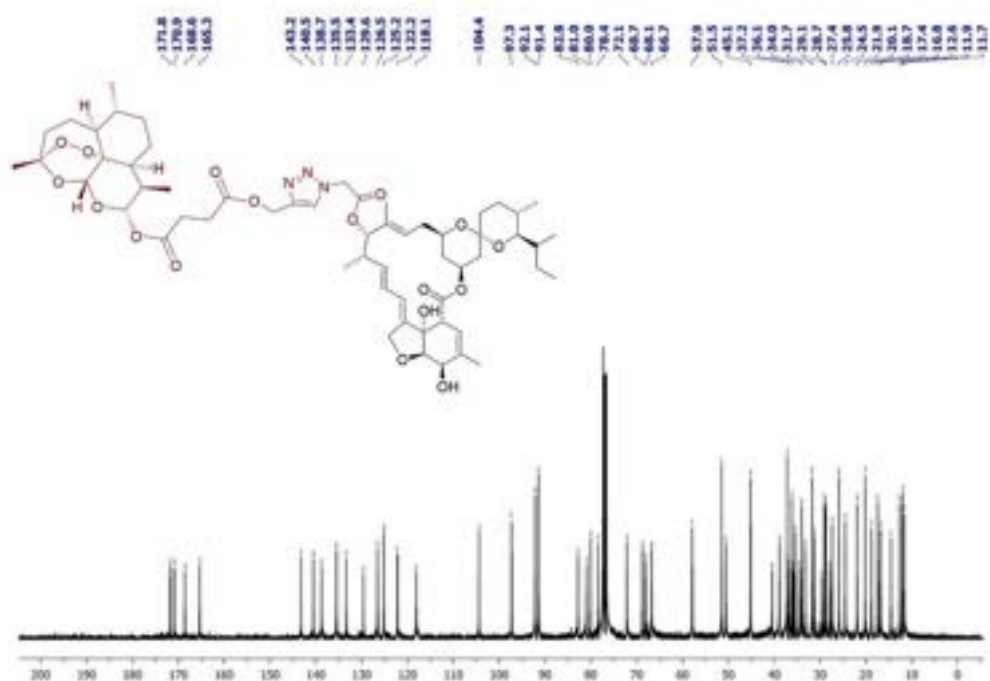
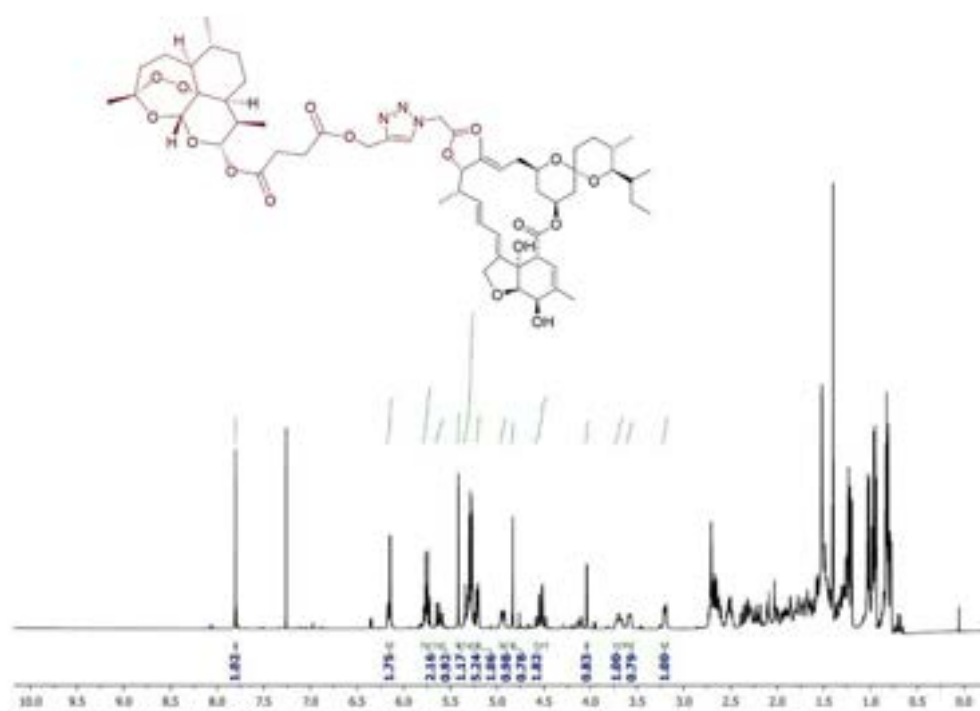


Figure S18. The ^1H NMR (400 MHz) spectrum of **15** in methylene chloride- d_2 .

Figure S19. The ^{13}C (^{1}H) NMR (101 MHz) spectrum of **16** in chloroform- d .Figure S20. The ^1H NMR (400 MHz) spectrum of **16** in chloroform- d .

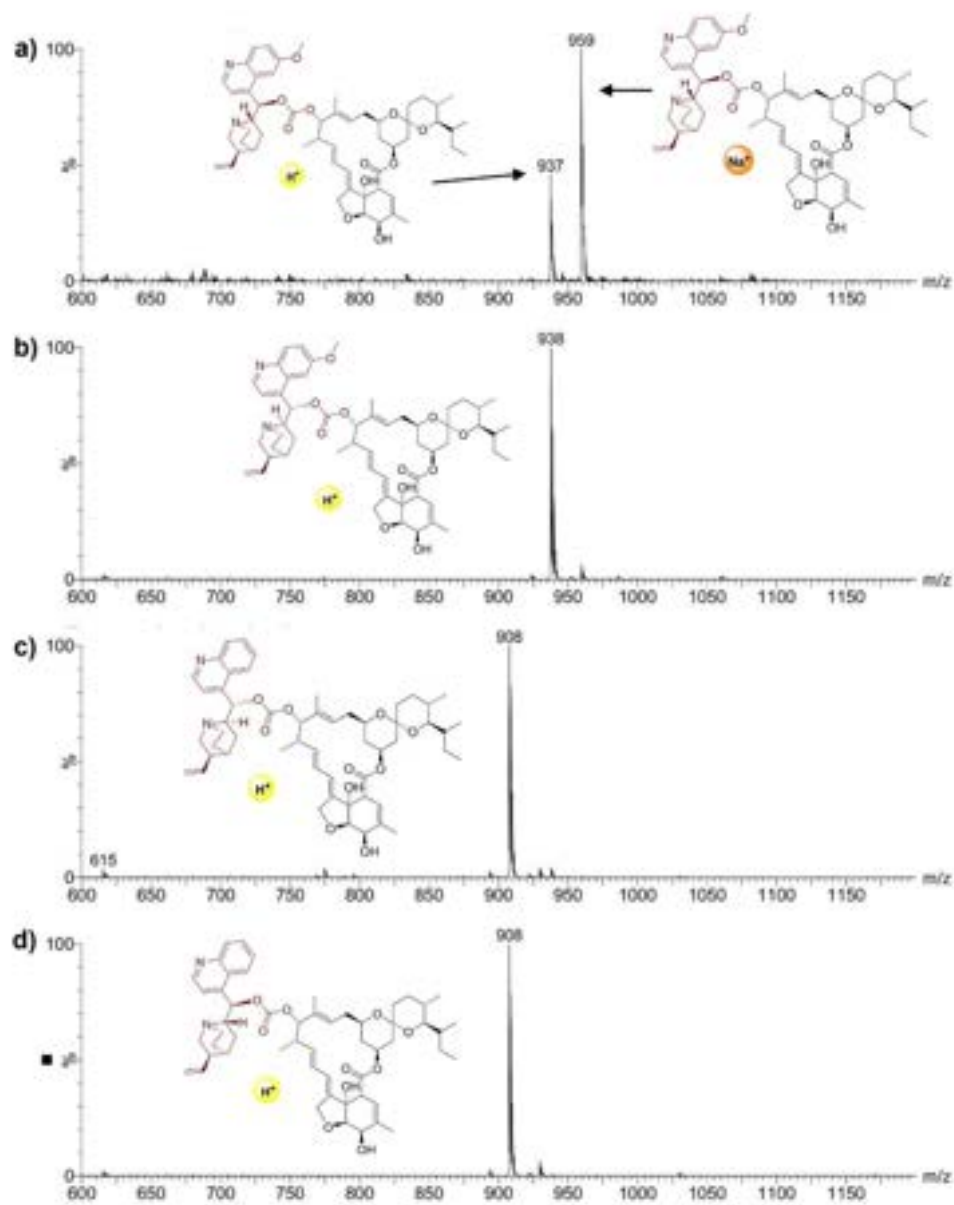


Figure S21. The ESI mass spectra of a mixture of **4** (a), **5** (b), **6** (c), and **7** (d), with $NaClO_4$ at $cv = 10$ V.

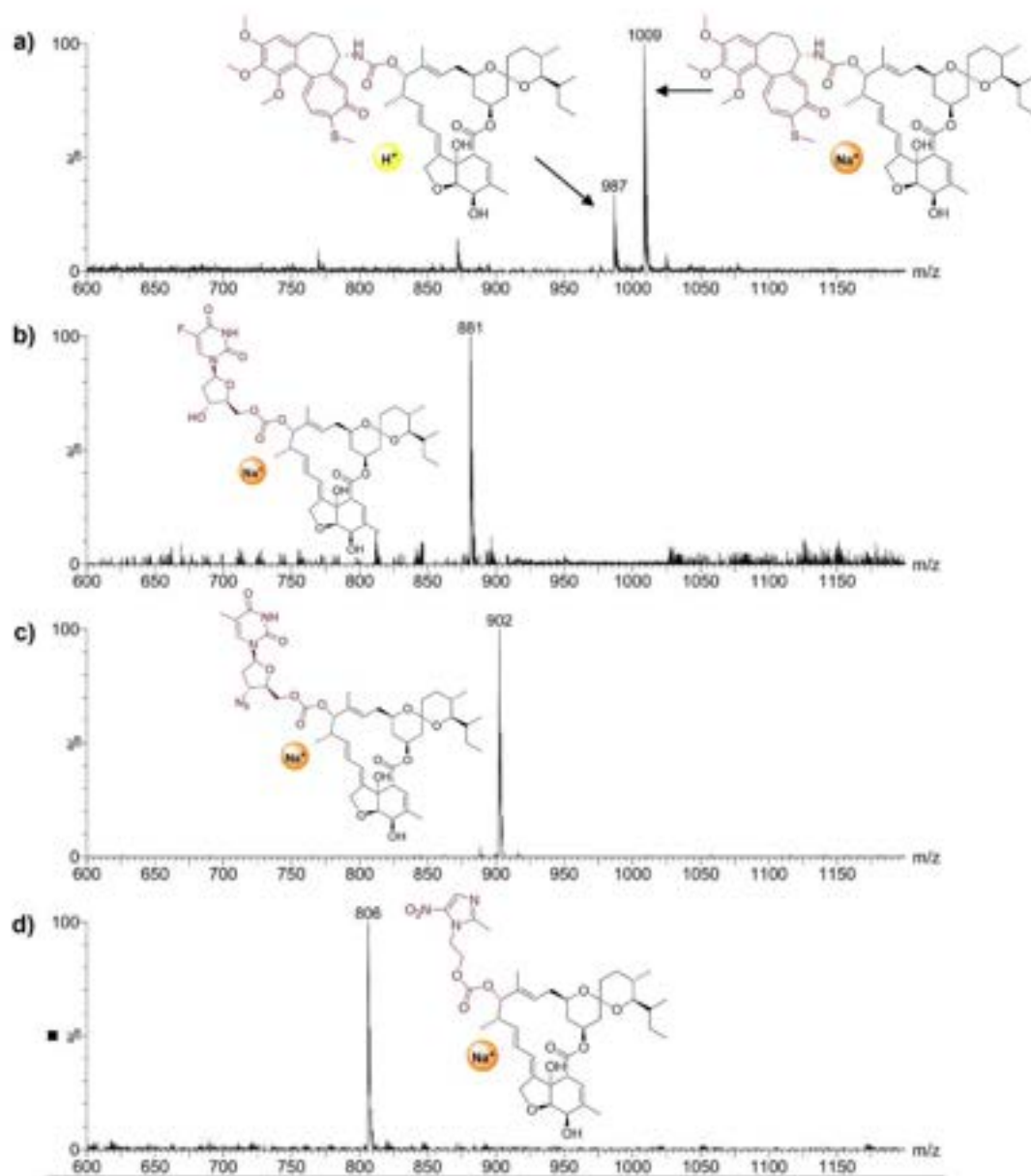


Figure S22. The ESI mass spectra of a mixture of **8** (a), **10** (b), **11** (c), and **12** (d), with NaClO_4 at $\text{cv} = 10 \text{ V}$.

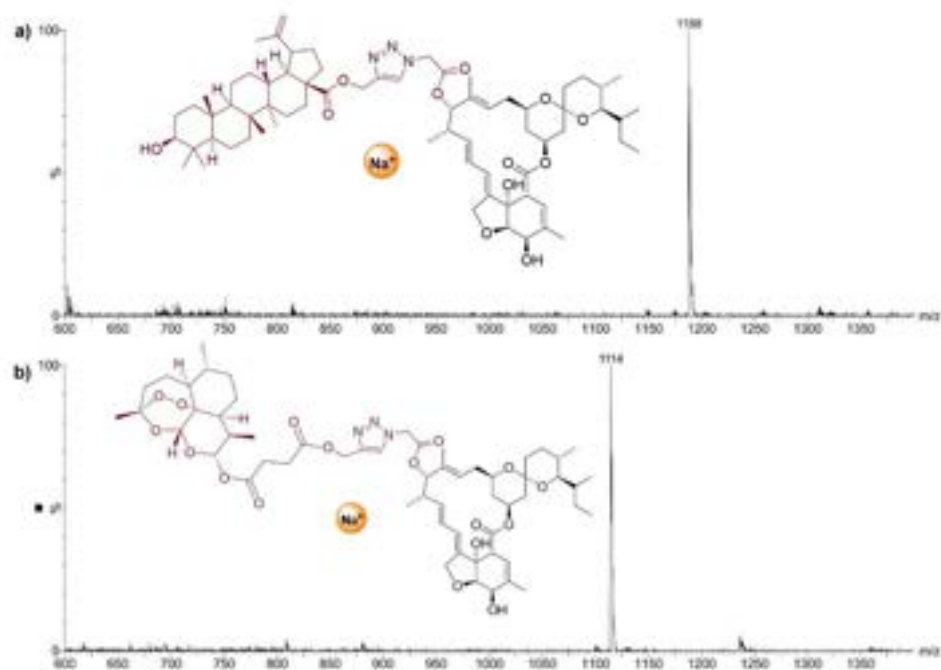


Figure S23. The ESI mass spectra of a mixture of **15** (a), and **16** (b) with NaClO_4 , at -10 V.

11.5. Publikacja V

Michał Sulik, Marta Jędrzejczyk, Magdalena Mielczarek-Puta, Gwan Yong Lim,
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*Mitochondria-targeting phosphonium salts of monensin A as the source of novel
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Opis wkładu doktoranta w powstanie publikacji: Jestem w pełni odpowiedzialny za syntezę oraz oczyszczanie wszystkich soli fosfoniowych monenzyny opisanych w pracy. Przeprowadziłem też analizę otrzymanych wyników, w tym tych z badań biologicznych oraz biofizycznych. Odpowiadałem za napisanie manuskryptu.



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Mitochondria-targeting phosphonium salts of monensin A as the source of novel antiproliferative agents

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Black lipid membrane

ABSTRACT

Monensin A is a representative of natural ionophores, which exhibit a broad spectrum of biological properties, with the most fascinating being its potent anticancer activity. A very intriguing research direction is the rational chemical modification of the monensin A skeleton to obtain analogs with higher antiproliferative activity and selectivity indices. In this paper, synthetic access to monensin A conjugates with mitochondria-targeting chemical moieties, such as the triphenylphosphonium cation and its analogs, is presented. Furthermore, the obtained derivatives were screened for lead structures with improved anticancer properties and mitochondria-targeting ability. Some of the obtained compounds exhibited greater antiproliferative activity and selectivity than unmodified monensin. Furthermore, biological and biophysical studies have confirmed that the induction of apoptosis by the aforementioned hybrids is linked to targeting of mitochondria in cancer cells. These results prove that such a strategy is a promising approach for the search for new effective drug candidates.

1. Introduction

Although many innovative treatment methods have been proposed and a number of new drugs have been introduced, cancer remains one of the main causes of death in the modern world. According to the World Health Organization (WHO) data, in 2020, this disease was responsible for nearly one in six deaths worldwide (almost 10 million deaths) [1,2]. In the same year, 19 million new cases were diagnosed, with the most common types of cancer being that of breast, lung, colon and rectum, prostate, skin, and stomach [2]. Unfortunately, according to WHO forecasts, these values will grow rapidly in the coming years, reaching up to 30 million new cases by 2040 [1]. Considering the above reports, the search for new drugs that would be effective in the fight against different types of cancer is one of the key issues to address in modern science and medicine.

A crucial parameter determining the effectiveness of anticancer drug candidates is selectivity, that is the ability to attack tumors while being neutral towards healthy cells. Designing analogs with a high selectivity index is currently one of the most demanding challenges in cancer therapy. In this context, it is worth mentioning that many diseases

(cancer, diabetes, and neurodegenerative diseases) are related to the functioning of mitochondria [3–5]. These cell organelles have been proven to play an essential role in both extrinsic and intrinsic apoptotic pathways, which makes them significant therapeutic targets for designing new anticancer drugs. Mitochondrial membrane permeabilization is a phenomenon that causes cancer cells to enter the apoptosis pathway and consequently leads to cell death [3–5]. Many substances and chemical groups are known to target mitochondria in cancer cells including rhodamine or pyridinium cations (Fig. 1). The most widely applied is the triphenylphosphonium cation (TPP⁺), which readily accumulates and penetrates the mitochondrial membrane of cancer cells due to its highly negative mitochondrial membrane potential (Fig. 1) [3,5]. Therefore, bioconjugation of the TPP⁺ moiety with other highly bioactive chemical compounds such as ionophore antibiotics may be a source of novel derivatives with potent anticancer properties (Fig. 1).

Monensin A (MON, Fig. 2A) is a natural compound that belongs to the group of polyether ionophore antibiotics (ionophores). It was first isolated in 1967 from *Streptomyces cinnamonensis* and was the first ionophore whose structure has been thoroughly described and explained

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[9]. Like other ionophores, this compound can coordinate metal cations (with the highest selectivity for sodium cation) and transport them across cell membranes, leading to the disruptions in intracellular pH and ion concentration gradients, which in turn results in mitochondrial damage, vacuolization, and cell death (Fig. 2B) [10,11]. This is because of MON exhibits strong antimicrobial and anticancer activity. A significant potential of this ionophore in the treatment of cancer has been proven in screening studies aimed at finding potential drugs for prostate cancer. The effects of almost 5000 known drugs and drug-like compounds were examined. Four of them, including MON, selectively inhibited the growth of prostate cancer cells at nanomolar concentrations, while showing no significant effect on the growth of healthy prostate epithelial cells [12]. MON has also been shown to inhibit cell proliferation in many other types of cancer, including abnormal colon cells, lymphoma, and myeloma, through cell cycle arrest and loss of mitochondrial transmembrane potential [13–15]. Moreover, in recent years, many research groups have carried out chemical modification of MON, thus obtaining analogs with high biological activity and promising selectivity indices [16–22]. This indicates that rational modification of the chemical skeleton of MON might be an interesting and promising direction in the search for new drug candidates.

Noteworthy, our previous reports indicated that derivatives of another ionophore, salinomycin, obtained by bioconjugation with various TPP⁺ cations via an ester linker, exhibited greater antiproliferative activity towards different cell lines than the lead compound [8]. In addition, physicochemical studies have shown that some of the derivatives exhibit the mitochondria-targeting properties and support the K⁺/H⁺-exchange activity on artificial and natural membranes [8, 23]. Bearing in mind that MON exhibits generally higher anticancer activity than salinomycin [16,22], it is expected that its conjugation with various TPP⁺ cations will result in a library of novel analogs showing not only high antiproliferative activity but also the ability to target the mitochondria of cancer cells.

Despite the high anticancer activity of MON and its analogs, its primary use as a potential antimicrobial agent should not be ignored. The extraordinary antibacterial activity of MON has been confirmed in many studies, and it has also been proven that some of its derivatives (e.g. esters or urethanes) exhibit activity against various Gram-positive bacteria [24–26]. However, many years of ionophore antibiotics usage as veterinary feed additives have increased the problem of drug resistance [27]. Therefore, the search for new ionophore-based compounds that are effective against different types of bacteria is a top-priority task to address. Bioconjugates of biologically active compounds with TPP⁺ have been suggested to exhibit significantly greater antibacterial

properties than starting compounds. Thymol-TPP⁺ conjugates are a perfect example as they showed significantly improved inhibitory activity against Gram-positive bacteria compared to thymol itself [28]. TPP⁺ conjugates with chloramphenicol and various quinone derivatives were also proven to be effective against various types of bacteria [29, 30]. These reports allow us to conclude that conjugates of MON with TPP⁺ may also turn out to be potentially effective antibacterial compounds.

2. Results and discussion

2.1. Analogs design and synthesis

As mentioned above, a widely applied method for obtaining mitochondria-targeting compounds is the conjugation of their precursors with the triphenylphosphonium cation (TPP⁺). We decided to expand this idea to include triphenylphosphine derivatives with modified aromatic rings. This approach allows for checking the correlation between the distribution of electron density in the newly introduced substituent and the biological and biophysical properties of the obtained derivatives. In addition, the tributylphosphonium cation was included to assess whether the presence of aromatic rings was necessary to maintain the desired properties. According to our previously published protocol [8], we linked MON with TPP⁺ cations at position C1 using an ester bond as a linker. This choice can be justified on biochemical grounds, as the ester bond can be hydrolyzed under physiological conditions due to the presence of esterases in cancer cells [31], which may have a key impact on the biological activity of the obtained analogs. Additionally, the linkers used were chosen to have different carbon chain lengths (four or six carbon atoms) to check whether this property has a direct impact on the ability of the conjugates to enter cancer cells, which corresponds to their biological activity and mitochondria-targeting properties. Taking into account all the above choices, twelve MON-TPP⁺ conjugates were synthesized (3a–3f, 5a–5f, Scheme 1). Furthermore, the study also included the benzyl ester of MON (6) to assess whether a simple aromatic ester would demonstrate mitochondria-targeting abilities and high biological activity associated with this property.

The desired conjugates were synthesized mainly through a nucleophilic substitution between a halogenated MON precursor and the appropriate phosphine (Scheme 1). Starting from MON, key ester precursors with terminal bromine atom were obtained according to the protocols published previously with minor modifications [18]. Briefly, MON was subjected to esterification reaction with the respective alkyl halide (1,4-dibromobutane or 1,6-dibromohexane) in the presence of



Fig. 1. Typical mitochondria targeting cations used in medicinal chemistry and ionophore antibiotic conjugates – lasalocid and salinomycin linked to the triphenylphosphonium (TPP⁺) cation – along with their antiproliferative activity [6–8].

DBU, as a non-nucleophilic base, which resulted in the formation of **2** and **4**, with yields of 58 % and 50 %, respectively. The same method was used to synthesize the benzyl ester of **MON** (**6**), using benzyl bromide as the alkyl halide (Scheme 1). The expected product was obtained with a satisfactory yield of 77 %. Having access to halide precursors **2** and **4**, it was possible to obtain the desired conjugates **3a–3f** and **5b–5f** by substitution reactions with appropriate phosphines (Scheme 1). Depending on the length of the linker and the type of phosphine used, the reaction yields ranged from 14 % to 36 %. Surprisingly, this method was unsuitable for obtaining compound **5a**, which is a conjugate of **MON** with triphenylphosphine via a hexane-like linker. This is most likely due to the fact that triphenylphosphine does not have any electron-donating substituents in its structure, which would improve its nucleophilic properties, as in the case of other aromatic phosphines. To address this synthetic problem, a nucleophilic substitution reaction was performed between triphenylphosphine and an excess of 1,6-dibromohexane (to avoid double substitution). This compound was then treated as an alkyl halide and combined with **MON** through an esterification reaction as in previous cases (Scheme 2). The expected product **5a** was obtained with a yield of 43 %.

The purity and structure of the newly synthesized derivatives of **MON** (**3a–3f** and **5a–5f**) were determined using spectroscopic (^1H NMR, ^{13}C NMR, ^{31}P NMR) and spectrometric (HRMS) methods. In the ^{13}C NMR spectra of **MON** derivatives, the signal with the highest analytical significance was assigned to the carbonyl group of the ester moiety at position C1. Depending on the types of substituent and linker used, the signal from the ester group appeared at the narrow range of 176.0–176.3 ppm. In the ^{31}P NMR spectra, the signal from the phosphonium salt appeared in the range of 22.5–34.9 ppm, depending on the type of substituent used. The HRMS analysis confirmed the formation of the desired products, with $[\text{M}]^+$ as the main peak (intensity 100 %). The relative error associated with the analysis was small and ranged from 0.0 ppm to 2.0 ppm, depending on the compound tested. The NMR spectra and HRMS of all the novel **MON** derivatives are included in the Supplementary material (Fig. S1–S48).

2.2. Biological activity

2.2.1. Cytotoxic activity studies

A biological study was conducted to evaluate the cytotoxic activity of **MON** and its derivatives **3a–6** as well as their anti-malignant potential against seven selected cell lines. Cell viability was determined using the MTT colorimetric assay on five cancer cell lines (SW480, SW620, PC3, MDA-MB-231, and A549) and two non-cancerous cell lines (HaCaT and V79). The antiproliferative activity was determined by calculating the

half-maximal inhibitory concentration (IC_{50}). Furthermore, a ratio that measures cytotoxicity in neoplastic and normal cell lines, the selectivity index (SI), was used to measure specificity. Doxorubicin was used as the reference drug in this study.

Compounds **3b**, **3c**, **3d**, **3e**, and **5e** demonstrated significant cytotoxic activity against all tested cancer cell lines, with IC_{50} values $< 1 \mu\text{M}$ (Table 1).

Notably, compounds **3a**, **3c**, and **3d** exhibited more potent anticancer effects towards the PC3 cell line than the conventional anticancer agent, doxorubicin (Table 1). Compounds **3b** and **3c** showed greater anticancer efficacy than **MON** against all tested cancer cell lines, except for A549 (Table 1). The MDA-MB-231 cell line exhibited greater sensitivity to **MON** derivatives, except for compounds **3f** and **6**. Compound **6** had the lowest cytotoxicity, with IC_{50} value approximately 51 times and 16 times higher than those of compound **3d** and **MON**, respectively (Table 1). The selectivity index (SI) was calculated for all selected cancer cell lines in comparison with non-malignant human immortal keratinocyte (HaCaT) and Chinese hamster lung fibroblasts (V79). In the HaCaT cells standard, compound **3a** was classified as highly selective compared to the other derivatives showing SI values < 1 in at least one cell line (Table 2).

Among all derivatives studied, compound **6** showed the most favorable selectivity in SW480 cells ($\text{SI} = 3.0$) and the lowest selectivity in MDA-MB-231 cells ($\text{SI} = 0.3$), indicating that it preferentially targets normal human cells (Table 2). In contrast to HaCaT cells, compound **6** showed lower SI values for V79 cells in all the tested cell lines (Table 2). The huge difference in the SI value of compound **6** in SW480 ($\text{SI} = 3.0$ for HaCaT and $\text{SI} = 0.3$ for V79) may be attributed to the fact that V79 is a non-human cell line. It is worth emphasizing that, as the HaCaT line is of human origin, it should be prioritized in the search for new, effective anticancer agents. Notably, most of the analogs we obtained exhibited higher selectivity index (SI) values than doxorubicin, a drug commonly used in clinical practice – as this compound was highly cytotoxic towards HaCaT cells. Derivates **3a** and **3d** had selectivity index favourable for target cytotoxicity in all investigated cell lines. In the cases of other conjugates the SI varied from very low value to very high, like compound **6** which reaching value of 3.0 for SW480 cell line. It indicates that some derivatives have some specific interactions with particular cancer cell lines. Overall, the **MON** derivatives provide safer and more adequate antineoplastic effects than **MON** alone. Moreover, in most cases, 4-carbon linked conjugates showed greater activity and selectivity than their 6-carbon linked counterparts. Particularly, derivatives **3a** and **3f** not only inhibited the growth of PC3 and A549 cells separately, with low IC_{50} values, but also carried higher SI values, highlighting their prospective therapeutic benefits and potential clinical usage. However,

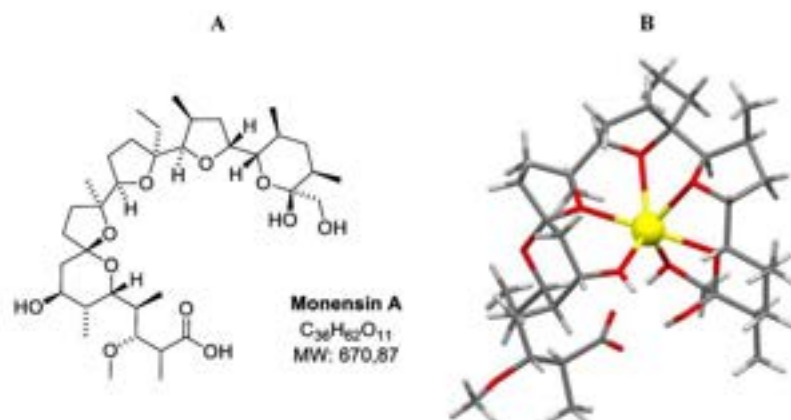
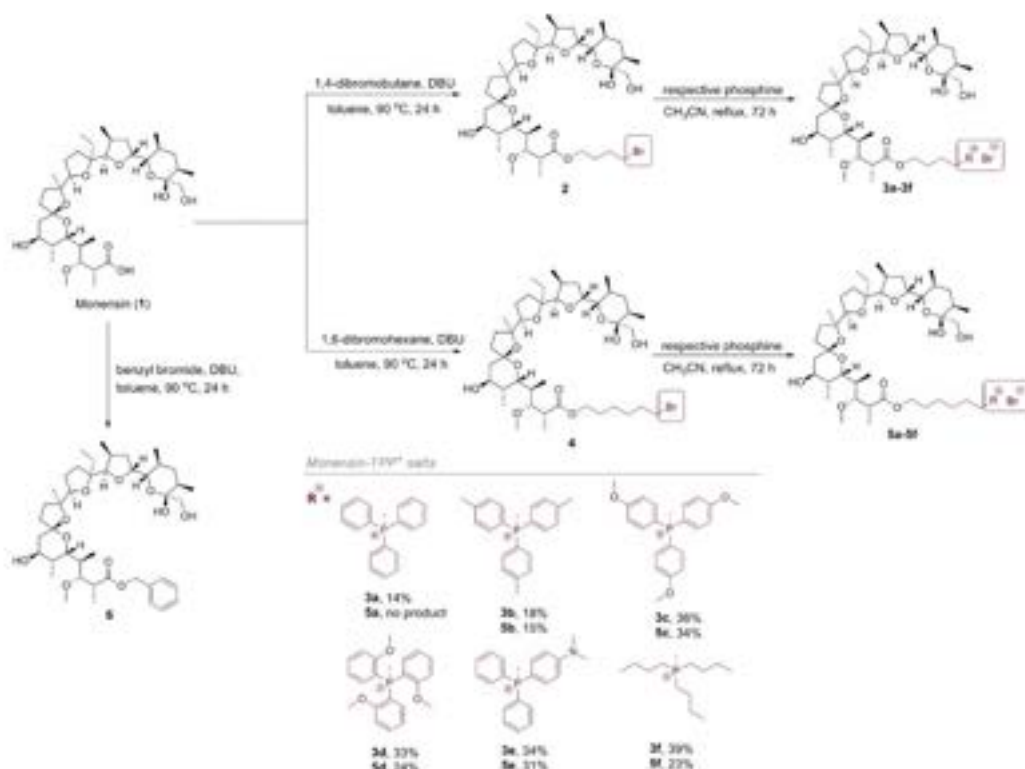
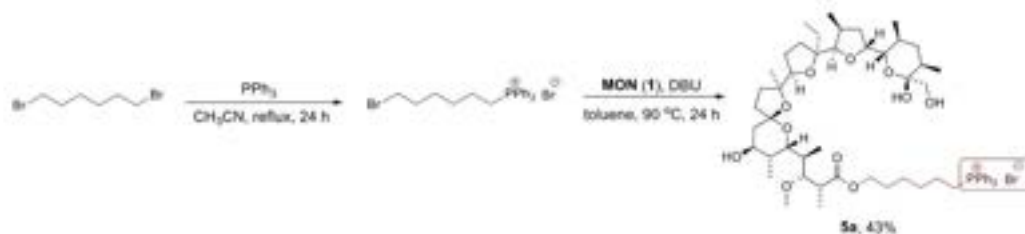


Fig. 2. (A) Chemical structure of monensin A, (B) Structure of monensin sodium salt.



Scheme 1. Synthesis of MON-TPP⁺ conjugates using various substituents via ester linker.



Scheme 2. Synthesis of the conjugate of MON with triphenylphosphine via a hexane-like linker (5a).

since ionophore antibiotics do not act through a single, well-defined molecular target, the possibility of off-target toxicity should be carefully considered. Therefore, further and more detailed studies of the obtained compounds are necessary to explore their potential anticancer mechanism beyond those investigated in this work.

2.2.2. Induction of cell cycle arrest

To evaluate the anticancer properties of MON and its derivatives (3a, 3f and 6), a PI-metric cell cycle analysis was performed using flow cytometry. This method facilitated the identification of cell cycle stage alterations across four phases: sub-G1, G0/G1, S, and G2. A549 and MDA-MB-231 cells were treated with MON and its derivatives (3a, 3f, and 6) at their respective IC₅₀ concentrations, and incubated for 24 h.

In A549 cells, MON treatment predominantly induced cell cycle arrest in G0/G1 phase (86 % compared to 47.1 % in control) and also significantly reduced the proportion of cells in S phase (from 29.2 % in control to 4.5 %) and G2 phase (from 21.0 % to 6.3 %) (Fig. 3). Compounds 3a and 3f demonstrated a similar trend, inducing G0/G1 arrest

in 65.9 % and 63.5 % of cells, respectively (Fig. 3). Additionally, derivative 3a led to an increase in sub-G1 cells relative to that observed after treatment with MON. In contrast, compound 6 exhibited a cell cycle distribution resembling that of the control group (Fig. 3).

In MDA-MB-231 cells, all tested compounds except derivative 6, caused G0/G1 phase arrest (ranging from 67.7 % to 69.8 %, compared to 56.7 % in control) and slightly reduced the proportion of cells in S and G2 phases (Fig. 3).

Overall, MON and its derivatives consistently increased the proportion of cells in the G0/G1 phase while reducing the S and G2 phase populations, suggesting that these compounds induce cell cycle arrest. Among the tested compounds, MON displayed the strongest anti-proliferative effect on A549 cells, while MON, 3a, and 3f exhibited comparable efficacy in inducing cell arrest in MDA-MB-231 cells.

2.2.3. Induction of apoptosis

The pre-apoptotic activities of MON and its derivatives (3a, 3f, and 6) were assessed via flow cytometry using an Annexin V-FITC/PI

Table 1
Antiproliferative activity (IC_{50} , μ M) of monensin (MON) and its derivatives estimated by the MTT assay [30].

Compound	Cancer cells					Normal cells	
	SW480	SW620	PC3	MDA-MB-231	A549	HaCaT	V79
	IC_{50}	IC_{50}	IC_{50}	IC_{50}	IC_{50}	IC_{50}	IC_{50}
(MON)	0.69 ± 0.15	0.92 ± 0.08	0.62 ± 0.04	2.11 ± 0.02	0.74 ± 0.03	0.62 ± 0.04	0.91 ± 0.07
3a	0.71 ± 0.02	1.05 ± 0.10	0.52 ± 0.12	0.97 ± 0.20	0.82 ± 0.09	1.23 ± 0.42	1.31 ± 0.15
3b	0.57 ± 0.06	0.89 ± 0.19	0.59 ± 0.19	0.72 ± 0.08	0.92 ± 0.13	0.84 ± 0.11	0.72 ± 0.02
3c	0.57 ± 0.01	0.96 ± 0.21	0.50 ± 0.11	0.93 ± 0.29	0.84 ± 0.01	0.91 ± 0.14	0.76 ± 0.01
3d	0.74 ± 0.03	0.65 ± 0.04	0.48 ± 0.09	0.66 ± 0.17	0.68 ± 0.01	0.74 ± 0.13	0.76 ± 0.11
3e	0.57 ± 0.02	0.86 ± 0.21	0.97 ± 0.22	0.69 ± 0.10	0.89 ± 0.20	0.89 ± 0.21	0.65 ± 0.08
3f	4.32 ± 1.40	1.35 ± 0.08	1.11 ± 0.06	2.66 ± 0.14	0.80 ± 0.10	1.72 ± 0.21	0.61 ± 0.07
5a	0.91 ± 0.37	0.75 ± 0.07	0.61 ± 0.07	0.84 ± 0.35	1.19 ± 0.22	0.80 ± 0.09	0.70 ± 0.04
5b	2.60 ± 1.15	0.90 ± 0.11	1.16 ± 0.15	1.38 ± 0.03	1.07 ± 0.01	1.19 ± 0.09	0.75 ± 0.02
5c	0.71 ± 0.07	0.74 ± 0.13	0.77 ± 0.13	0.88 ± 0.17	0.95 ± 0.05	0.88 ± 0.15	0.62 ± 0.07
5d	2.00 ± 0.03	0.88 ± 0.15	0.82 ± 0.05	0.91 ± 0.28	0.75 ± 0.01	0.88 ± 0.19	0.48 ± 0.04
5e	2.68 ± 1.16	1.12 ± 0.08	0.79 ± 0.08	1.24 ± 0.11	0.81 ± 0.03	1.24 ± 0.33	0.68 ± 0.14
5f	0.91 ± 0.33	0.94 ± 0.04	0.81 ± 0.17	1.71 ± 0.31	1.01 ± 0.01	1.31 ± 0.31	0.55 ± 0.02
6	2.87 ± 0.75	10.98 ± 1.0	5.22 ± 0.79	33.96 ± 1.6	4.97 ± 1.67	8.51 ± 0.79	1.71 ± 0.24
DOX	0.29 ± 0.08	0.31 ± 0.08	0.59 ± 0.02	0.83 ± 0.03	0.63 ± 0.2	0.29 ± 0.01	2.01 ± 0.03

Data are expressed as the mean $\pm$ SD. IC_{50} (μ M) is the concentration of the compound that corresponds to a 50 % growth inhibition of cell line (as compared to the control) after culturing the cells for 72 h with the studied compound. Human primary colon cancer cells (SW480); human metastatic colon cancer cells (SW620); human metastatic prostate cancer cells (PC3); human breast cancer cells (MDA-MB-231); human lung cancer cells (A549); human immortal keratinocyte cells (HaCaT); Chinese hamster lung fibroblasts (V79). Doxorubicin (DOX) – the selected reference compound commonly used in cancer treatment.

Table 2
The calculated values of the selectivity indices (SI) for HaCaT (left side) and V79 (right side) non-tumor cell lines.

Compound	HaCaT					V79				
	SW480	SW620	PC3	MDA-MB-231	A549	SW480	SW620	PC3	MDA-MB-231	A549
1 (MON)	0.9	0.7	1.0	0.3	0.8	1.3	1.0	1.5	0.4	1.2
3a	1.7	1.2	2.3	1.2	1.5	1.8	1.2	2.5	1.4	1.6
3b	1.5	0.9	1.4	1.1	0.9	1.3	0.8	1.4	1.0	0.8
3c	1.6	0.9	1.8	1.0	1.1	1.3	0.8	1.5	0.8	0.9
3d	1.0	1.1	1.8	1.1	1.1	1.0	1.2	1.6	1.2	1.1
3e	1.6	1.0	0.9	1.3	1.0	1.1	0.8	0.7	0.9	0.7
3f	0.4	1.3	1.5	0.6	2.2	0.1	0.5	0.5	0.2	0.8
5a	0.9	1.1	1.3	0.9	0.7	0.8	0.9	1.1	0.8	0.6
5b	0.5	1.3	1.0	0.9	1.1	0.3	0.8	0.6	0.5	0.7
5c	1.2	1.2	1.1	1.0	0.9	0.9	0.8	0.8	0.7	0.7
5d	0.4	1.0	1.1	1.0	1.2	0.2	0.5	0.6	0.5	0.6
5e	0.5	1.1	1.6	1.0	1.5	0.3	0.6	0.9	0.5	0.8
5f	1.4	1.4	1.6	0.8	1.3	0.6	0.6	0.7	0.3	0.5
6	3.0	0.8	1.6	0.3	1.7	0.6	0.2	0.3	0.1	0.3
DOX	1.0	0.9	0.5	0.3	0.5	6.9	6.5	3.4	2.4	3.2

SI = IC_{50} for normal cell line/ IC_{50} for cancer cell line.

binding assay under 72 h treatment conditions in A549 and MDA-MB-231 cell lines. This study quantitatively evaluated early apoptosis, late apoptosis, and necrosis to provide a comprehensive profile of the cytotoxic effects exerted by these compounds.

In both A549 and MDA-MB-231 cell lines, the treatment with MON demonstrated potent pro-apoptotic effects. MON induced a substantial increase in late apoptosis in A549 and 45.38 % in MDA-MB-231, alongside a marked elevation in early apoptosis (20.26 % and 22.54 %, respectively) (Figs. 4 and 5). These findings underscore MON's robust cytotoxic efficacy across both cell lines.

For derivative 3a, late apoptosis was notably elevated relative to controls, accounting for 25 % in MDA-MB-231 and 12.77 % in A549 cells. Compound 3f induced apoptosis primarily in MDA-MB-231 cells, with significant contributions from both early (16.43 %) and late (27.76 %) apoptosis (Figs. 4 and 5). Interestingly, while derivatives 3f and 6 showed only a modest induction of apoptosis in A549 cells, compound 6 in MDA-MB-231 cells emerged as a significant inducer of late apoptosis (12.9 %), suggesting that its cytotoxic effects may be more specific to this cell line (Figs. 4 and 5).

To sum up, MON demonstrated pronounced cytotoxicity characterized by the strong induction of both early and late apoptosis in A549 and MDA-MB-231 cells. Compound 3a showed pro-apoptotic activity in both cancer cell lines while derivative 3f exhibited cell line-specific effects, with enhanced efficacy in MDA-MB-231 cells. These observations highlight the potential of 3a and 3f as therapeutic agents with robust apoptotic induction, albeit with variable efficacies across different cancer cell types. Further studies are needed to elucidate the molecular mechanisms underlying their selective cytotoxicity and optimize their potential for clinical applications.

2.2.4. Mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\psi_m$) determined by the fluorescence-based method in the JC-10 assay is a critical parameter reflecting mitochondrial function and health in cells. This assay is widely employed in studies on apoptosis, and mitochondrial dysfunction as well as in drug screening, as the loss of mitochondrial membrane potential is an early indicator of apoptosis. The present study revealed a decrease in $\Delta\psi_m$ after 48 h of treatment with MON and its derivatives,

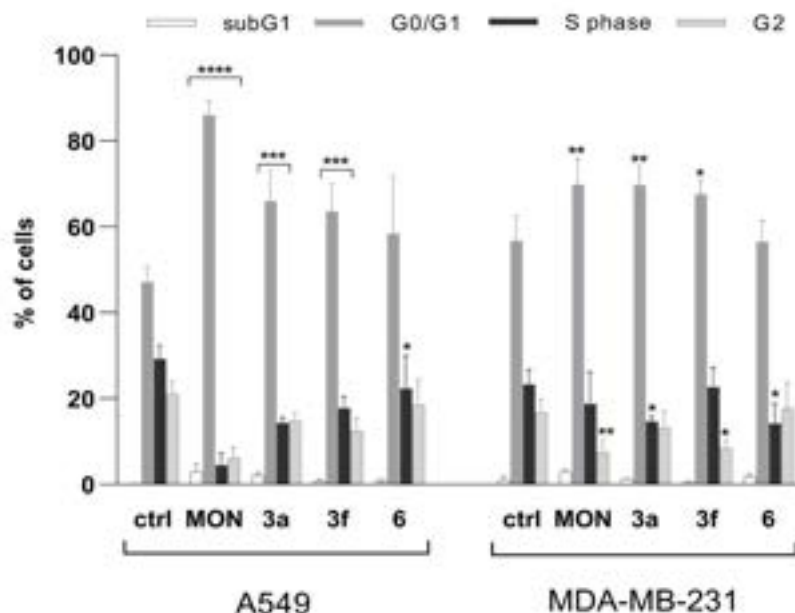


Fig. 3. The effect of MON and its derivatives on the cell cycle of A549 and MDA-MB-231 cells. The bars represent the effect of MON, 3a, 3f and 6 on the percent of cells in particular cell cycle phases. The cells were incubated for 24 h with the compounds at their IC_{50} concentrations, stained with PI and analyzed by flow cytometry. Untreated cells were used as the control. The results are presented by means $\pm$ SD, $n = 4$. Statistical significance was determined at $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***) and $p < 0.0001$ (****) by 1-way ANOVA.

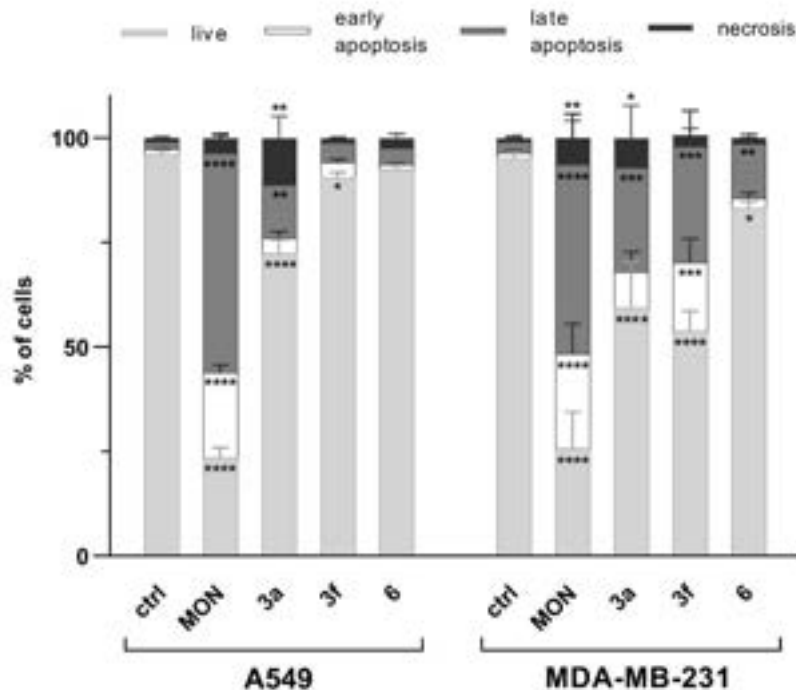


Fig. 4. The effect of MON and its derivatives on the apoptosis of A549 and MDA-MB-231 cells. The bars represent % of live cells, the cells at the early and late stage of apoptosis and necrosis. The cells were incubated for 72 h with the compounds at their IC_{50} concentrations, stained with Annexin V-FITC and PI and analyzed by flow cytometry. Untreated cells were used as the control. The results are presented by means $\pm$ SD, $n = 4$. Statistical significance was determined at $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***) and $p < 0.0001$ (****) by 1-way ANOVA.

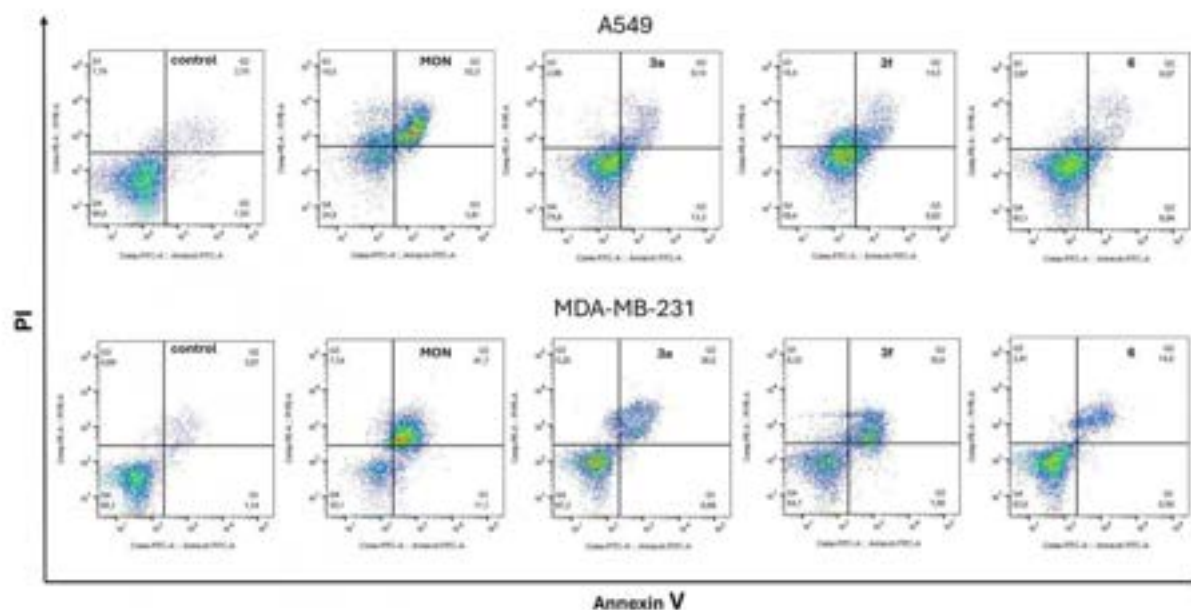


Fig. 5. Representative results (%) as dot plots showing apoptosis of A549 and MDA-MB-231 cells after treatment with MON and its derivatives 3a, 3f, 6, analyzed by flow cytometry.

which is a hallmark of mitochondrial dysfunction and early apoptosis.

Elevated green fluorescence, informing about the content of JC-10 monomers, was observed mainly with MDA-MB-231 cells, indicating a pronounced sensitivity of these cells to the treatment with MON and its derivatives (Figs. 6 and 7). The highest percentage of green fluorescent monomers was observed for compound 3f at 87.9 %, followed by 3a at

78.26 % and MON at 76.65 % (Figs. 6 and 7). The sensitivity of A549 cells to treatment with MON was similar (84 %), with a lower decrease in $\Delta\psi_m$ after treatment with 3a and 3f (21.33 % and 11.34 % of green monomers, respectively) (Figs. 6 and 7). Notably, compound 6 exhibited a higher effect on MDA-MB-231 (26.67 %) than A549 cells (4.74 %) (Figs. 6 and 7). This finding aligns with the results of the Annexin V-

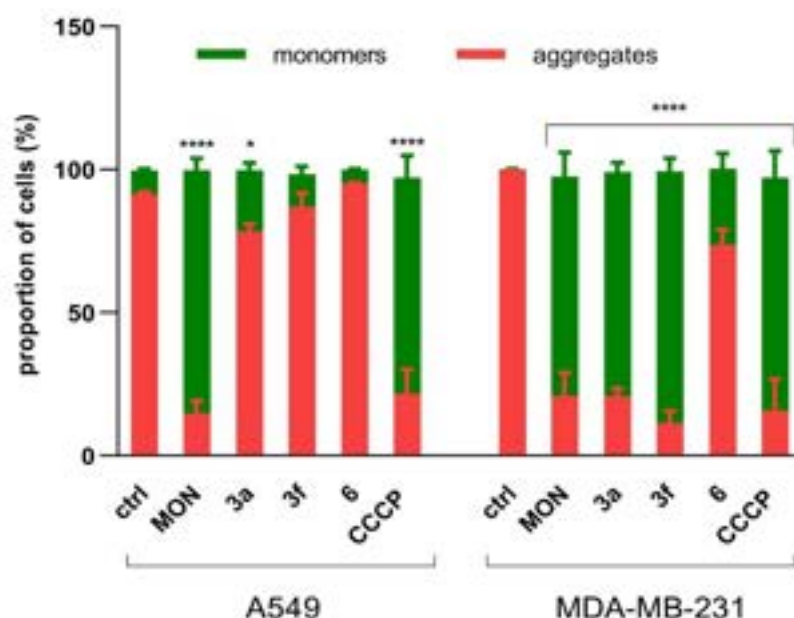


Fig. 6. Flow cytometry analysis of changes in the mitochondrial membrane potential in A549 and MDA-MB-231 cells after treatment with MON, its derivatives 3a, 3f, 6 and carbonylcyanide-m-chlorophenylhydrazone (CCCP) for 48 h, at their EC_{50} concentrations. Untreated cells were used as the control. The bars represent the proportion of cells (%) showing green or red fluorescence. The results are presented by means $\pm$ SD, $n = 4$. Statistical significance was determined at $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***) and $p < 0.0001$ (****) by 1-way ANOVA.

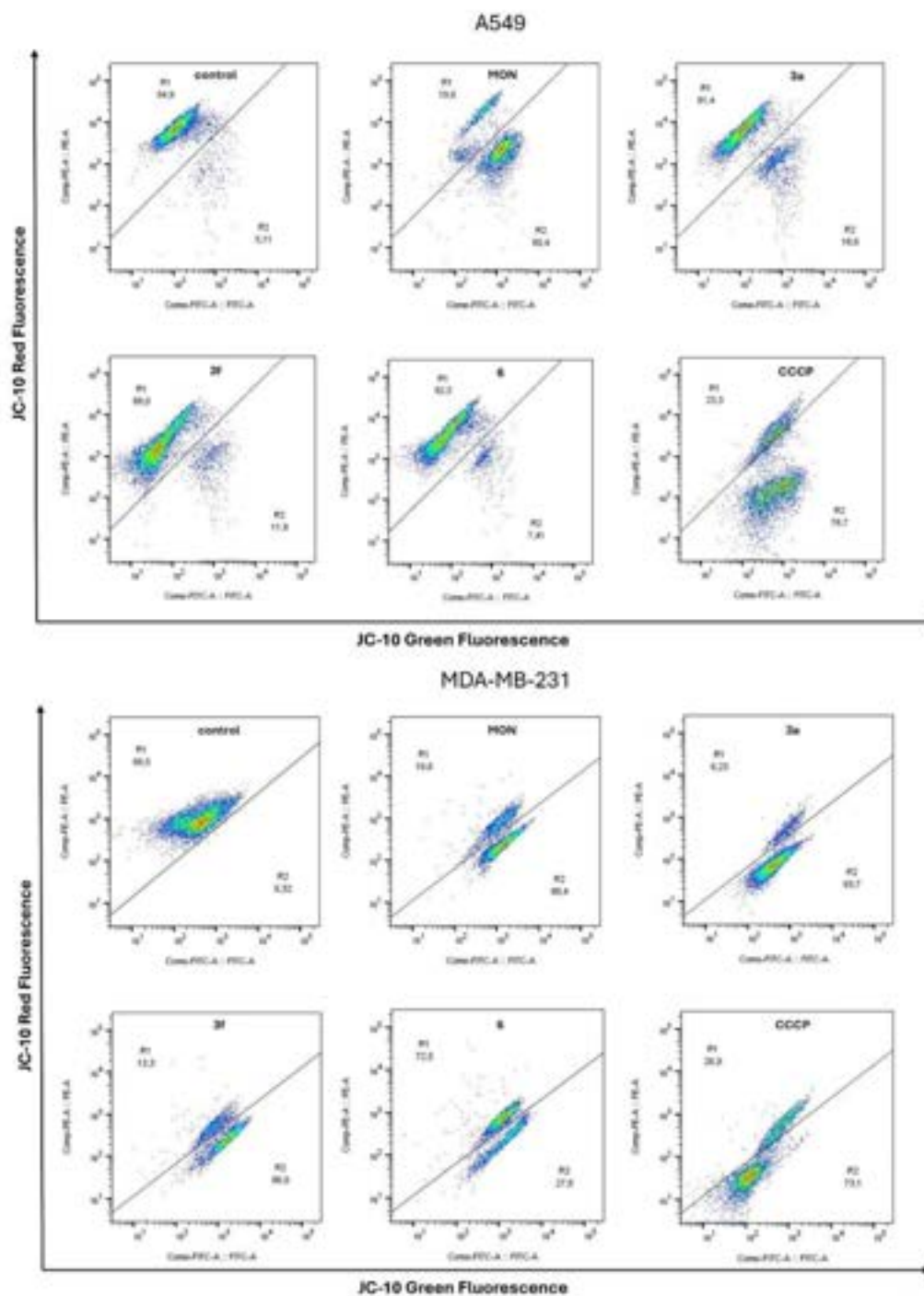


Fig. 7. Results of a representative experiment of JC-10 fluorescence assays in A549 and MDA-MB-231 cells, after treatment with MON and its derivatives 3a, 3f, 6, analyzed by flow cytometry.

FITC/PI binding assay, further supporting the heightened susceptibility of MDA-MB-231 cells to mitochondrial depolarization and apoptotic induction by MON and its derivatives.

To recapitulate, different cancer cells display changes in $\Delta\psi_m$ depending on their metabolic properties. Variations in mitochondrial dynamics, such as mitochondrial fusion and fission, also have impact on $\Delta\psi_m$ and uptake of JC-10 dye across different types of cancer cells.

2.2.5. Mitochondria activity

MitoTracker Red and Green staining were used in flow cytometry to evaluate mitochondrial function ($\Delta\psi_m$) and mass. The obtained results revealed that MTGreen staining of A549 cells treated with MON and MDA-MB-231 cells treated with 3a and 3f demonstrated the highest, 8-fold increase in mean fluorescence intensity (MFI) in comparison to controls, and the percentages of MT Green stained positive cells were 83.6 %, 95 % and 88.6 %, respectively (Figs. 8 and 9). Additionally, above 70 % of MTGreen positive cells were observed in response to MON in MDA-MB-231 cells and to 3a in A549 cells (Figs. 8 and 9). Moreover, MDA-MB-231 cells were also sensitive to the treatment with compound 6 showing 51 % of MT Green positive cells, while for A549 cell line a moderately elevated level of MT Green positive cells was found (Figs. 8 and 9). Consistent with the apoptosis and JC-10 assay results, the MitoTracker Green data showed that MDA-MB-231 cells exhibited a more dynamic response, which could indicate a higher sensitivity to treatment with MON and its derivatives (3a, 3f, and 6) compared to A549 cells.

In contrast to the MT Green dye, only a slight, insignificant increase in MitoTracker CMXRos staining was observed in response to all tested compounds in both cancer cell lines. It was found, that both mean fluorescence intensity (MFI) and the percentage of CMXRos-positive cells were similar to the levels in the controls (Figs. 8 and 9).

In conclusion, the high MFI and increased percentage of MitoTracker Green-positive cells, combined with MitoTracker Red levels comparable to those of the controls, might suggest that mitochondrial mass increased, without a significant improvement in mitochondrial membrane potential ($\Delta\psi_m$). The observed increase in MitoTracker Green fluorescence, possibly a result of mitochondrial fission, indicated an increase in mitochondrial mass or density, which is often associated with specific cellular processes. These findings may reflect a defensive mechanism of cancer cells against the action of MON and its derivatives. However, MitoTracker Red levels comparable to those in the controls

suggest that the membrane potential ($\Delta\psi_m$) remains unchanged, implying that the newly formed mitochondria are not functioning at the same energy state as the control cells.

2.2.6. ROS production

In this study the CellROX Green Reagent was used to quantitatively assess reactive oxygen species (ROS) in live cells. Upon oxidation, this reagent becomes DNA-bound, resulting in its fluorescence signal being predominantly localized in the nucleus and mitochondria, which are key sites of oxidative stress.

The experimental results revealed that ROS levels were the highest after treatment with MON alone, in both cancer cell lines (Fig. 10). In turn, the treatment with compounds 3a and 3f led to an elevated ROS generation with a 2-fold increase for A549 and a 1.5-fold increase for MDA-MB-231 cells, in comparison to the levels in the control cells (Fig. 10). Conversely, treatment with compound 6 resulted in only a marginal and statistically insignificant elevation in ROS levels in both cell lines (Fig. 10).

These findings suggest differential cellular responses to MON derivatives. A549 cells exhibited pronounced ROS generation in response to MON and its derivatives, potentially indicating higher oxidative stress sensitivity. However, subsequent analyses, including assessments of mitochondrial membrane potential (MMP) and apoptosis, suggest that despite their heightened ROS production, A549 cells demonstrate greater resistance to MON and its derivatives than MDA-MB-231 cells. This apparent paradox underscores the complexity of cancer cell responses to oxidative stress.

2.2.7. Antimicrobial activity

Because MON, like the other ionophores, exhibits high antibacterial activity, all the compounds obtained were tested for their antimicrobial properties against eight bacterial strains: five standard Gram-positive bacteria (*S. aureus* NCTC 4163, ATCC 25923, ATCC 6538, and ATCC 29213; *S. epidermidis* ATCC 12228 and ATCC 35984) and two Gram-negative strains (*E. coli* ATCC 25922 and *P. aeruginosa* ATCC 15442). Similar to MON, all analogs (3a–3f) exhibited high antimicrobial activity against Gram-positive bacteria and no activity against Gram-negative bacterial strain (Table 3). However, compounds 3b and 5b (tri(p-tolyl)phosphine derivatives) were more active against the Gram-negative bacterial strain *P. aeruginosa* than all other analogs and MON itself (Table 3). Moreover, all the obtained salts were more active than

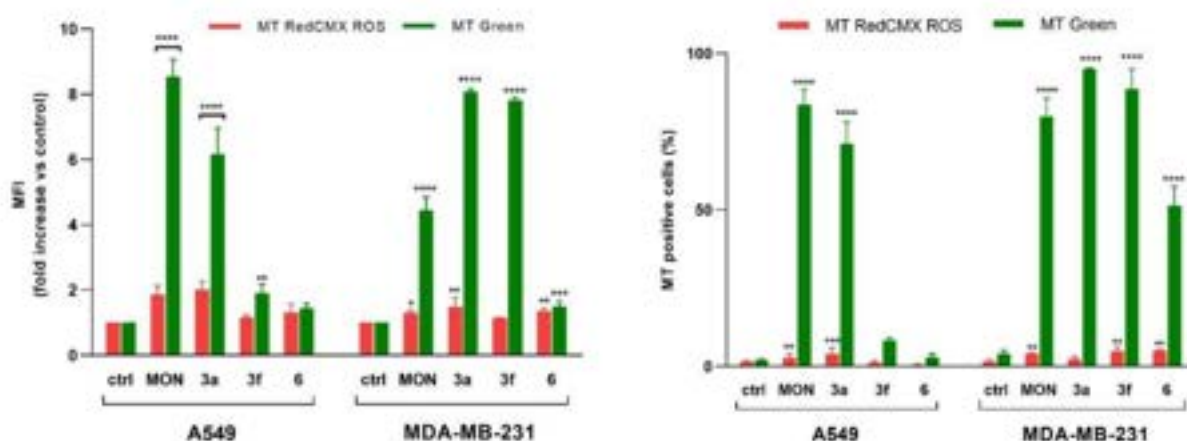


Fig. 8. Flow cytometry analysis of MitoTracker Red CMXRos and MitoTracker Green FM mitochondrial staining in A549 and MDA-MB-231 cells after treatment with MON and its derivatives 3a, 3f, and 6 for 48 h at their IC_{50} concentrations. Untreated cells were used as the control. The bars represent the fold change in mean fluorescent intensity (left panel) and % of MitoTrackers positive cells as compared to the control (right panel). The results are presented by means $\pm$ SD, $n = 4$. Statistical significance was determined at $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***) and $p < 0.0001$ (****) by 1-way ANOVA.

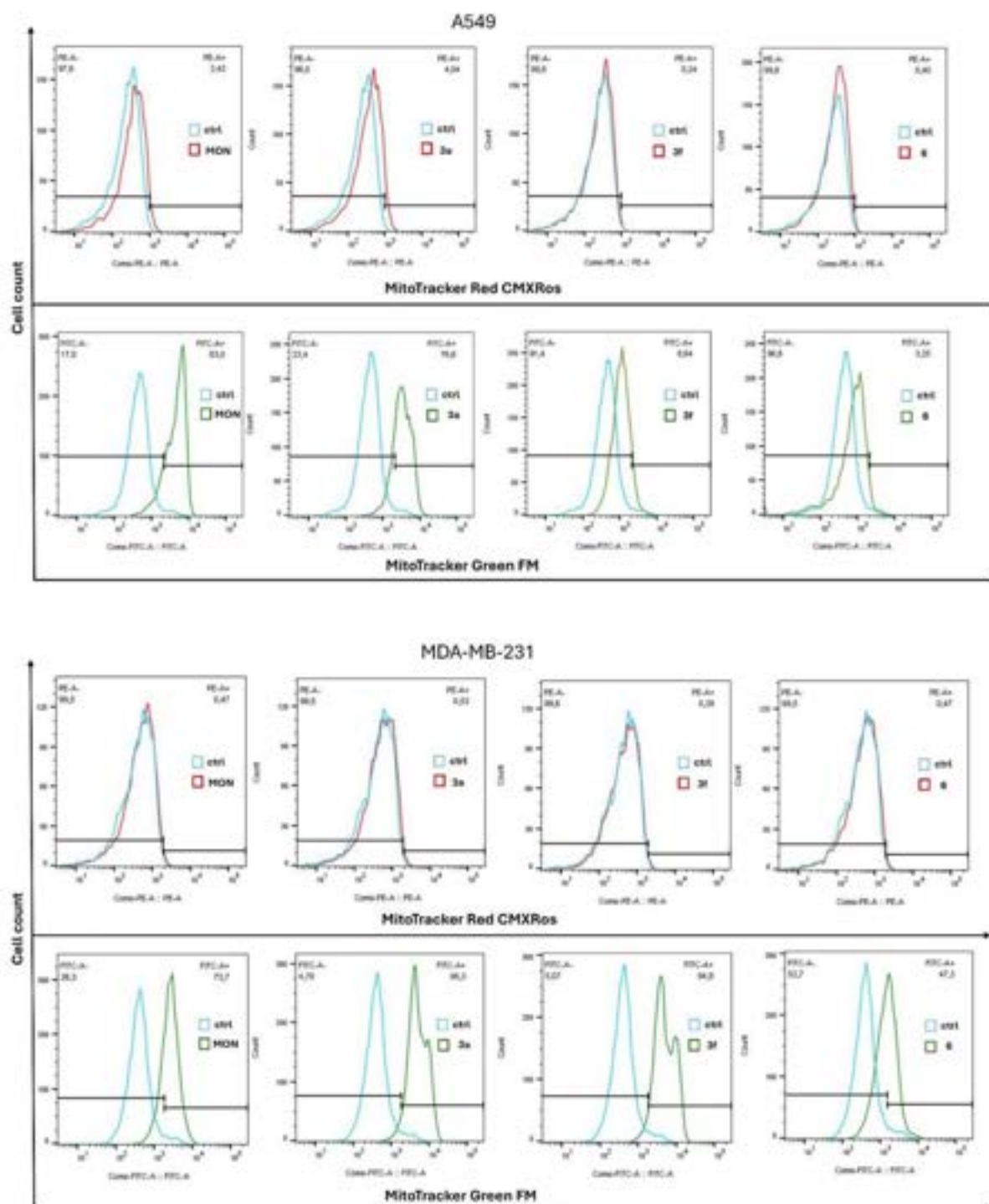


Fig. 9. Results of a representative experiment by MitoTracker Red CMXRos and MitoTracker Green FM fluorescence assays of A549 and MDA-MB-231 cells after treatment with MON and its derivatives **3a**, **3f**, **6**, analyzed by flow cytometry.

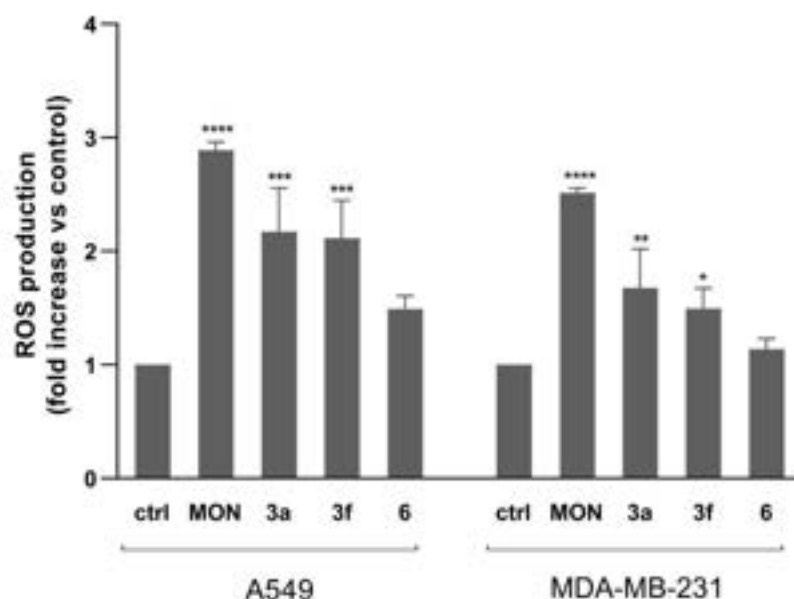


Fig. 10. The effect of MON and its derivatives 3a, 3f, and 6 on ROS production in A549 and MDA-MB-231 cells. The bars show the fold change in mean fluorescent intensity as compared to the control. The cells were incubated for 6 h with the compounds at their EC_{50} concentrations, labeled with CellROX Green Reagent and analyzed by microplate-based fluorimetry. Untreated cells were used as the control. The results are presented by means $\pm$ SD, $n = 4$. Statistical significance was determined at $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***) and $p < 0.0001$ (****), by 2-way ANOVA.

Table 3

The MIC values (in $\mu\text{g mL}^{-1}$) of monensin, its benzyl ester, and all conjugates against Gram-positive and Gram-negative bacteria strains.

Compound	Reference strains of Gram(+) and Gram(-) bacteria							
	<i>S. aureus</i> NCTC 4163	<i>S. aureus</i> ATCC 25923	<i>S. aureus</i> ATCC 6538	<i>S. aureus</i> ATCC 29213	<i>S. epidermidis</i> ATCC 12228	<i>S. epidermidis</i> ATCC 35984	<i>E. coli</i> ATCC 25922	<i>P. aeruginosa</i> ATCC 15442
	MIC	MIC	MIC	MIC	MIC	MIC	MIC	MIC
1 (MON)	2	1	2	1	2	2	na	na
3a	2	2	2	2	2	2	>256	>256
3b	1	2	2	2	2	2	256	128
3c	2	2	2	2	2	2	>256	>256
3d	2	2	2	2	2	2	>256	>256
3e	2	2	2	2	2	2	256	>256
3f	4	4	4	4	4	4	>256	>256
5a	1	1	1	1	1	1	256	256
5b	2	2	2	2	2	2	>256	128
5c	2	2	2	2	2	2	>256	>256
5d	2	2	2	2	2	2	>256	>256
5e	2	2	2	4	2	2	256	256
5f	4	4	4	4	4	4	>256	>256
6	>256	>256	>256	>256	>256	>256	>256	>256
CIP	0.125	0.25	0.125	0.25	0.125	0.125	0.0075	0.125

na = no activity.

MON benzyl ester 6. The conjugates with triphenylphosphine or its analogs (3a–3e and 5a–5e) were more active than their aliphatic counterparts 3f and 5f, suggesting the necessity of an aromatic moiety for the antimicrobial activity of such hybrids (Table 3). Some of the analogs showed even higher activity than MON, particularly compound 5a, which showed an MIC value of $1 \mu\text{g mL}^{-1}$ for all the tested strains of Gram-positive bacteria (Table 3). However, none of the compounds showed higher activity than ciprofloxacin, which is commonly used in treatment (Table 3).

2.3. Biophysical studies

2.3.1. Mitochondrial respiration rate

Changes in cellular respiration in the presence of MON and its derivatives were examined using an Oxygraph-2K system. To determine the respiration rate of A549 cells, the high-resolution respirometry was applied to measure oxygen consumption. The measurements were conducted in live non-permeabilized cells to analyze the effect of MON and its analogs on mitochondria and their ability to aggregate in these organelles reflected by changes in the respiration rate of A549 cells. FCCP was administered to achieve the maximal respiration rate. As shown in

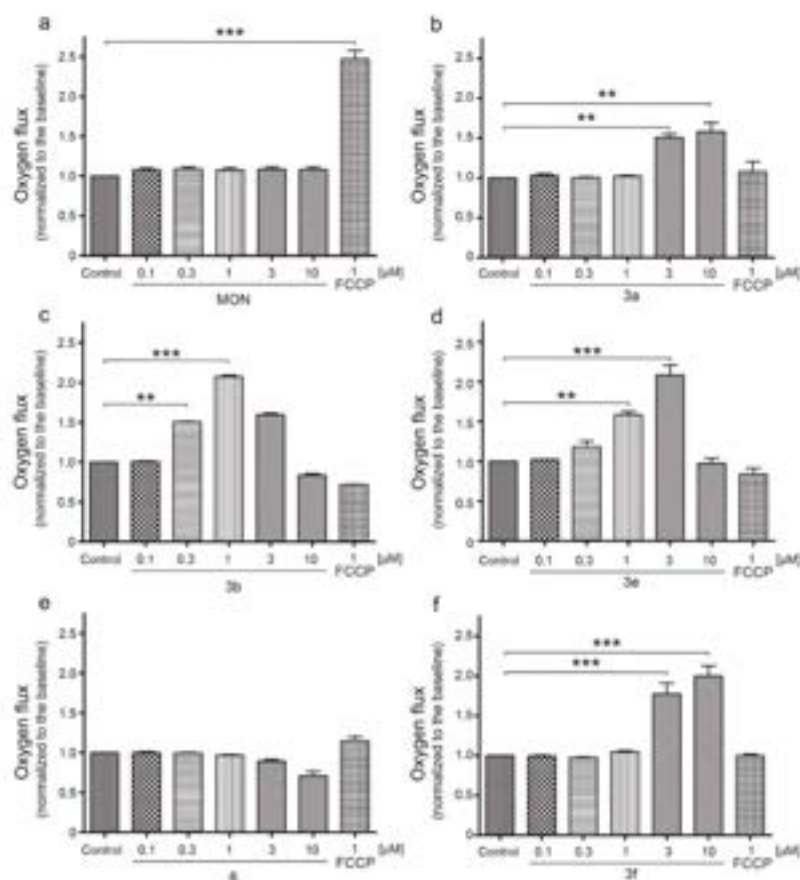


Fig. 11. The effect of MON and its derivatives on the oxygen consumption rate in the non-perforated A549 cell model in starving DMEM (Dulbecco Minimal Essential Medium). The bars represent the effect of the tested compounds at different concentrations: 0.1, 0.3, 1, 3, and 10 μM on A549 cell oxygen consumption rate, measured by the high-resolution respirometry. The effect of the tested compounds: a) monensin A (MON), b) 3a, c) 3b, d) 3c, e) 6, f) 3f was compared to the oxygen consumption rate measured after stabilization before addition of any compounds. Complete uncoupling was induced by 1 μM FCCP. The results are represented by means $\pm$ SD, $n = 4$. Statistical significance was determined at $p < 0.01$ (**) and $p < 0.001$ (***) by 1-way ANOVA.

Fig. 11a, MON did not change the oxygen respiration rate at any of the applied concentration. FCCP was used to show the proper functioning of cells by the full uncoupling of mitochondria. A slight decrease in the oxygen consumption rate was observed upon exposure to compound 6 (Fig. 11e), while FCCP did not perform full uncoupling of mitochondria. Compound 3e (Fig. 11d) caused an increase in oxygen consumption at concentrations of up to 10 μM, which resulted in mitochondria disability by a decreased respiration rate in concentrations of 3 and 10 μM. A similar decrease in oxygen consumption rate was observed in the presence of compound 3b (Fig. 11c), which was the most active among the compounds examined at a concentration of 1 μM. Conjugates 3a (Figs. 11b) and 3f (Fig. 11f) were effective at concentrations of 3 and 10 μM, respectively, with fully uncoupled mitochondria before the addition of FCCP.

2.3.2. Interaction of monensin A and its derivatives with lipid membranes

To prove the electrophysiological activity of the tested compounds, we performed black lipid membrane experiments in gradient solutions 50/150 mM NaCl (*cis/trans*). The applied experimental voltages varied from -60 mV to $+60$ mV. After assessment of the activity of the tested compounds by oxygraph examination, we chose three compounds 3a (Fig. 12b), 3e (Figs. 12c), and 6 (Fig. 12d), to check their ion transport capability. Unmodified MON (Fig. 12a) did not change the ionic current

flow through the azolectin bilayer membrane at concentration up to 10 μM. A similar effect was observed for the uncharged benzyl ester of MON (compound 6, Fig. 12d). Also, no significant decrease in ion current flow was noted for the concentration of 10 μM as expected on the basis of oxygraph experiments. The other compounds studied were capable of current generation in a dose-dependent manner. Similar to the results of the oxygraph experiments, the increase in ionic current flow was pronounced for conjugates 3a and 3b at concentrations above 3 μM and 1 μM, respectively. Based on the high values of ionic current, conjugate 3e showed far more electrophysiological activity than 3a. Examples of recorded signals are presented in Fig. 11a, 11b, 11c, and 11d. The two compounds alternated the basal ionic current but did not perform any open or close states as observed in typical channels. The current flow was smooth without any jagged traces.

3. Conclusions

The syntheses of a series of novel phosphonium salts of monensin A (MON) are described. These compounds can be obtained by simple nucleophilic substitution or esterification reactions, which provide convenient access to an extensive library of derivatives that are interesting for medicinal chemistry and cell biology. The desired compounds were obtained with the yields of 14–43 %. Furthermore, for comparative

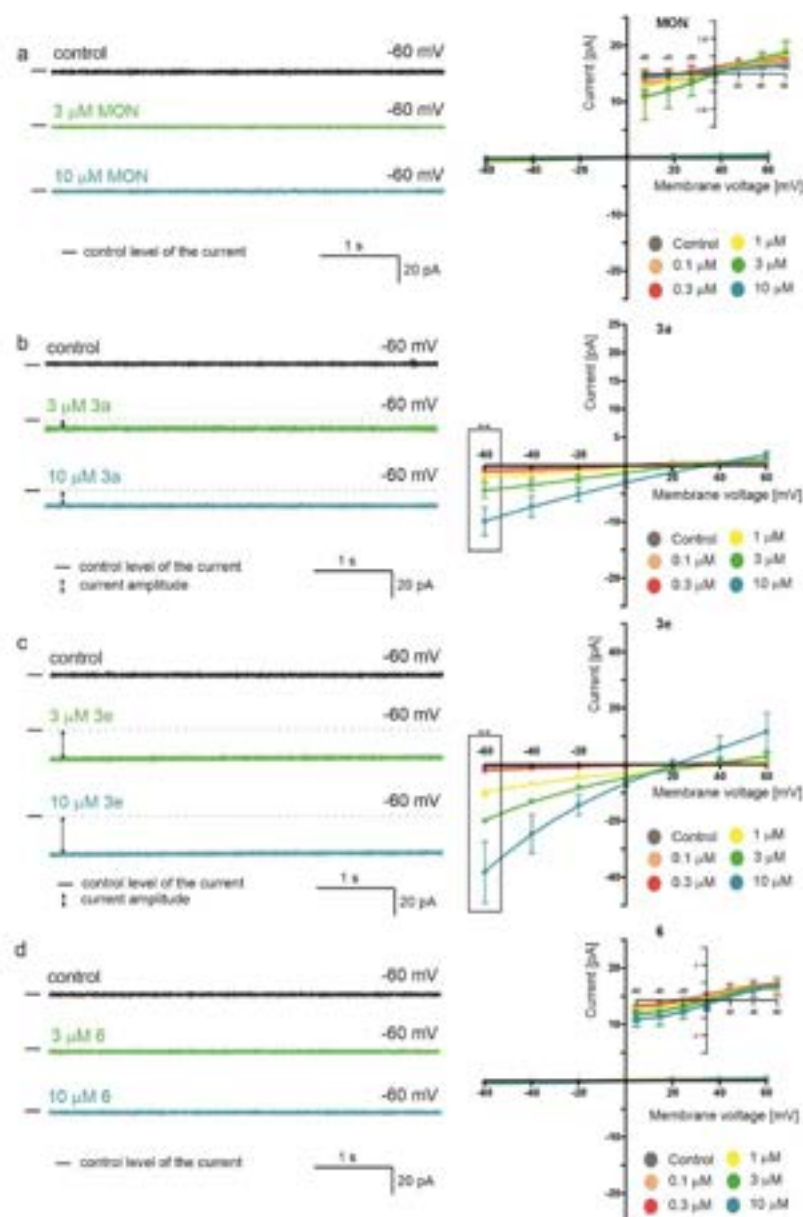


Fig. 12. Current-time recordings of ionic current flow through the lipid bilayer membranes and current-voltage (*i-v*) relationship measured in a gradient of 50/150 mM NaCl (cis/trans). The left panel shows representative recordings in the control and in the presence of 3 or 10 μ M (a) monensin A (MON), and (b, c, d) its derivatives at a potential of -60 mV. The right panel represents current-voltage characteristics in the presence of (a) monensin A (MON), (b) **3a**, (c) **3e**, and (d) **6** at concentrations of 0.1, 0.3, 1, 3, and 10 μ M at potentials of -60 , -40 , -20 , 0, 20, 40, and 60 mV. The average current values at the voltages and their standard deviations (SD) are marked on the chart. The experiment was performed in four repetitions. The symbol “—” indicates the control level of the current in the absence of the compounds studied, the symbol “|” indicates the representative amplitude of the measured current in the presence of each conjugate studied. Statistical significance was determined at $p < 0.001$ (***) by 1-way ANOVA.

purposes, the synthesis of MON benzyl ester (compound **6**) was also performed, with a yield of 77 %. With respect to the antiproliferative activity, it should be noted that many of the obtained compounds exhibit lower IC_{50} values and higher selectivity indices than MON. Good examples are compounds **3a** and **3f**, which not only inhibited the growth of prostate cancer cells (PC3) and human lung cancer cells (A549) but were also characterized by higher SI values, highlighting their prospective

therapeutic use. Moreover, compounds **3a**, **3e**, and **3d** exhibited more potent anticancer effects towards the PC3 cell line than doxorubicin – a commonly used anticancer drug. Additionally, the benzyl ester of MON (compound **6**) exhibited the lowest activity among the tested analogs, proving the necessity of TPP⁺ moiety in the structure of the MON derivatives to obtain the desired effects. The steric factor is also important in the design of new derivatives, as 4-carbon linked conjugates showed

higher activity and selectivity than their 6-carbon linked counterparts. Since, the main objective of the conducted research was to determine whether the obtained conjugates were targeted to the mitochondria of cancer cells, studies on mitochondrial membrane potential and activity were carried out. MON caused mitochondrial potential dissipation in both the A549 and MDA-MB-231 cell lines. However, derivatives **3a** and **3f** induced green fluorescence of JC-10 mainly in the MDA-MB-231 line, indicating its high sensitivity to MON derivatives. These data are in good correlation with the Annexin V-FITC/PI binding assay, confirming that the studied conjugates induce apoptosis in cancer cells, among other mechanisms, by dissipating their mitochondrial potential. Furthermore, MitoTracker Green data confirmed the increased sensitivity of the MDA-MB-231 cell line to the tested compounds compared to that of the A549 cell line. MDA-MB-231 cells treated with **3a** and **3f** demonstrated an 8-fold increase in mean fluorescence intensity compared to that of the controls. The mitochondrial activity of the tested compounds was also confirmed in biophysical studies. The study conducted using the Oxygraph-2K system showed that unmodified MON did not change the oxygen respiration rate of A549 cells. Its derivatives, on the other hand, demonstrated very potent activity; compounds **3a** and **3f** induced changes in the oxygen respiration rate at concentrations of 3 and 10 μM , respectively, while the highest effectiveness was observed for compound **3b**, which led to a decrease in the oxygen consumption rate after reaching the max level at a concentration of 1 μM . The results of the study on the Oxygraph-2K system were also confirmed by the black lipid membrane experiment. MON and its benzyl ester **6** did not change the ionic current flow through the azolectin bilayer membrane at concentration of up to 10 μM . On the other hand, conjugates **3a** and **3e** were capable of current generation in a dose-dependent manner, confirming their capability for ionic transport. In summary, it has been demonstrated that the conjugates are efficiently transported into the mitochondria of cancer cells, where they induce mitochondrial uncoupling and an increased rate of respiration, ultimately leading to apoptosis. The strategy of bioconjugation may contribute to development of novel, effective drug candidates that, owing to their high efficacy and selectivity, hold potential for application in chemotherapy.

4. Experimental

4.1. General procedures

Monensin A (MON) was isolated in the form of sodium salt (MON-Na) from premix Coxidin® (commercially available veterinary feed additive). MON was obtained from MON-Na by the extraction with H_2SO_4 (pH = 1.5) in CH_2Cl_2 as previously described [33,34]. All other reagents were commercially available and purchased from Merck or Trimem Chemicals S.A., and were used without further purification. Compounds **2**, **4**, and **6** were synthesized according to the protocol published previously [10]. A comprehensive description of the general procedures, equipment, and measurement parameters can be found in the Supplementary material.

4.2. Synthesis of 4-bromobutyric ester of monensin A (compound 2)

MON (1.0 g, 1.49 mmol, 1.0 equiv.) was dissolved in toluene (30 mL) and the solution was then heated to 90 °C. After 15 min, DBU (386 mg, 2.53 mmol, 1.7 equiv.) was added dropwise to the reaction mixture, and after another 20 min 1,4-dibromobutane (965 mg, 4.47 mmol, 3.0 equiv.) was added dropwise. After a few minutes, the color of the solution changed to brown. The resulting solution was stirred for the next 24 h. The reaction mixture was then concentrated on a rotary evaporator. Purification on silica gel using the CombiFlash system (0%–50% EtOAc/hexane) gave the pure ester **2** as a colorless oil. After twice co-evaporation to dryness with n-pentane, the product remained in an oily form (700 mg, 58 % yield). The spectroscopic data were in agreement with previously published data [10].

4.3. General procedure for preparation of phosphonium salts of monensin A with butane-like linker (analogs 3a–3f)

Compound **2** (400 mg, 0.50 mmol, 1.0 equiv.) was dissolved in CH_3CN (30 mL) and the respective phosphine (1.50 mmol, 3.0 equiv.) was added upon stirring. The solution was heated under reflux for 72 h. Subsequently, the reaction mixture was concentrated on a rotary evaporator. Purification on silica gel using the CombiFlash system (0%–40% acetone/ CHCl_3) gave the phosphonium salts of MON as colorless oils. After twice co-evaporation to dryness with n-pentane, the oily products were completely converted into white amorphous solids.

Phosphonium salt 3a: 74 mg, 14 % yield. Isolated as a white amorphous solid, a single spot by TLC. UV-active and stains green with PMA; ^1H NMR (400 MHz, CD_3CN) δ 7.92–7.68 (m, 15H), 5.78 (d, J = 1.3 Hz, 1H), 5.53–5.50 (m, 1H), 4.37 (ddd, J = 9.5, 7.2, 4.1 Hz, 1H), 4.20–4.03 (m, 4H), 4.03–3.92 (m, 2H), 3.84–3.79 (m, 1H), 3.69 (dd, J = 5.3, 2.9 Hz, 1H), 3.67–3.53 (m, 3H), 3.49–3.41 (m, 1H), 3.38 (ddd, J = 10.1, 4.0, 1.7 Hz, 1H), 2.83 (ddd, J = 13.8, 6.8, 2.8 Hz, 1H), 2.60 (s, 1H), 2.34 (s, 1H), 2.31–2.22 (m, 2H), 2.17–0.63 (m, 48H) ppm; ^{13}C NMR (101 MHz, CD_3CN) δ 176.0, 135.8, 135.8, 134.6, 134.5, 131.1, 131.0, 119.6, 118.7, 108.4, 99.0, 86.6, 86.4, 84.6, 81.8, 81.6, 76.3, 74.7, 69.6, 69.1, 67.0, 63.8, 58.4, 55.2, 41.3, 39.9, 36.8, 36.5, 36.3, 35.5, 35.1, 34.6, 33.4, 33.2, 32.2, 30.4, 30.1, 30.0, 29.7, 29.6, 28.3, 26.8, 22.6, 22.1, 20.0, 20.0, 16.4, 16.1, 14.5, 13.2, 11.5, 10.8, 8.1 ppm; ^{31}P NMR (122 MHz, CD_3CN) δ 25.2 ppm; HRMS (ESI^+) m/z : [M] $^+$ Calcd for $\text{C}_{58}\text{H}_{86}\text{O}_{11}\text{P}^+$ 987.5746; Found 987.5745.

Phosphonium salt 3b: 99 mg, 18 % yield. Isolated as a white amorphous solid, a single spot by TLC. UV-active and stains green with PMA; ^1H NMR (401 MHz, CD_3CN) δ 7.67–7.48 (m, 12H), 5.79–5.75 (m, 1H), 5.51 (dd, J = 3.0, 1.6 Hz, 1H), 4.36 (ddd, J = 9.4, 7.2, 4.1 Hz, 1H), 4.17–4.09 (m, 1H), 4.09–4.00 (m, 2H), 4.00–3.90 (m, 3H), 3.80–3.76 (m, 1H), 3.67 (dd, J = 5.2, 2.8 Hz, 1H), 3.66–3.56 (m, 2H), 3.52–3.44 (m, 1H), 3.36 (ddd, J = 9.7, 3.8, 1.5 Hz, 1H), 3.30–3.19 (m, 1H), 2.86–2.78 (m, 1H), 2.58 (s, 1H), 2.27 (s, 9H), 2.17–0.65 (m, 51H) ppm; ^{13}C NMR (101 MHz, CD_3CN) δ 176.3, 147.4, 147.4, 134.7, 134.6, 132.0, 131.9, 116.8, 115.9, 108.7, 99.3, 86.9, 86.7, 84.9, 82.1, 81.8, 76.6, 75.1, 69.9, 69.5, 67.3, 64.1, 58.5, 55.4, 41.6, 40.2, 37.0, 36.6, 36.5, 35.8, 35.4, 34.9, 33.6, 33.5, 32.5, 30.6, 30.4, 30.3, 29.9, 29.8, 28.5, 27.1, 23.1, 22.6, 21.9, 21.9, 20.3, 20.2, 16.6, 16.3, 14.7, 13.5, 11.7, 10.9, 8.3 ppm; ^{31}P NMR (122 MHz, CD_3CN) δ 24.1 ppm; HRMS (ESI^+) m/z : [M] $^+$ Calcd for $\text{C}_{61}\text{H}_{90}\text{O}_{11}\text{P}^+$ 1029.6215; Found 1029.6215.

Phosphonium salt 3c: 207 mg, 36 % yield. Isolated as a white amorphous solid, a single spot by TLC. UV-active and stains green with PMA; ^1H NMR (400 MHz, CD_3CN) δ 7.70–7.61 (m, 6H), 7.23–7.16 (m, 6H), 5.77 (d, J = 1.4 Hz, 1H), 5.49 (dd, J = 3.3, 1.8 Hz, 1H), 4.36 (ddd, J = 9.6, 7.2, 4.0 Hz, 1H), 4.17–4.01 (m, 3H), 4.00–3.92 (m, 2H), 3.89 (s, 9H), 3.80–3.75 (m, 1H), 3.70–3.66 (m, 1H), 3.65–3.55 (m, 2H), 3.44–3.33 (m, 2H), 2.82 (ddd, J = 13.8, 6.9, 2.6 Hz, 1H), 2.40–0.62 (m, 54H) ppm; ^{13}C NMR (101 MHz, CD_3CN) δ 176.1, 165.4, 165.4, 136.5, 136.4, 116.8, 116.6, 110.7, 109.8, 108.5, 99.1, 86.7, 86.4, 84.7, 81.9, 81.6, 76.4, 74.8, 69.7, 69.3, 67.1, 64.0, 58.4, 56.6, 55.2, 41.3, 40.0, 36.8, 36.4, 36.3, 35.6, 35.1, 34.7, 33.4, 33.3, 32.3, 32.1, 30.4, 30.2, 30.0, 29.7, 29.6, 28.3, 26.9, 23.5, 23.0, 20.0, 20.0, 16.5, 16.1, 14.6, 13.3, 11.5, 10.8, 8.1 ppm; ^{31}P NMR (122 MHz, CD_3CN) δ 22.8 ppm; HRMS (ESI^+) m/z : [M] $^+$ Calcd for $\text{C}_{61}\text{H}_{90}\text{O}_{14}\text{P}^+$ 1077.6063; Found 1077.6064.

Phosphonium salt 3d: 190 mg, 33 % yield. Isolated as a white amorphous solid, a single spot by TLC. UV-active and stains green with PMA; ^1H NMR (400 MHz, CD_3CN) δ 7.82–7.74 (m, 3H), 7.32–7.21 (m, 6H), 7.21–7.13 (m, 3H), 5.85 (d, J = 1.1 Hz, 1H), 5.58 (dd, J = 3.0, 1.7 Hz, 1H), 4.35 (ddd, J = 9.5, 7.2, 4.0 Hz, 1H), 4.11 (dt, J = 11.0, 6.1 Hz, 1H), 4.04 (d, J = 3.4 Hz, 1H), 4.02 (s, 1H), 4.00–3.91 (m, 3H), 3.86–3.80 (m, 1H), 3.72 (s, 9H), 3.67–3.62 (m, 2H), 3.62–3.56 (m, J = 10.0, 5.8 Hz, 2H), 3.37 (d, J = 9.8 Hz, 1H), 3.30–3.11 (m, 3H), 2.81 (ddd, J = 13.8, 6.9, 2.7 Hz, 1H), 2.43–0.63 (m, 56H) ppm; ^{13}C NMR (101 MHz, CD_3CN) δ 176.0, 162.5, 162.5, 137.6, 137.6, 135.8, 135.7, 122.9, 122.7, 113.7,

113.6, 108.5, 107.4, 106.5, 99.1, 86.7, 86.4, 84.6, 81.8, 81.6, 76.3, 74.8, 69.9, 69.7, 69.3, 67.1, 64.5, 58.3, 56.9, 55.2, 41.5, 40.0, 36.7, 36.5, 36.3, 35.5, 35.2, 34.7, 33.5, 33.3, 32.3, 32.1, 30.4, 30.4, 30.2, 29.7, 29.6, 28.3, 26.9, 24.4, 23.9, 21.6, 21.5, 16.4, 16.1, 14.6, 13.2, 11.5, 10.9, 8.1 ppm; ^{31}P NMR (122 MHz, CD_3CN) δ 26.3 ppm; HRMS (ESI^+) m/z : $[\text{M}]^+$ Calcd for $\text{C}_{65}\text{H}_{90}\text{O}_{14}\text{P}^+$ 1077.6063; Found 1077.6076.

Phosphonium salt 3e: 187 mg, 34 % yield. Isolated as a white amorphous solid, a single spot by TLC. UV-active and stains green with PMA; ^1H NMR (400 MHz, CD_3CN) δ 7.88–7.62 (m, 9H), 7.57–7.39 (m, 3H), 6.95–6.82 (m, 2H), 5.78 (d, $J = 1.5$ Hz, 1H), 5.53 (dd, $J = 3.2, 1.7$ Hz, 1H), 4.34 (ddd, $J = 9.6, 7.2, 4.1$ Hz, 1H), 4.20 (dd, $J = 3.7, 3.0$ Hz, 1H), 4.16–4.00 (m, 3H), 4.00–3.90 (m, 2H), 3.83–3.77 (m, 1H), 3.60 (dd, $J = 5.4, 2.6$ Hz, 1H), 3.66–3.54 (m, 2H), 3.52–3.20 (m, 4H), 3.04 (s, 6H), 2.81 (ddd, $J = 13.7, 6.8, 2.6$ Hz, 1H), 2.39 (s, 1H), 2.31–2.20 (m, 2H), 2.17–0.60 (m, 48H) ppm; ^{13}C NMR (101 MHz, CD_3CN) δ 176.0, 154.9, 135.7, 135.6, 135.3, 135.3, 134.3, 134.2, 130.9, 130.8, 121.4, 120.6, 113.3, 113.2, 108.4, 99.0, 86.7, 86.4, 84.6, 81.8, 81.5, 76.3, 74.7, 69.6, 69.3, 67.0, 63.9, 58.4, 41.2, 40.1, 39.9, 36.8, 36.4, 36.3, 35.5, 35.1, 34.6, 33.4, 33.2, 32.2, 30.4, 30.2, 29.7, 29.6, 28.4, 26.9, 23.1, 22.6, 20.04, 20.00, 16.4, 16.1, 14.5, 13.2, 11.4, 10.8, 8.2 ppm; ^{31}P NMR (122 MHz, CD_3CN) δ 23.5 ppm; HRMS (ESI^+) m/z : $[\text{M}]^+$ Calcd for $\text{C}_{66}\text{H}_{90}\text{NO}_{11}\text{P}^+$ 1030.6168; Found 1030.6180.

Phosphonium salt 3f: 195 mg, 39 % yield. Isolated as a white amorphous solid, a single spot by TLC. UV-active and stains green with PMA; ^1H NMR (400 MHz, CD_3CN) δ 5.82 (s, 1H), 5.56 (s, 1H), 4.34 (ddd, $J = 9.6, 7.4, 4.1$ Hz, 1H), 4.24 (s, 1H), 4.10 (t, $J = 5.9$ Hz, 2H), 4.04 (d, $J = 3.4$ Hz, 1H), 4.00 (d, $J = 1.8$ Hz, 1H), 3.98 (d, $J = 1.9$ Hz, 1H), 3.97–3.91 (m, 1H), 3.86–3.82 (m, 1H), 3.76 (dd, $J = 4.9, 3.4$ Hz, 1H), 3.65 (dd, $J = 9.9, 1.9$ Hz, 1H), 3.62–3.55 (m, 1H), 3.40–3.33 (m, 1H), 3.29 (s, 2H), 2.89 (dd, $J = 6.8, 3.0$ Hz, 1H), 2.46–0.69 (m, 78H) ppm; ^{13}C NMR (101 MHz, CD_3CN) δ 176.1, 108.5, 99.1, 86.8, 86.5, 84.7, 81.9, 76.4, 74.9, 69.8, 69.1, 67.1, 63.9, 58.5, 41.7, 40.0, 36.7, 36.6, 36.3, 35.6, 35.2, 34.7, 33.4, 33.3, 32.3, 30.5, 30.4, 30.3, 29.7, 28.4, 28.3, 27.8, 26.9, 24.8, 24.7, 24.5, 24.4, 23.9, 23.9, 19.4, 19.2, 18.9, 18.7, 16.5, 16.1, 14.6, 13.8, 13.6, 13.3, 11.9, 11.0, 8.1 ppm; ^{31}P NMR (122 MHz, CD_3CN) δ 34.9 ppm; HRMS (ESI^+) m/z : $[\text{M}]^+$ Calcd for $\text{C}_{62}\text{H}_{90}\text{O}_{11}\text{P}^+$ 927.6685; Found 927.6689.

4.4. Synthesis of 6-bromohexanoic ester of monensin A (compound 4)

MON (2.25 g, 3.35 mmol, 1.0 equiv.) was dissolved in toluene (60 mL) and the solution was heated to 90 °C. After 15 min, DBU (868 mg, 5.70 mmol, 1.7 equiv.) was added dropwise to the reaction mixture, and after another 20 min, 1,6-dibromohexane (2.45 g, 10.06 mmol, 3.0 equiv.) was added dropwise. After a few minutes, the color of the solution changed to brown. The resulting solution was stirred for the next 24 h. The reaction mixture was then concentrated on a rotary evaporator. Purification on silica gel using the CombiFlash system (0 %–50 % EtOAc/hexane) gave the pure ester **4** as a colorless oil. After twice co-evaporation to dryness with *n*-pentane, the product remained in an oily form (1.40 g, 50 % yield). The spectroscopic data were in agreement with previously published data [10].

4.5. Synthesis of triphenylphosphonium salt of monensin A with hexane-like linker (analog 5a)

1,6-dibromohexane (1.0 g, 4.10 mmol, 1.0 equiv.) was dissolved in CH_3CN (30 mL) and then triphenylphosphine (538 mg, 2.05 mmol, 0.5 equiv.) was added upon stirring. The solution was then refluxed for 72 h and subsequently concentrated on a rotary evaporator. The resulting oily residue was used without purification for the next step.

MON (200 mg, 0.30 mmol, 1 equiv.) was dissolved in toluene (20 mL) and the solution was heated to 90 °C. After 15 min, DBU (77 mg, 0.51 mmol, 1.7 equiv.) was added dropwise to the reaction mixture, and after another 20 min (6-bromohexyl)triphenylphosphonium bromide obtained in the previous step (452 mg, 0.90 mmol, 3.0 equiv.) was

added. The resulting solution was stirred for the next 24 h. The reaction mixture was then concentrated on a rotary evaporator. Purification on silica gel using the CombiFlash system (0 %–40 % acetone/ CHCl_3) gave the analog **5a** as a colorless oil. After twice co-evaporation to dryness with *n*-pentane, the oily product was completely converted into white amorphous solid.

Phosphonium salt 5a: 151 mg, 43 % yield. Isolated as a white amorphous solid, a single spot by TLC. UV-active and stains green with PMA; ^1H NMR (401 MHz, CD_3CN) δ 7.95–7.60 (m, 15H), 5.82 (s, 1H), 5.60 (s, 1H), 4.34 (ddd, $J = 9.6, 7.3, 4.1$ Hz, 1H), 4.10 (t, $J = 3.3$ Hz, 1H), 4.07–3.91 (m, 5H), 3.78–3.68 (m, 2H), 3.68–3.54 (m, 3H), 3.52–3.31 (m, 4H), 2.92–2.78 (m, 1H), 2.37 (s, 1H), 2.31–2.19 (m, 2H), 2.17–0.67 (m, 51H) ppm; ^{13}C NMR (101 MHz, CD_3CN) δ 176.3, 134.8, 134.7, 131.3, 131.2, 119.9, 119.1, 108.6, 99.3, 86.9, 86.6, 84.8, 82.2, 82.0, 76.5, 74.9, 69.9, 69.4, 67.3, 64.8, 58.7, 55.5, 41.7, 40.2, 37.1, 36.7, 36.5, 35.7, 35.3, 34.9, 33.6, 33.4, 32.4, 32.3, 30.7, 30.5, 29.9, 29.8, 29.0, 28.5, 27.0, 26.1, 23.1, 23.1, 23.0, 22.5, 16.6, 16.2, 14.7, 13.6, 11.8, 11.1, 8.3 ppm; ^{31}P NMR (122 MHz, CD_3CN) δ 25.3 ppm; HRMS (ESI^+) m/z : $[\text{M}]^+$ Calcd for $\text{C}_{60}\text{H}_{90}\text{O}_{11}\text{P}^+$ 1015.6059; Found 1015.6064.

4.6. General procedure for preparation of phosphonium salts of monensin A with hexane-like linker (analog 5b–5f)

Compound **4** (250 mg, 0.30 mmol, 1.0 equiv.) was dissolved in CH_3CN (30 mL) and the respective phosphine (0.90 mmol, 3.0 equiv.) was added upon stirring. The solution was then refluxed for 72 h. After this time, the reaction mixture was concentrated on a rotary evaporator. Purification on silica gel using the CombiFlash system (0 %–40 % acetone/ CHCl_3) gave the phosphonium salts of **MON** as colorless oils. After twice co-evaporation to dryness with *n*-pentane, the oily products were completely converted into white amorphous solids.

Phosphonium salt 5b: 51 mg, 15 % yield. Isolated as a white amorphous solid, a single spot by TLC. UV-active and stains green with PMA; ^1H NMR (401 MHz, CD_3CN) δ 7.70–7.46 (m, 11H), 7.20 (dd, $J = 9.0, 2.6$ Hz, 1H), 5.99–5.84 (m, 1H), 5.72–5.51 (m, 1H), 4.46–4.23 (m, 1H), 4.11–3.94 (m, 6H), 3.89 (s, 2H), 3.88–3.82 (m, 1H), 3.75 (dd, $J = 4.5, 2.6$ Hz, 1H), 3.69–3.63 (m, 2H), 3.60 (dd, $J = 10.0, 6.0$ Hz, 1H), 3.36 (dd, $J = 9.0, 3.3$ Hz, 1H), 2.86 (dd, $J = 6.8, 3.3$ Hz, 1H), 2.46 (s, 9H), 2.35–0.68 (m, 55H) ppm; ^{13}C NMR (101 MHz, CD_3CN) δ 176.1, 147.2, 147.2, 136.6, 136.5, 134.5, 134.4, 131.8, 131.6, 116.7, 115.8, 108.5, 99.2, 86.9, 86.5, 84.7, 82.1, 81.9, 76.4, 74.9, 69.8, 69.3, 67.3, 64.7, 58.6, 56.6, 41.7, 40.1, 36.9, 36.5, 36.3, 35.6, 35.2, 34.8, 33.5, 33.3, 32.3, 30.7, 30.5, 30.2, 29.7, 29.0, 28.3, 26.9, 26.1, 21.7, 21.7, 16.5, 16.1, 14.6, 13.5, 11.9, 10.9, 8.1 ppm; ^{31}P NMR (122 MHz, CD_3CN) δ 24.2 ppm; HRMS (ESI^+) m/z : $[\text{M}]^+$ Calcd for $\text{C}_{60}\text{H}_{94}\text{O}_{11}\text{P}^+$ 1057.6528; Found 1057.6527.

Phosphonium salt 5c: 121 mg, 34 % yield. Isolated as a white amorphous solid, a single spot by TLC. UV-active and stains green with PMA; ^1H NMR (600 MHz, CD_3CN) δ 7.67–7.60 (m, 6H), 7.22–7.18 (m, 6H), 5.87 (d, $J = 1.3$ Hz, 1H), 5.59 (dd, $J = 2.9, 1.9$ Hz, 1H), 4.37 (ddd, $J = 9.6, 7.2, 4.2$ Hz, 1H), 4.10–3.99 (m, 5H), 3.97 (dd, $J = 9.4, 1.9$ Hz, 1H), 3.90 (s, 9H), 3.85 (t, $J = 3.8$ Hz, 1H), 3.78–3.74 (m, 1H), 3.68–3.65 (m, 2H), 3.61 (dd, $J = 10.0, 5.9$ Hz, 1H), 3.50–3.47 (m, 1H), 3.37 (ddd, $J = 9.8, 4.3, 1.7$ Hz, 1H), 3.26–3.25 (m, 3H), 3.24–3.15 (m, 2H), 3.07 (qd, $J = 7.4, 5.1$ Hz, 1H), 2.86 (qd, $J = 6.8, 3.3$ Hz, 1H), 2.58 (s, 1H), 2.34–0.71 (m, 49H) ppm; ^{13}C NMR (151 MHz, CD_3CN) δ 176.2, 165.5, 165.5, 136.6, 136.5, 116.7, 116.7, 110.7, 110.1, 108.6, 99.2, 86.9, 86.5, 84.7, 82.1, 81.9, 79.2, 76.4, 74.9, 70.2, 69.8, 69.3, 67.3, 64.8, 58.6, 56.6, 56.4, 55.2, 41.7, 40.1, 36.9, 36.5, 36.3, 35.6, 35.2, 34.8, 33.5, 33.3, 32.3, 32.1, 30.6, 30.5, 30.4, 29.8, 29.7, 29.0, 28.3, 26.9, 26.2, 23.8, 23.5, 23.1, 23.0, 16.4, 16.1, 14.6, 13.6, 11.9, 11.0, 8.1 ppm; ^{31}P NMR (122 MHz, CD_3CN) δ 22.8 ppm; HRMS (ESI^+) m/z : $[\text{M}]^+$ Calcd for $\text{C}_{60}\text{H}_{94}\text{O}_{14}\text{P}^+$ 1105.6376; Found 1105.6390.

Phosphonium salt 5d: 85 mg, 24 % yield. Isolated as a white amorphous solid, a single spot by TLC. UV-active and stains green with PMA; ^1H NMR (400 MHz, CD_3CN) δ 7.82–7.76 (m, 2H), 7.55–7.50 (m, 1H),

7.42 (ddd, $J = 14.7, 7.8, 1.7$ Hz, 1H), 7.28–7.13 (m, 7H), 7.04–6.99 (m, 1H), 5.87 (d, $J = 1.5$ Hz, 1H), 5.61 (dd, $J = 3.3, 1.9$ Hz, 1H), 4.36 (ddd, $J = 9.6, 7.2, 4.1$ Hz, 1H), 4.04 (dd, $J = 8.4, 4.4$ Hz, 2H), 4.02–3.95 (m, 2H), 3.87 (t, $J = 3.6$ Hz, 1H), 3.79 (dt, $J = 6.4, 3.3$ Hz, 1H), 3.74 (dd, $J = 5.4, 3.1$ Hz, 1H), 3.72 (s, 9H), 3.62 (ddd, $J = 15.8, 9.8, 4.4$ Hz, 2H), 3.53–3.50 (m, 2H), 3.45 (s, 1H), 3.36 (ddd, $J = 9.8, 4.1, 1.7$ Hz, 1H), 3.23 (s, 2H), 3.15–3.05 (m, 2H), 2.83 (ddd, $J = 13.9, 6.9, 3.0$ Hz, 1H), 2.63 (s, 1H), 2.50 (s, 1H), 2.35–0.70 (m, 50H) ppm; ^{13}C NMR (101 MHz, CD_3CN) δ 176.1, 162.5, 162.5, 137.6, 137.6, 135.8, 135.7, 122.8, 122.7, 113.7, 113.6, 108.5, 107.6, 106.7, 99.1, 86.8, 86.5, 84.7, 82.0, 81.9, 79.2, 76.4, 74.9, 70.1, 69.7, 69.3, 67.2, 64.8, 58.4, 56.8, 56.4, 56.0, 55.2, 41.6, 40.0, 36.9, 36.6, 36.3, 35.5, 35.2, 34.7, 33.5, 33.3, 32.3, 30.9, 30.7, 30.4, 30.2, 29.8, 29.6, 29.0, 28.3, 26.9, 26.1, 16.4, 16.1, 14.6, 13.5, 11.7, 11.0, 8.1 ppm; ^{31}P NMR (122 MHz, CD_3CN) δ 26.6 ppm; HRMS (ESI^+) m/z : $[\text{M}]^+$ Calcd for $\text{C}_{60}\text{H}_{104}\text{O}_{14}\text{P}^+$ 1105.6376; Found 1105.6381.

Phosphonium salt 5e: 106 mg, 31 % yield. Isolated as a white amorphous solid, a single spot by TLC. UV-active and stains green with PMA; ^1H NMR (600 MHz, CD_3CN) δ 7.84–7.79 (m, 2H), 7.75–7.66 (m, 8H), 7.49–7.43 (m, 2H), 6.91–6.87 (m, 2H), 5.87 (s, 1H), 5.61 (s, 1H), 4.36 (ddd, $J = 9.6, 7.2, 4.1$ Hz, 1H), 4.10–3.98 (m, 5H), 3.97 (dd, $J = 9.4, 1.9$ Hz, 1H), 3.87 (t, $J = 3.5$ Hz, 1H), 3.76 (dd, $J = 5.1, 3.2$ Hz, 1H), 3.72–3.64 (m, 2H), 3.60 (dd, $J = 10.0, 5.9$ Hz, 1H), 3.51 (s, 1H), 3.45 (s, 1H), 3.37 (ddd, $J = 9.9, 4.2, 1.7$ Hz, 1H), 3.25–3.16 (m, 2H), 3.06 (s, 6H), 2.86 (qd, $J = 6.9, 3.1$ Hz, 1H), 2.63 (s, 2H), 2.58 (s, 3H), 2.33–0.68 (m, 48H) ppm; ^{13}C NMR (151 MHz, CD_3CN) δ 176.2, 155.1, 155.0, 135.8, 135.7, 135.4, 134.39, 134.30, 134.33, 134.32, 131.0, 130.9, 113.34, 113.25, 108.6, 99.2, 86.9, 86.5, 84.7, 82.0, 81.9, 76.4, 74.9, 70.2, 69.9, 69.8, 69.4, 67.3, 64.8, 58.5, 56.5, 55.2, 41.6, 40.1, 36.9, 36.5, 36.3, 35.6, 35.2, 34.8, 33.5, 33.3, 32.3, 32.1, 30.7, 30.6, 30.5, 29.8, 29.7, 29.0, 28.3, 26.9, 26.1, 23.1, 16.5, 16.1, 14.6, 13.5, 11.8, 11.0, 8.1 ppm; ^{31}P NMR (122 MHz, CD_3CN) δ 23.6 ppm; HRMS (ESI^+) m/z : $[\text{M}]^+$ Calcd for $\text{C}_{62}\text{H}_{103}\text{NO}_{11}\text{P}^+$ 1058.6481; Found 1058.6480.

Phosphonium salt 5f: 71 mg, 23 % yield. Isolated as a white amorphous solid, a single spot by TLC. UV-active and stains green with PMA; ^1H NMR (401 MHz, CD_3CN) δ 5.85 (s, 1H), 5.64 (s, 1H), 4.34 (t, $J = 9.6$ Hz, 1H), 4.12–3.91 (m, 8H), 3.83 (s, 1H), 3.76 (s, 1H), 3.69–3.57 (m, 3H), 3.37 (dd, $J = 10.2, 3.0$ Hz, 1H), 2.86 (s, 1H), 2.39–0.66 (m, 82H) ppm; ^{13}C NMR (101 MHz, CD_3CN) δ 176.2, 108.5, 99.1, 86.9, 86.5, 84.8, 82.2, 76.5, 69.9, 69.2, 67.2, 64.9, 58.6, 41.7, 40.0, 37.0, 36.7, 36.3, 35.2, 34.8, 33.4, 30.9, 30.7, 29.7, 28.9, 25.9, 24.5, 24.37, 23.92, 23.9, 21.9, 21.8, 19.5, 19.2, 19.1, 19.0, 18.74, 18.66, 16.46, 13.8, 13.5, 11.9, 11.0, 8.1 ppm; ^{31}P NMR (122 MHz, CD_3CN) δ 34.9 ppm; HRMS (ESI^+) m/z : $[\text{M}]^+$ Calcd for $\text{C}_{54}\text{H}_{100}\text{O}_{11}\text{P}^+$ 955.6998; Found 955.7017.

4.7. Synthesis of benzyl ester of monensin A (compound 6)

MON (0.5 g, 0.75 mmol, 1.0 equiv.) was dissolved in toluene (20 mL) and the solution was heated to 90 °C. After 15 min, DBU (193 mg, 1.27 mmol, 1.7 equiv.) was added dropwise to the reaction mixture, and after another 20 min, benzyl bromide (382 mg, 2.24 mmol, 3.0 equiv.) was added dropwise. After a few minutes, the color of the solution changed to brown. The resulting solution was stirred for the next 24 h. The reaction mixture was then concentrated on a rotary evaporator *in vacuo*. Purification on silica gel using the CombiFlash system (0 %–50 % EtOAc /hexane) gave the pure ester 6 as a colorless oil. After twice co-evaporation to dryness with *n*-pentane, the oily product was completely converted into white amorphous solid (438 mg, 77 % yield). The spectroscopic data were in agreement with previously published data [18].

4.8. In vitro biological studies

4.8.1. Culture cell lines

The study was conducted on various cell lines, including human primary (SW480) and metastatic (SW620) colon cancer, human

metastatic prostate cancer (PC3), human breast cancer (MDA-MB-231), human lung cancer (A549), human immortal keratinocytes (HaCat), and Chinese hamster lung fibroblasts (V79). All cell lines, obtained from the repository of the Medical University of Warsaw, were cultured in the recommended medium supplemented with 10 % fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 $\mu\text{g}/\text{L}$) at 37 °C with 5 % CO_2 . Once the cells reached 80 % confluence, they were harvested using 0.25 % trypsin (Gibco Life Technologies), seeded into 96-, 12-, and 6-well plates, and incubated for 24 h at 37 °C with 5 % CO_2 for optimal attachment before being used in further assays.

4.8.2. MTT assay

The cytotoxic activity of monensin (**MON**) and its derivatives was assessed by MTT assay. The cells were seeded (5×10^3 cells per well) in 96-well plates, and after 24 h of incubation, the cells were treated with the studied compounds at concentrations ranging from 0.5 to 40 μM . After 72 h of incubation at 37 °C/5 % CO_2 , the culture medium was removed and MTT solution (0.5 mg/mL) was added to each well. Next, the cells were incubated for additional 4 h at 37 °C/5 % CO_2 . The developed formazan crystals were dissolved in DMSO and isopropanol mixture (1:1, v/v). The optical density of the solution was measured at a wavelength of 570 nm using a Multiscan Go spectrophotometer (ThermoScientific). Cell viability was assessed by calculating the percentage of MTT reduction in treated cells compared with that in untreated control cells. The relative MTT level was determined as a percentage using formula $[A]/[B] \times 100$, where $[A]$ represents the absorbance of the tested samples and $[B]$ stands for the absorbance of the controls. The IC_{50} values were determined using GraphPad Prism 8.0.1 software (GraphPad Software).

4.8.3. Cell cycle assay

A549 and MDA-MB-231 cells (1×10^5 cells per well) were seeded in 6-well plates and treated with the studied compounds at their IC_{50} concentrations for 24 h. Following treatment, both adherent and detached cells were collected and centrifuged at 450 $\times g$ for 5 min at 4 °C. The cells were then washed twice with PBS and fixed overnight in cold 70 % ethanol at 4 °C. Before analysis, the fixed cells were centrifuged at 800 $\times g$ for 5 min at 4 °C and washed again with PBS. The cells were then incubated with RNase (100 $\mu\text{g}/\text{mL}$) and stained with propidium iodide (PI, 50 $\mu\text{g}/\text{mL}$) at 37 °C for 30 min in the dark. After staining, 200 μL of PBS was added to each sample. The cell cycle stages (sub-G1, G0/G1, S, and G2/M phases) were analyzed using a flow cytometer (Becton Dickinson FACS Verse).

4.8.4. Mitochondrial activity

Cells (5×10^4 per well) were seeded in 12-well plates, treated with **MON** and selected derivatives at their IC_{50} concentrations, and incubated for 48 h at 37 °C with 5 % CO_2 . After incubation, both suspended and adherent cells were collected, centrifuged at 800 $\times g$ for 5 min at 4 °C, washed twice with PBS, and stained with JC-10 (Abcam), MitoTracker Red CMXRos, and MitoTracker Green FM dyes (Invitrogen), following the manufacturers' protocols. Flow cytometry analysis (Becton Dickinson FACS Verse) was performed, where JC-10 aggregates (red fluorescence) were observed in the mitochondria during potential-dependent aggregation, while JC-10 monomers (green fluorescence) increased in the cytosol after mitochondrial membrane depolarization. MitoTracker Green FM binds to the mitochondrial membrane, indicating mitochondrial mass, and MitoTracker Red CMXRos localizes to the matrix of functional mitochondria, reflecting an intact mitochondrial membrane potential.

4.8.5. Annexin V-FITC/PI binding assay

A549 and MDA-MB-231 cells (5×10^4 cells per well) were seeded in 12-well plates and treated with **MON** and its selected derivatives (3a, 3f, 6) at their IC_{50} for 72 h. After treatment, both adherent and detached cells were collected, centrifuged at 800 $\times g$ for 5 min at 4 °C, and washed

twice with cold PBS. The cells were then labeled with Annexin V-FITC and propidium iodide (PI) following the manufacturer's protocol (BD Biosciences Pharmingen). Stained cells were analyzed by flow cytometry (Becton Dickinson FACS Verse) to identify early apoptotic cells (Annexin V+/PI-) and late apoptotic or necrotic cells (Annexin V+/PI+).

4.8.6. ROS detection assay

A549 and MDA-MB-231 cells (5×10^4 cells per well) were seeded into black 96-well plates and treated with MON and its derivatives at their respective IC_{50} concentrations. Following the treatment, the cells were incubated for 6 h at 37°C with 5 % CO_2 . Intracellular reactive oxygen species (ROS) levels were quantified using the CellROX™ Green Reagent (Invitrogen), following the protocol provided by the manufacturer. This cell-permeant probe remained weakly fluorescent in its reduced state and exhibited bright, photostable green fluorescence upon oxidation with ROS and subsequent interaction with DNA.

To validate the assay, H_2O_2 (1.5 mM) was used as a positive control to ensure robust ROS induction. The fluorescence intensity, indicative of ROS levels, was measured using a BioTek Synergy Microplate Spectrofluorometer (BioTek Instruments).

4.9. Biophysical studies

4.9.1. Cell culture

Oxygraph-2K experiments were performed on adenocarcinomic human alveolar basal epithelial cells (A549, Merck, Darmstadt, Germany). The cells were cultured on T75 flasks (Corning) in Dulbecco Minimal Essential Medium (DMEM; Merck, Darmstadt, Germany) supplemented with 10 % fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and antibiotics – 100 U/mL penicillin and 100 mg/mL streptomycin (Merck, Darmstadt, Germany). The cells were maintained in a humidified 5 % CO_2 atmosphere at 37°C and reseeded when they reached 90 % confluence.

4.9.2. High-resolution respirometry

High-resolution respirometry was performed using an Oxygraph-2K system (Oroboros Instruments, Innsbruck, Austria) according to the commonly used method [35]. Briefly, A549 cells were harvested, centrifuged, and resuspended in starved DMEM (1 % FBS) in 1×10^6 cells/ cm^3 . Cells were added to the respiratory chambers to measure basal respiration. MON and its derivatives in the concentration of 0.1, 0.3, 1, 3, and $10 \mu\text{M}$ were added to chambers after stabilization of the respiration. The maximum uncoupled electron transfer system (ETS) was measured using carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP; $1 \mu\text{M}$). The data are reported as mean values $\pm$ SD (SD, standard deviation).

4.9.3. Black lipid membrane technique (BLM)

Azolectin dissolved in *n*-decane at a final concentration of 25 mg/mL was used as a model biological membrane and was applied using a polyethylene brush to the 250 μm diameter opening of the Teflon cup to separate the two chambers (cis/trans) [36,37]. To improve the lipid bilayer's stability, the aperture's outline was coated with a lipid solution and N_2 -dried before bilayer formation. Overall, 1 mL of the solutions containing 50/150 mM NaCl (cis/trans) and 10 mM HEPES at pH = 7.2 were added to the measurement chambers and stirred using magnetic stirrers. Silver-chloride (Ag/AgCl) electrodes were introduced into the chambers and connected to the BLM-120 amplifier (Bio-Logic, Seyssinet-Pariset, France). The PowerLab 2/25 (ADInstruments, Sydney, Australia) converter processed the electric signal, recorded using LabChart5 and analyzed in Clampfit 8. The experiments were performed in Faraday's cage to prevent external electromagnetic interferences. All measurements were carried out at room temperature (25°C). MON and the studied compounds were added to trans side chamber in the concentration of 0.1, 0.3, 1, 3, and $10 \mu\text{M}$.

The bilayer formation and thinning were monitored by the

capacitance measurements and optical observations. The final accepted capacitance values ranged from 110 pF to 180 pF. Electrical connections were made by Ag/AgCl electrodes and agar salt bridges (3 M KCl) to minimize the liquid junction potentials. Voltage was applied to the cis compartment and the trans compartment was grounded.

The single-channel data were filtered at 500 Hz. The current was digitized at a sampling rate of 100 kHz. The single-channel currents were recorded at different voltages in steps of 20 mV. The data are reported as mean value $\pm$ SD (SD, standard deviation).

CRediT authorship contribution statement

Michał Sulik: Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology, Investigation, Data curation, Conceptualization. **Marta Jędrzejczyk:** Investigation, Magdalena Mielczarek-Puta: Writing – original draft, Visualization, Methodology, Investigation, Data curation. **Gwan Yong Lim:** Investigation. **Małgorzata Podsiad:** Investigation. **Jakub Hoser:** Writing – original draft, Visualization, Methodology, Investigation, Data curation. **Piotr Bednarczyk:** Writing – review & editing, Writing – original draft, Validation, Methodology, Conceptualization. **Marta Struga:** Writing – review & editing, Writing – original draft, Validation, Supervision, Conceptualization. **Adam Huczynski:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ejmech.2025.100275>.

Data availability

Data will be made available on request.

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Supplementary material

Mitochondria-targeting phosphonium salts of monensin A as the source of novel antiproliferative agents

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General procedures

All reagents, were purchased from two sources – Merck or Trimen Chemicals S.A., and used without further purification. CD₃CN spectral grade was stored over 3Å molecular sieves for several days. Reaction mixtures were stirred using Teflon-coated magnetic stir bars and were monitored by thin layer chromatography (TLC) using aluminum-backed plates (Merck 60 F₂₅₄). TLC plates were visualized by UV-light (254 nm), followed by treatment with phosphomolybdic acid (PMA) (5% in absolute ethanol) and gentle heating. Products of the reactions were purified using CombiFlash® Rf+ Lumen Flash Chromatography System (Teledyne Isco) with integrated ELS and UV detectors. All solvents used in flash chromatography were of HPLC grade (Merck) and were used as received. Solvents were removed using a rotary evaporator.

NMR spectra were recorded on a Varian 400 (¹H NMR at 400 MHz and ¹³C NMR at 101 MHz, ³¹P 122 MHz) magnetic resonance spectrometer, Bruker AvanceNEO 600 (¹H NMR at 600 MHz and ¹³C NMR at 151 MHz) magnetic resonance spectrometer or Mercury 300 (³¹P 162 MHz) magnetic resonance spectrometer. ¹H NMR spectra are reported in chemical shifts downfield from TMS using the respective residual solvent peak as internal standard (CD₃CN δ 1.94 ppm). ¹H NMR spectra are reported as follows: chemical shift (δ, ppm), multiplicity (s = singlet, d = doublet, t = triplet, dd = doublet of doublets, dt = doublet of triplets, ddd = doublet of doublet of doublets, qd = quartet of doublets, m = multiplet), coupling constant(s) in Hz, and integration. Significant peaks are reported within the overlapping ~2.00–0.50 ppm region of the ¹H NMR spectra. ¹³C NMR spectra are reported in chemical shifts downfield from TMS using the respective residual solvent peak as internal standard (CD₃CN δ 1.32 ppm, 118.26 ppm). ³¹P NMR spectra are reported in chemical shifts. Line broadening parameters were 0.5 or 1.0 Hz, while the error of chemical shift value was 0.1 ppm.

Electrospray ionization (ESI) high resolution mass spectra were recorded on a QTOF (Impact HD, Bruker Daltonics) mass spectrometer in the positive ion detection mode. Samples were prepared in dry acetonitrile. The mass range for ESI experiments was from $m/z = 400$ to $m/z = 1350$.

Spectroscopic and spectrometric analysis of newly synthesized compounds

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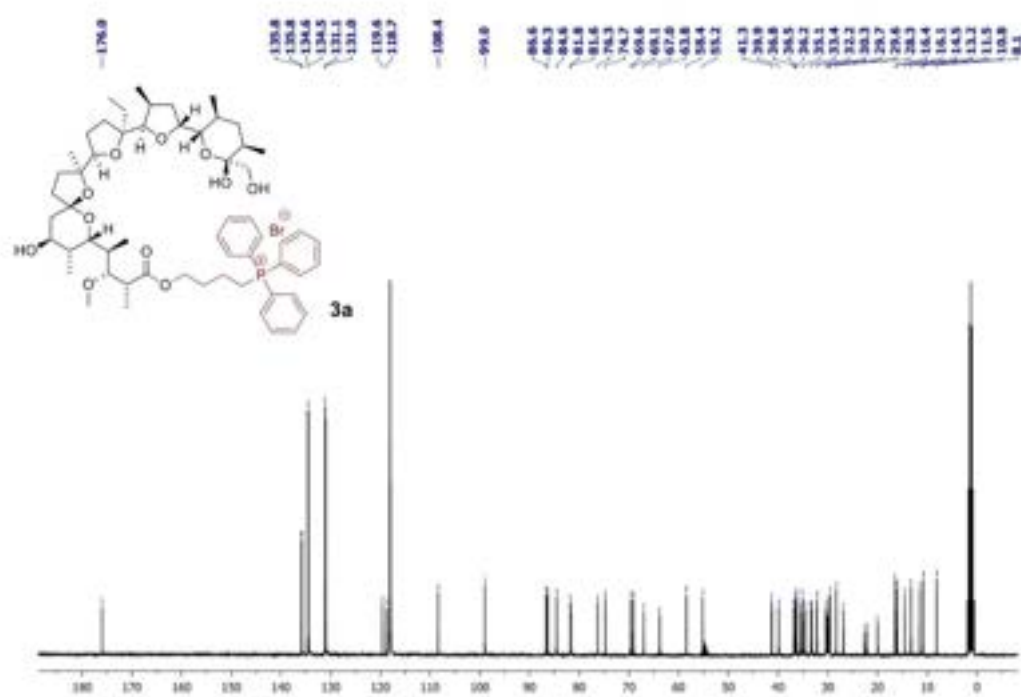


Figure S1. The ^{13}C NMR spectrum of **3a** in acetonitrile- d_3 .

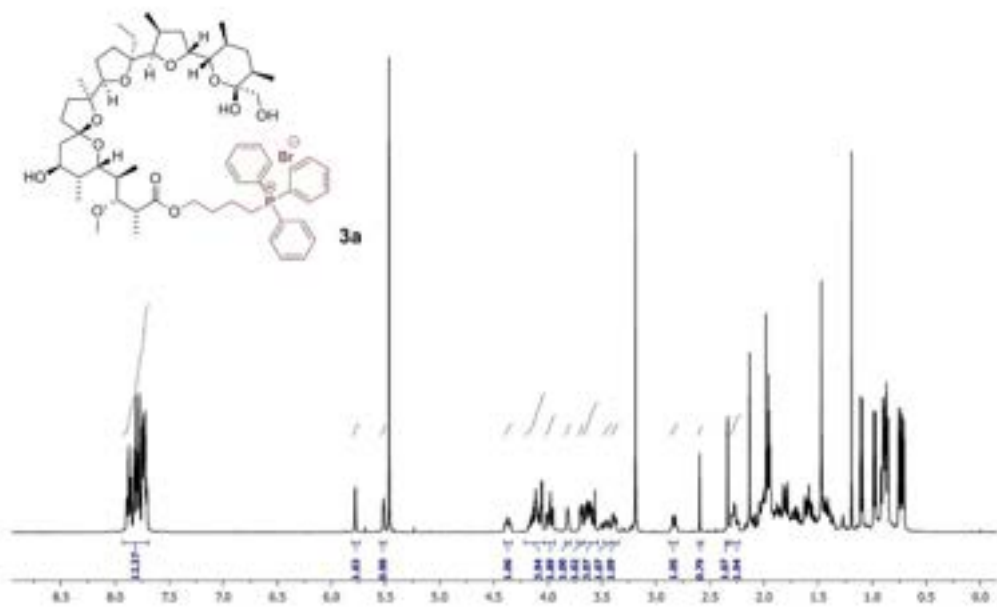
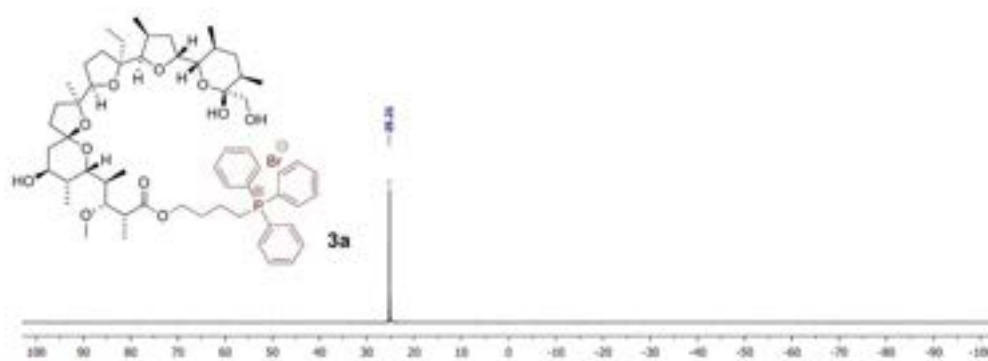
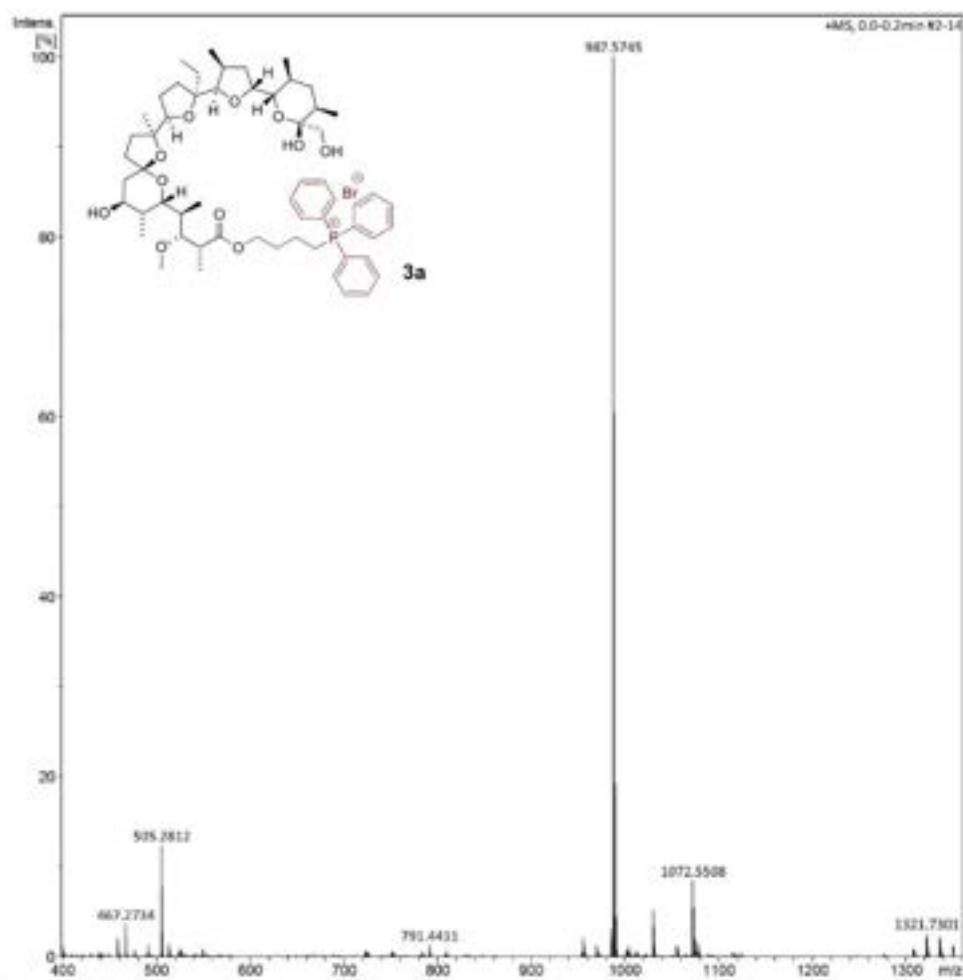


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Figure S3. The ^{31}P NMR spectrum of **3a** in acetonitrile- d_3 .Figure S4. The ESI high resolution mass spectrum of **3a**.

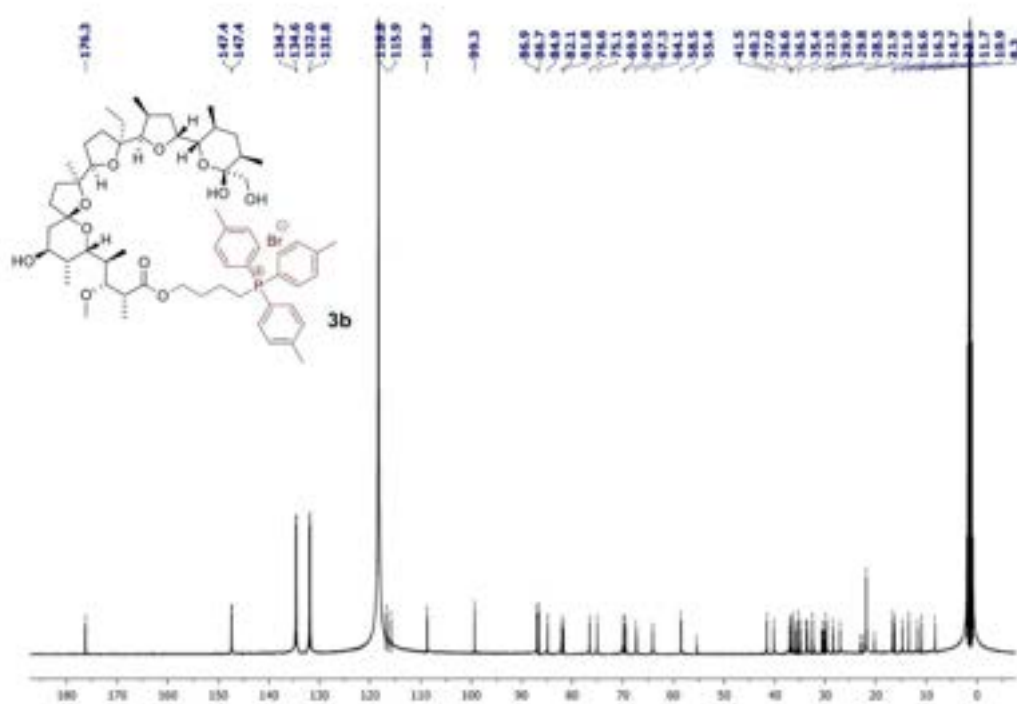


Figure S5. The ^{13}C NMR spectrum of **3b** in acetone- d_3 .

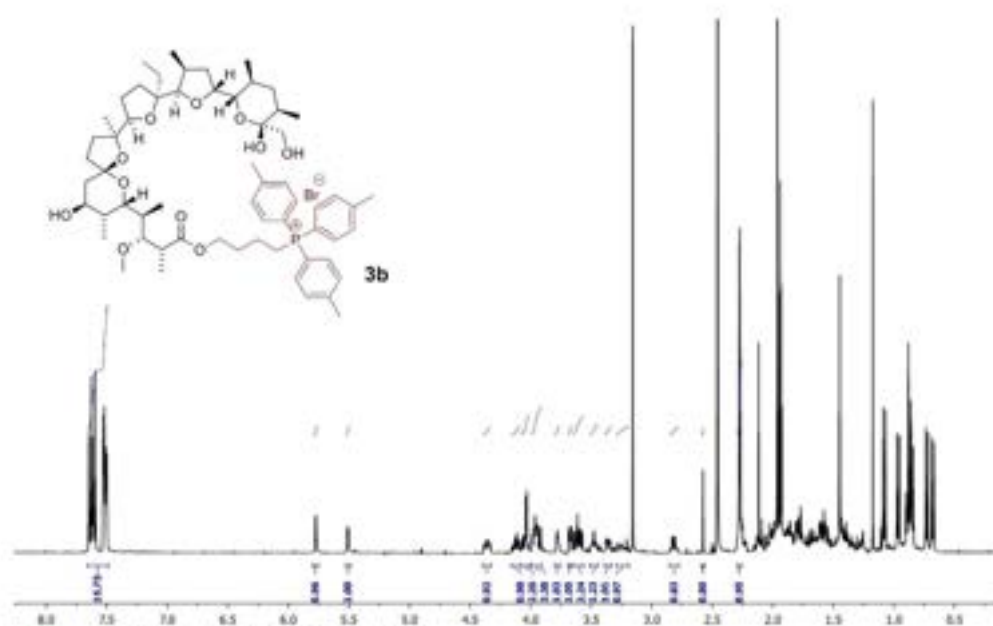


Figure S6. The ^1H NMR spectrum of **3b** in acetonitrile- d_3 .

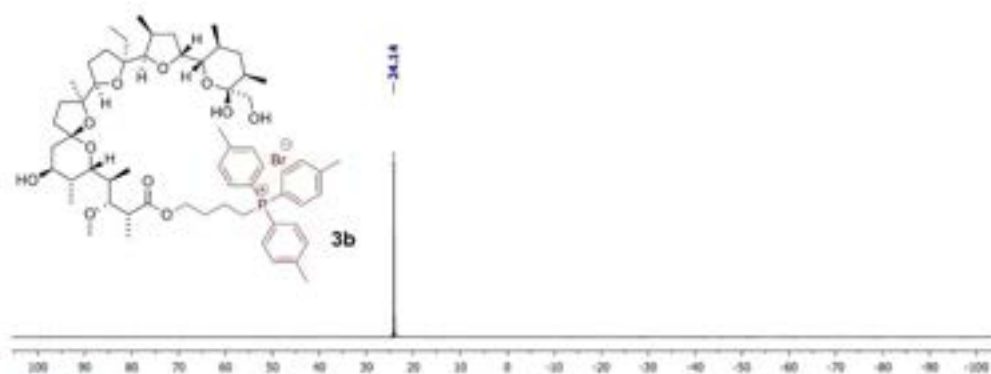


Figure S7. The ^{31}P NMR spectrum of **3b** in acetonitrile- d_3 .

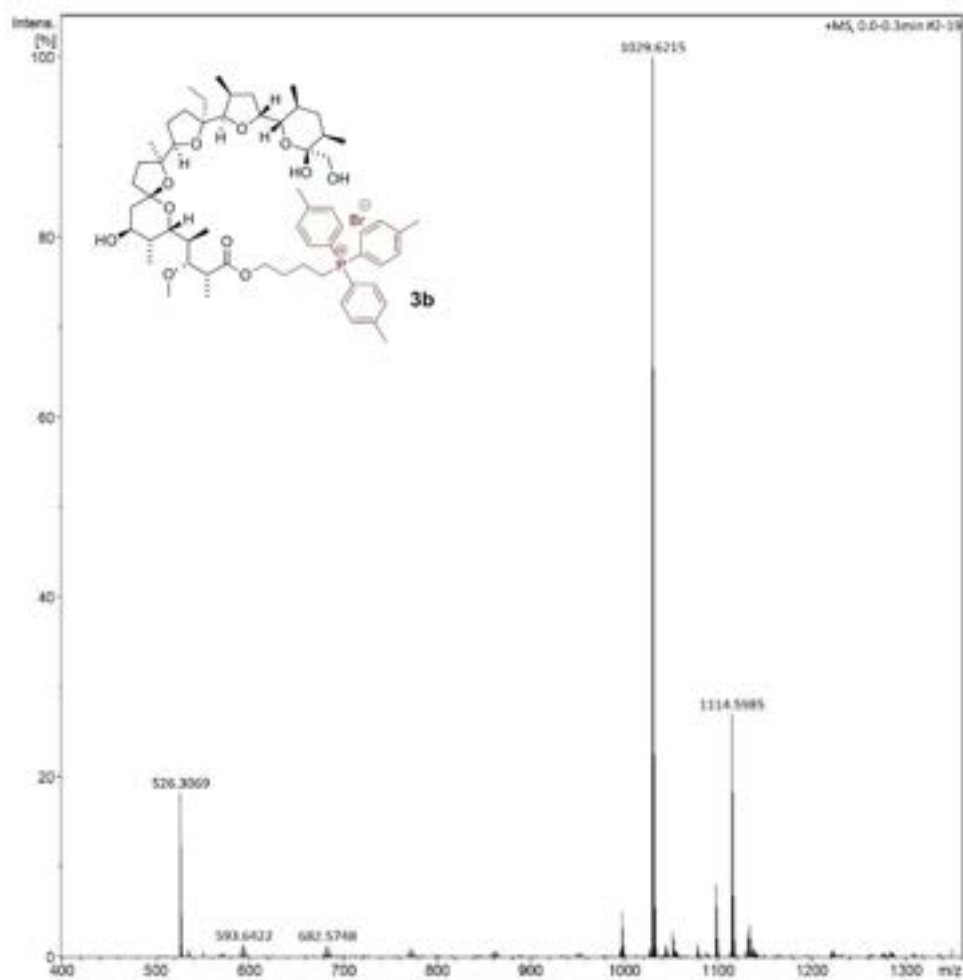
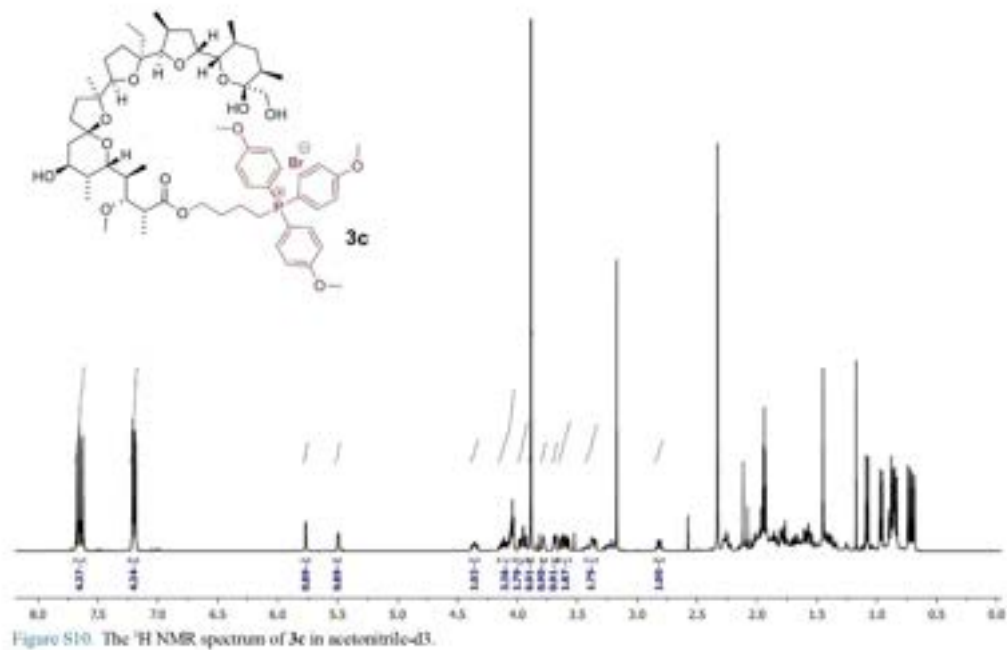
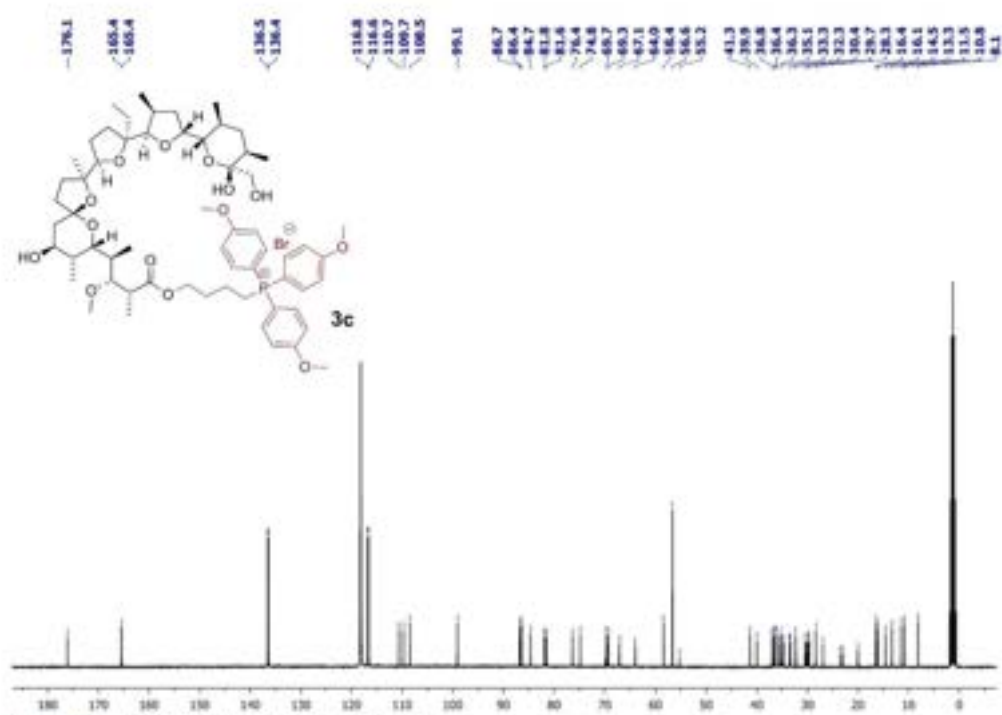
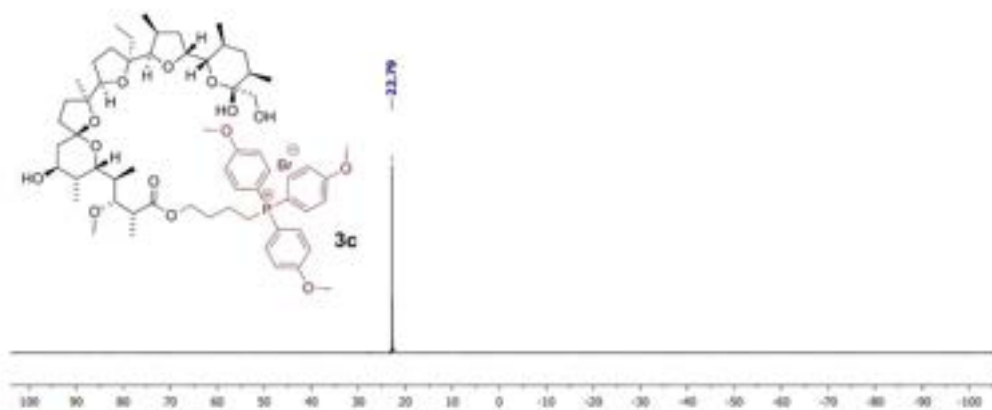
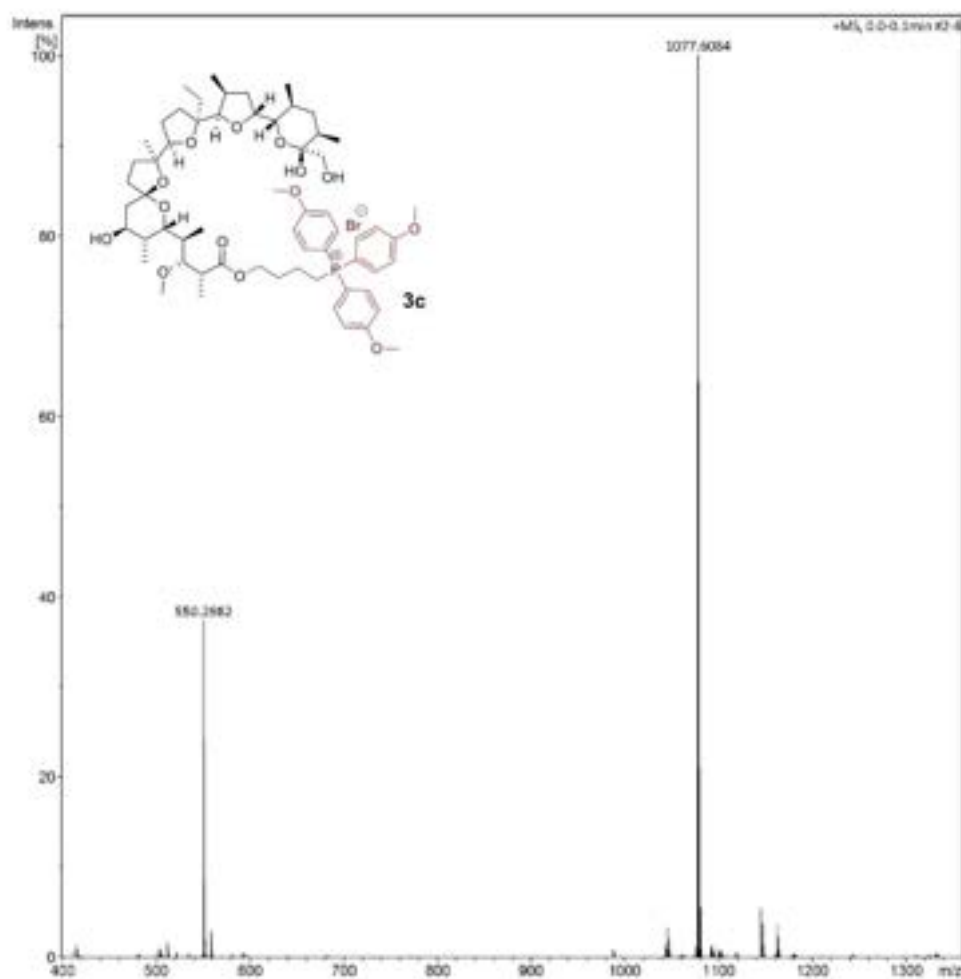


Figure S8. The ESI high resolution mass spectrum of **3b**.



Figure S11. The ^{31}P NMR spectrum of **3c** in acetone- d_3 .Figure S12. The ESI high resolution mass spectrum of **3c**.

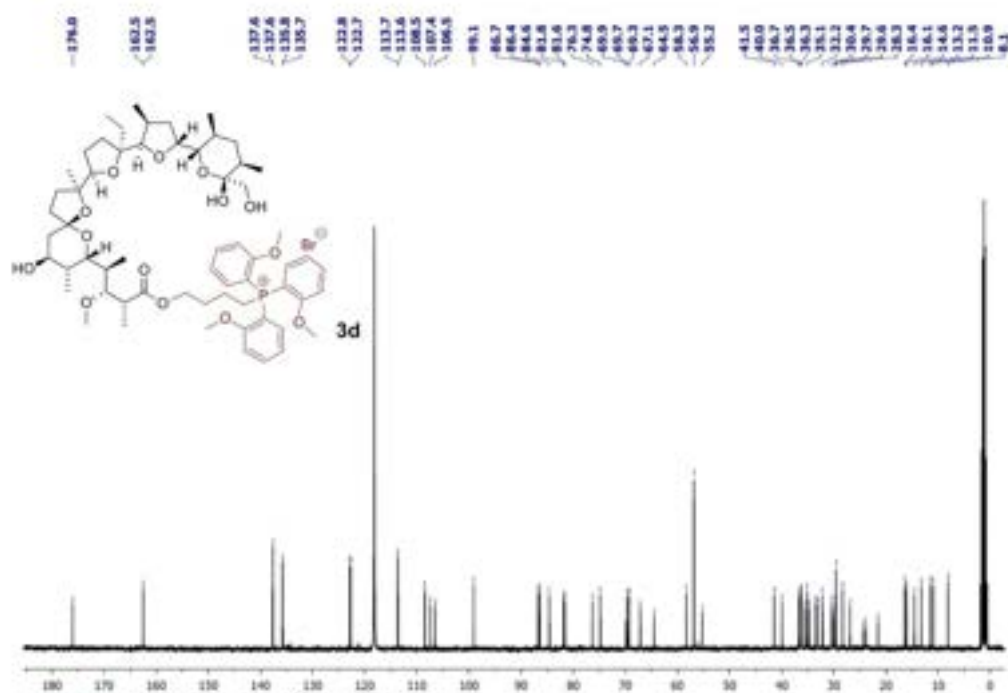


Figure S13. The ¹³C NMR spectrum of **3d** in acetonitrile-d₃.

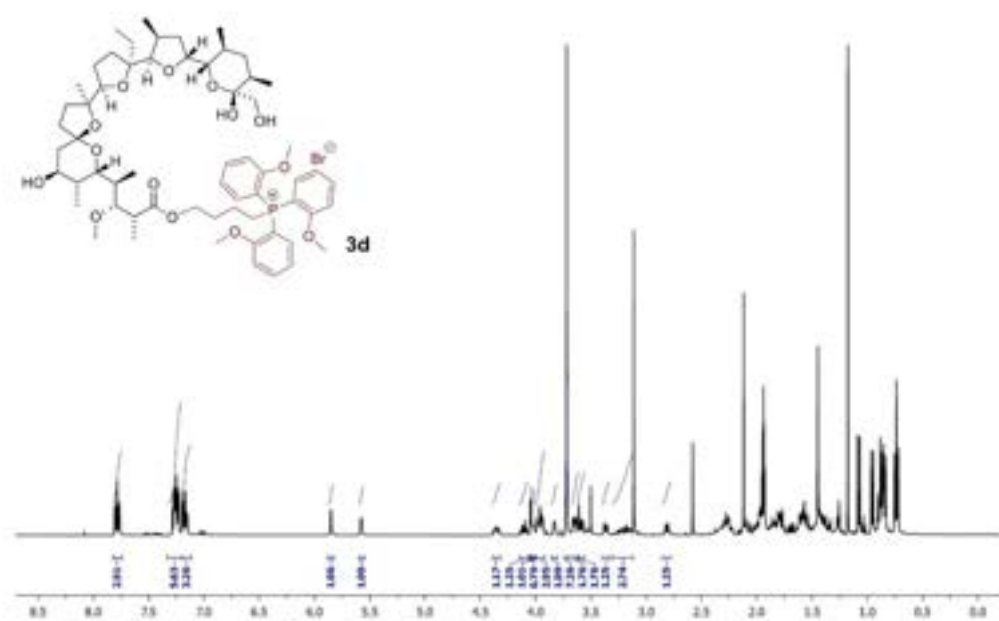
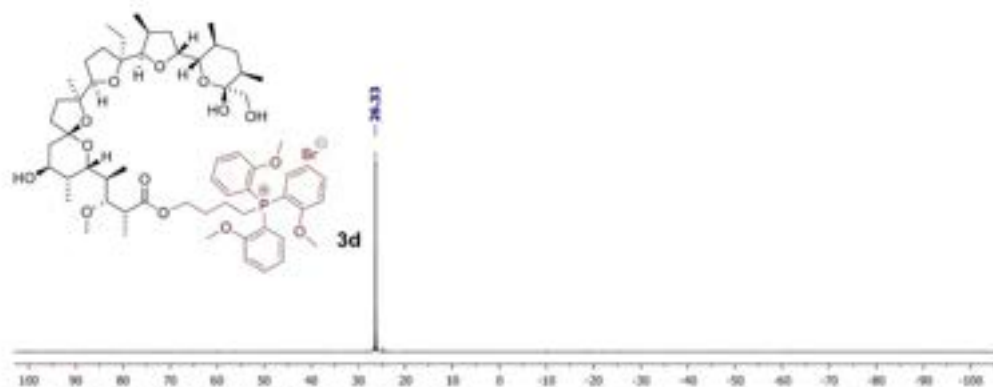
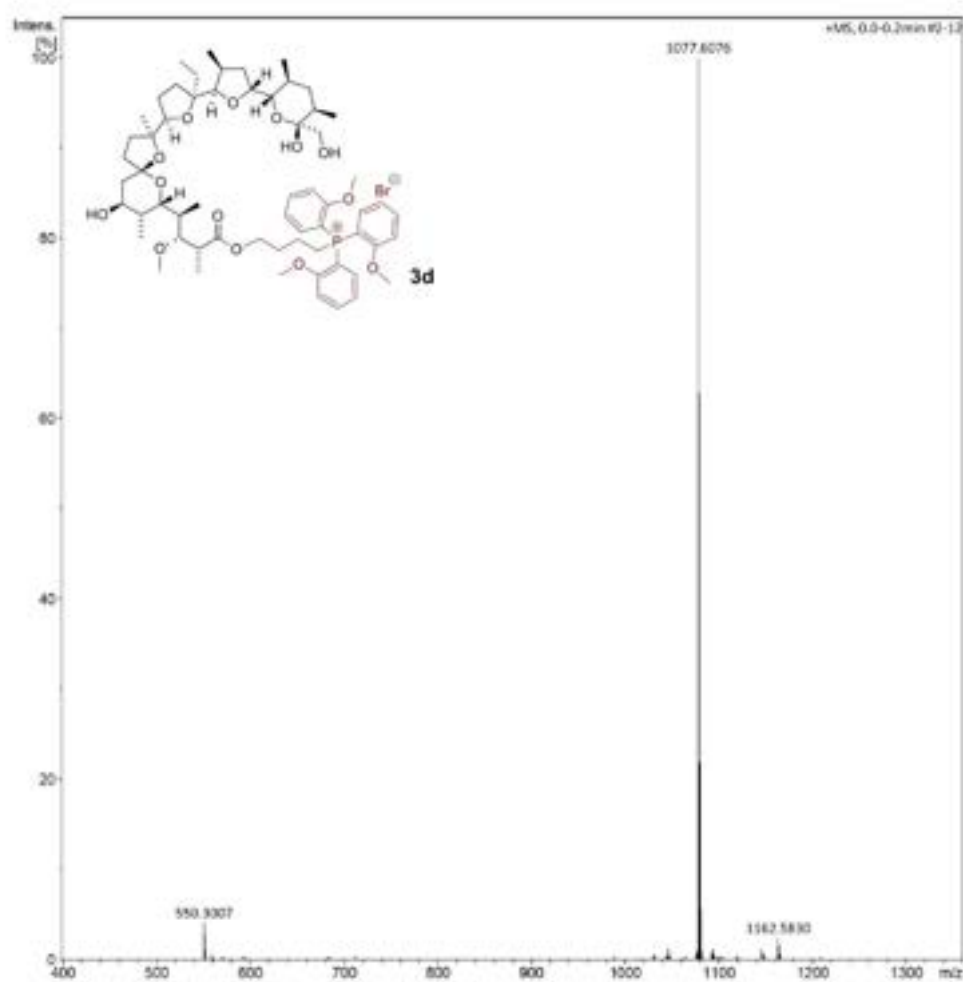
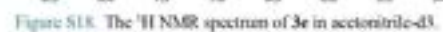
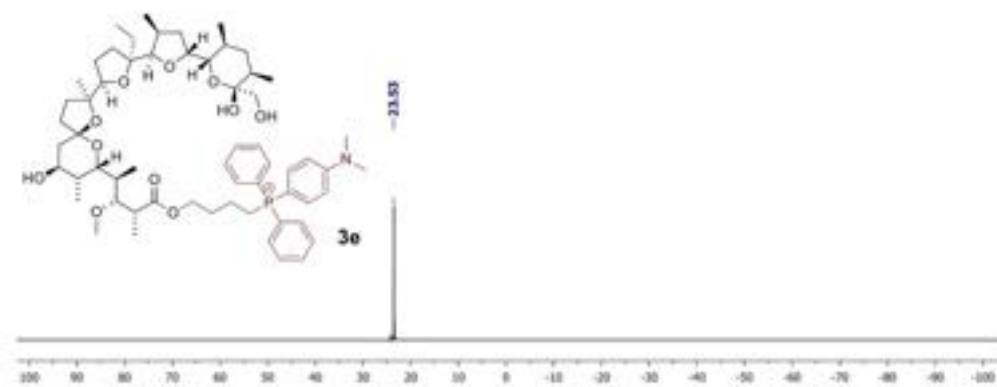
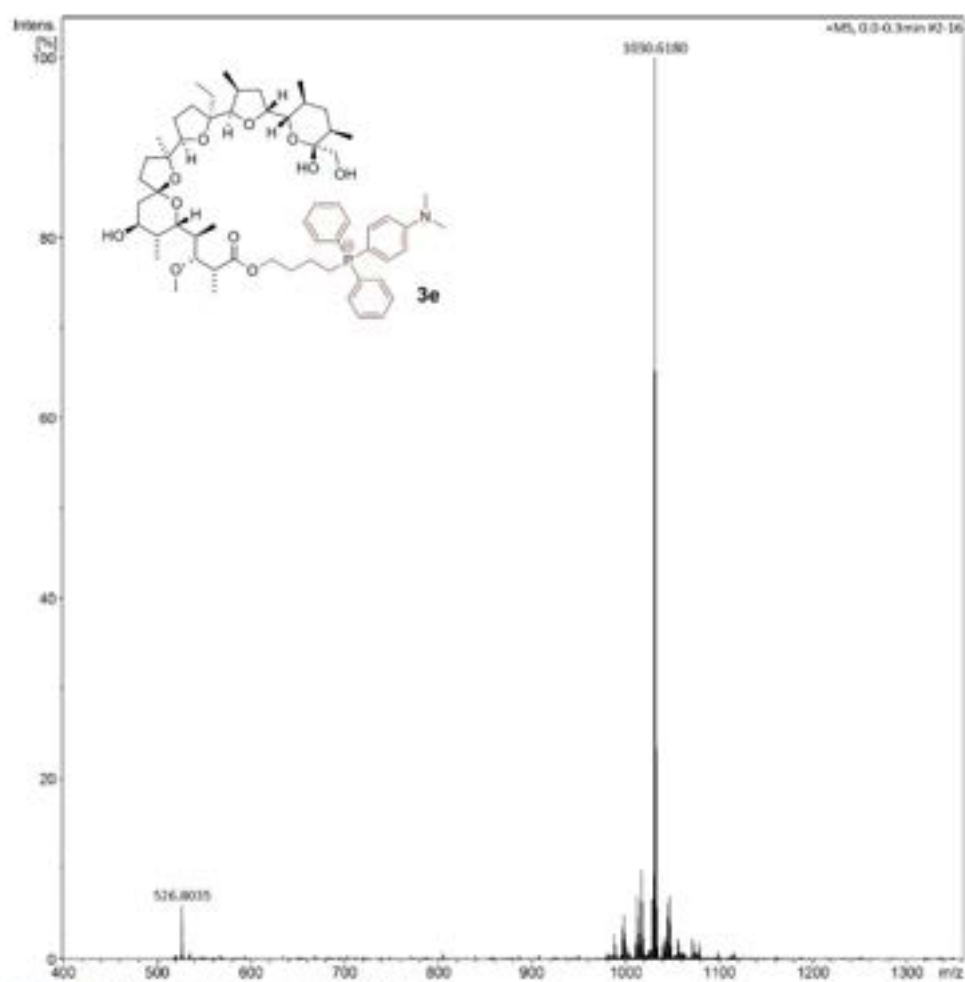
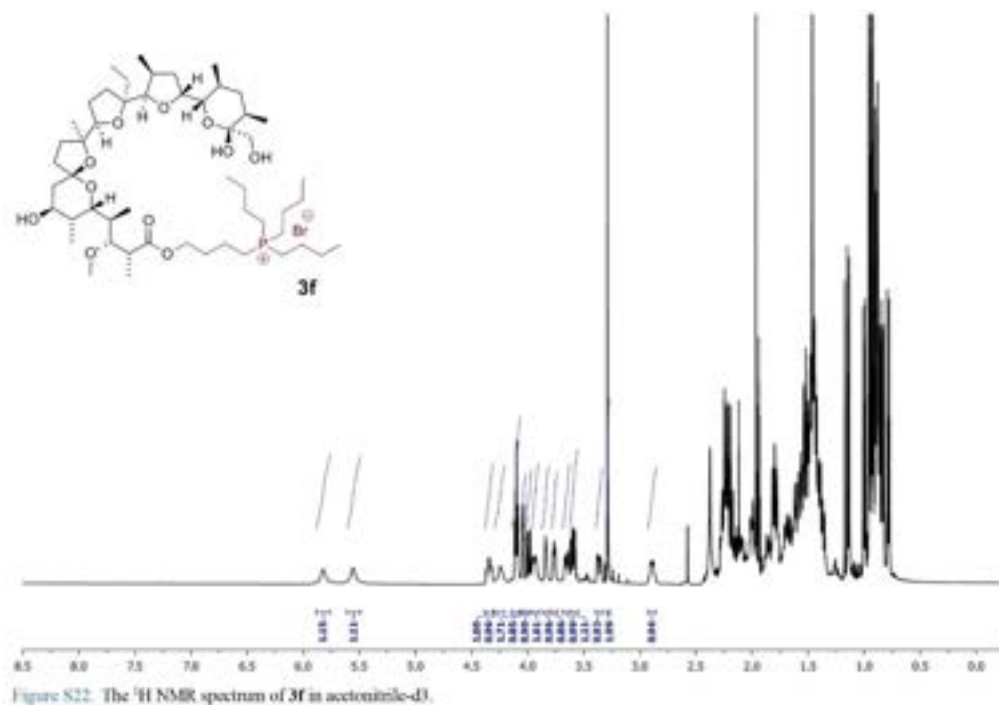
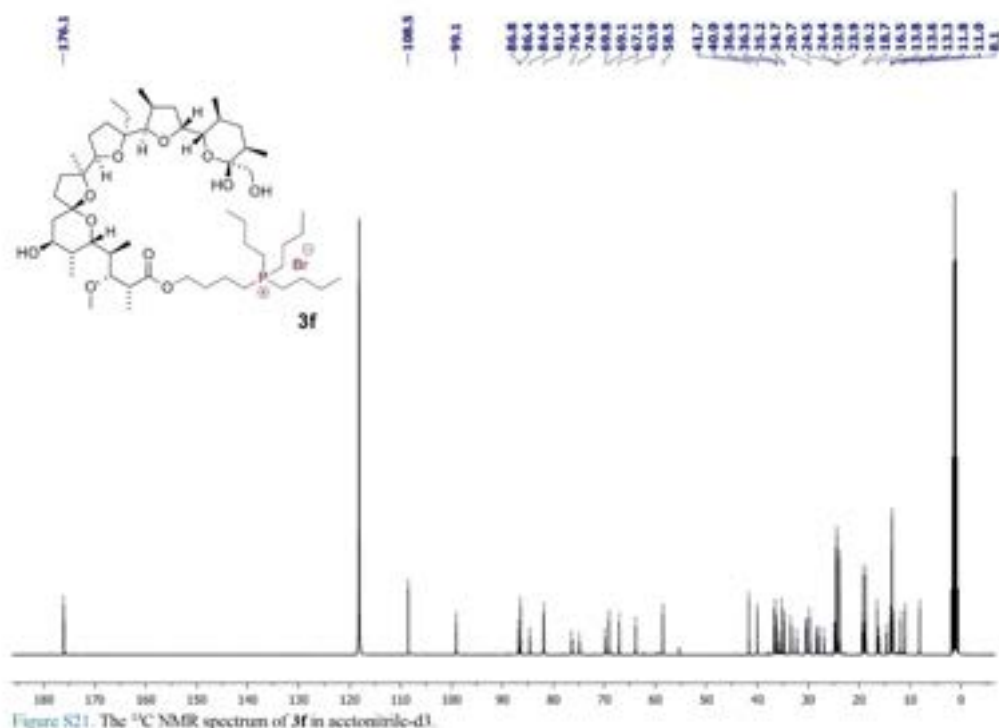


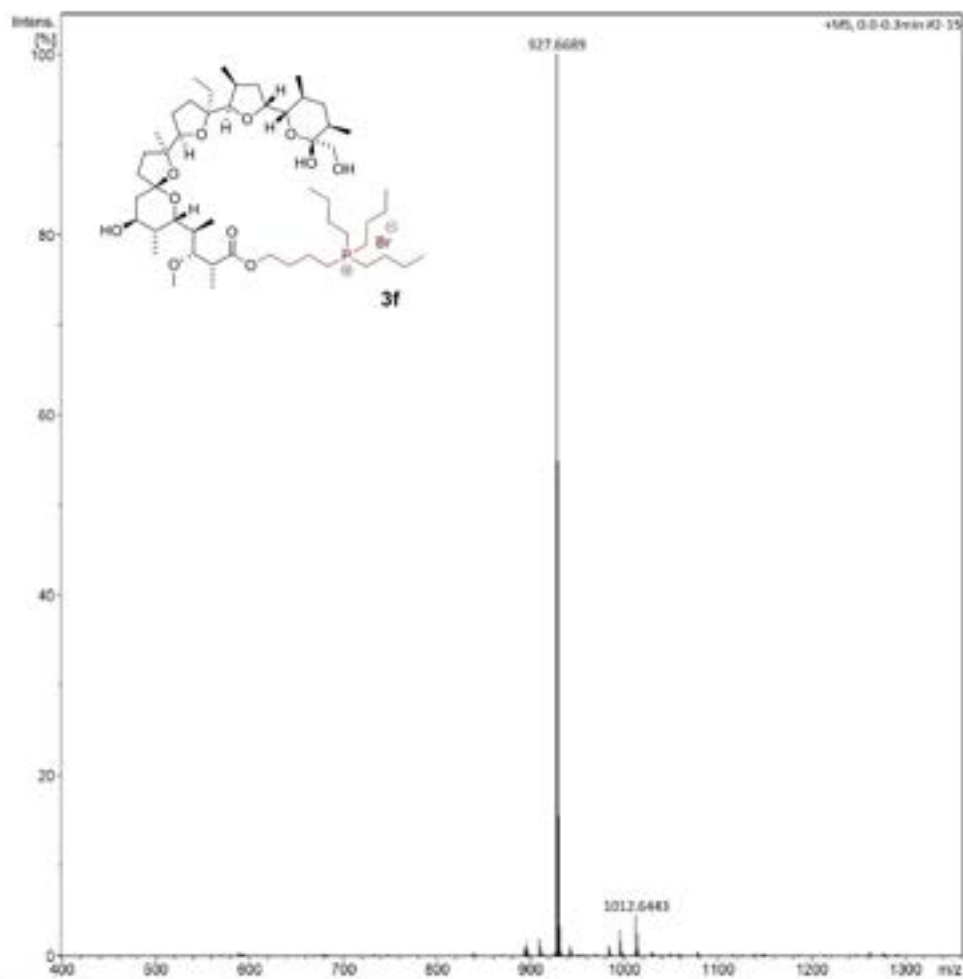
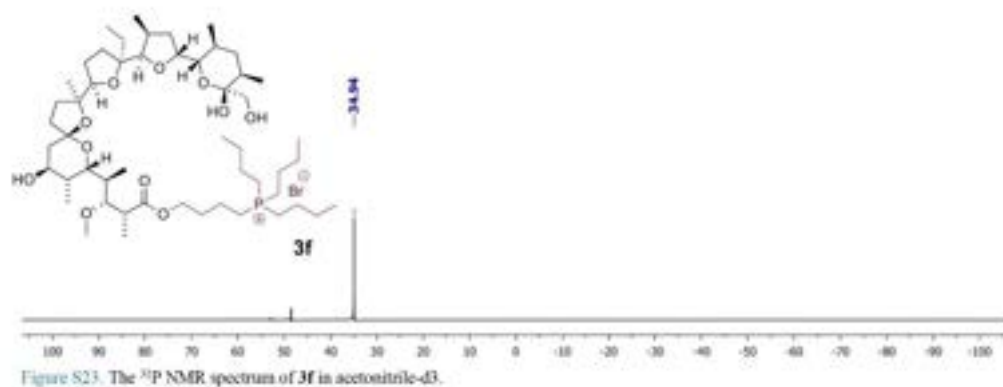
Figure S14. The ¹H NMR spectrum of **3d** in acetonitrile-d₃.

Figure S15. The ^{31}P NMR spectrum of **3d** in acetonitrile- d_3 .Figure S16. The ESI high resolution mass spectrum of **3d**.



Figure S19. The ^{31}P NMR spectrum of **3e** in acetonitrile- d_3 .Figure S20. The ESI high resolution mass spectrum of **3e**.







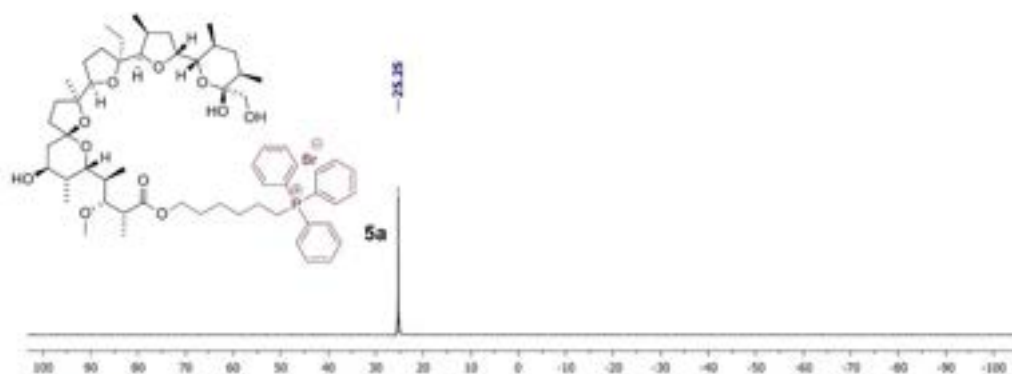


Figure S27. The ^{31}P NMR spectrum of **5a** in acetonitrile- d_3 .

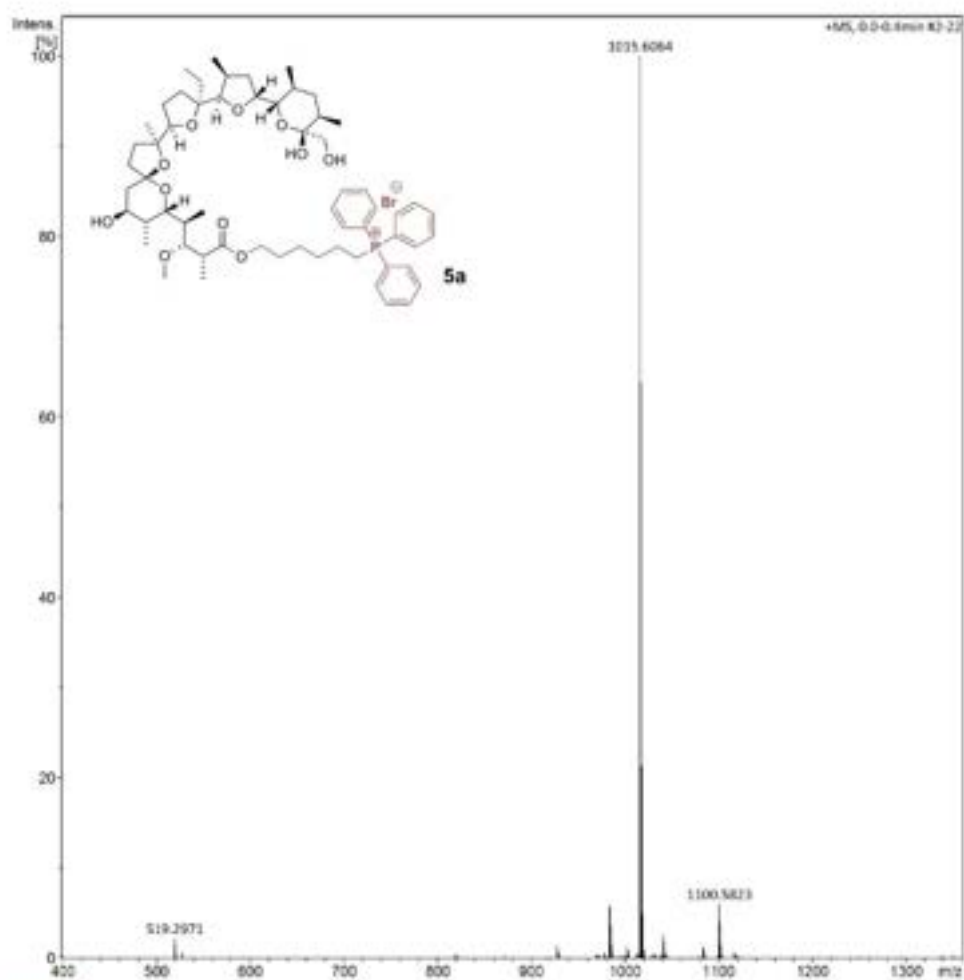
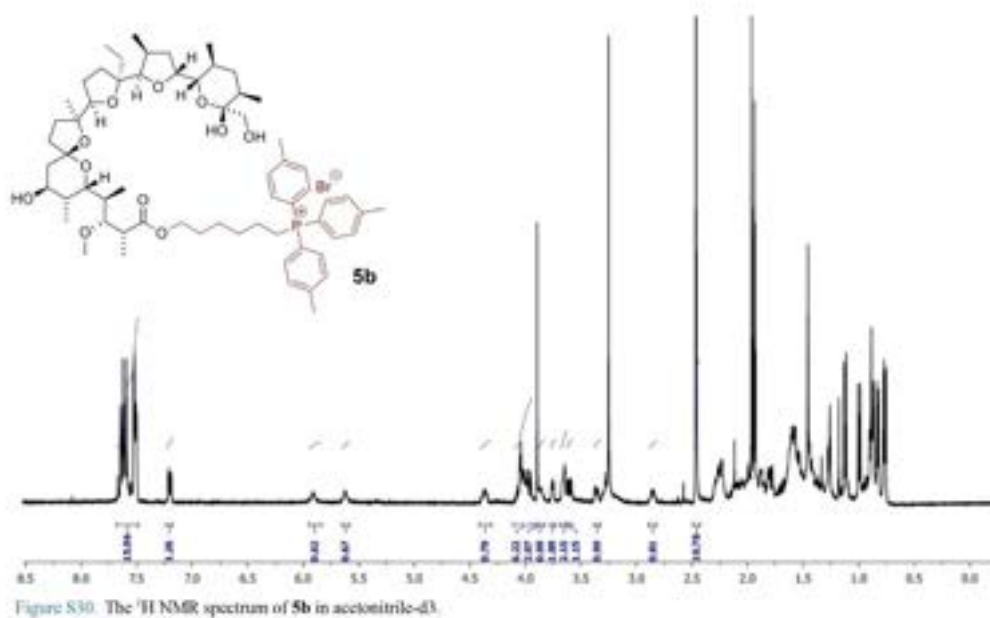
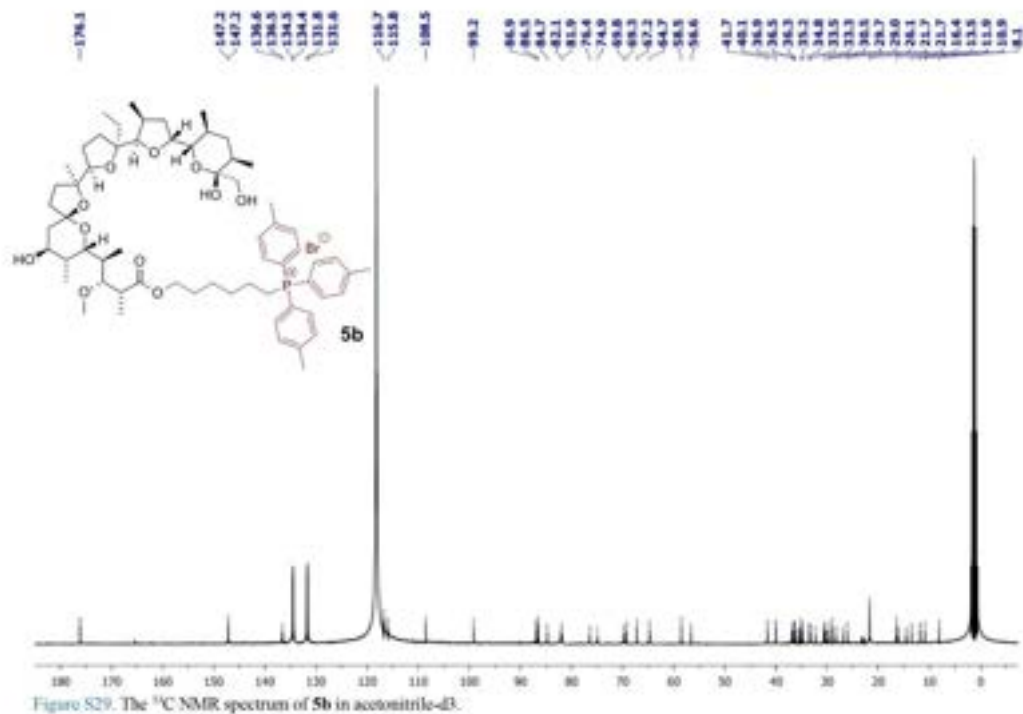


Figure S28. The ESI high resolution mass spectrum of **5a**.



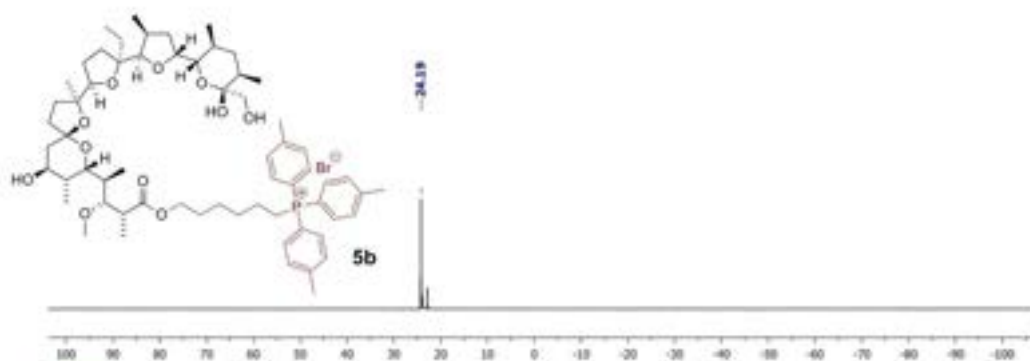


Figure S31. The ^{31}P NMR spectrum of **5b** in acetonitrile- d_3 .

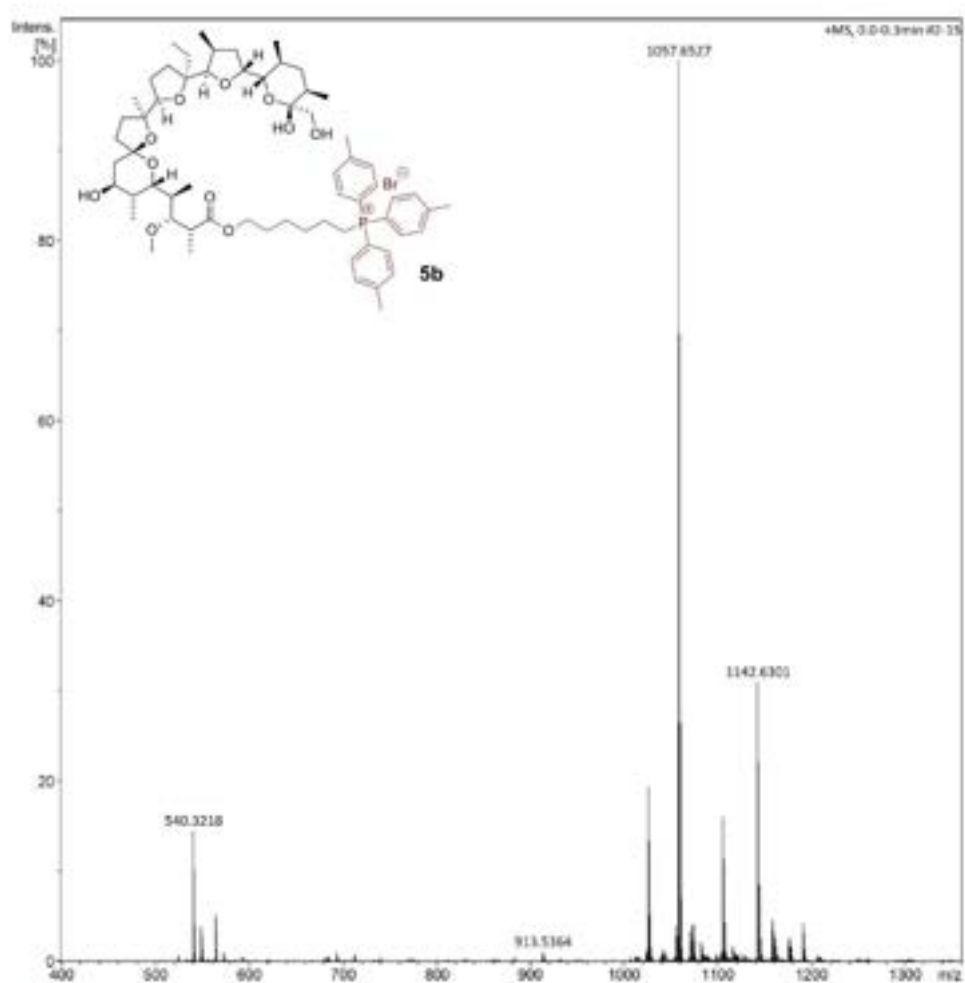


Figure S32. The ESI high resolution mass spectrum of **5b**.

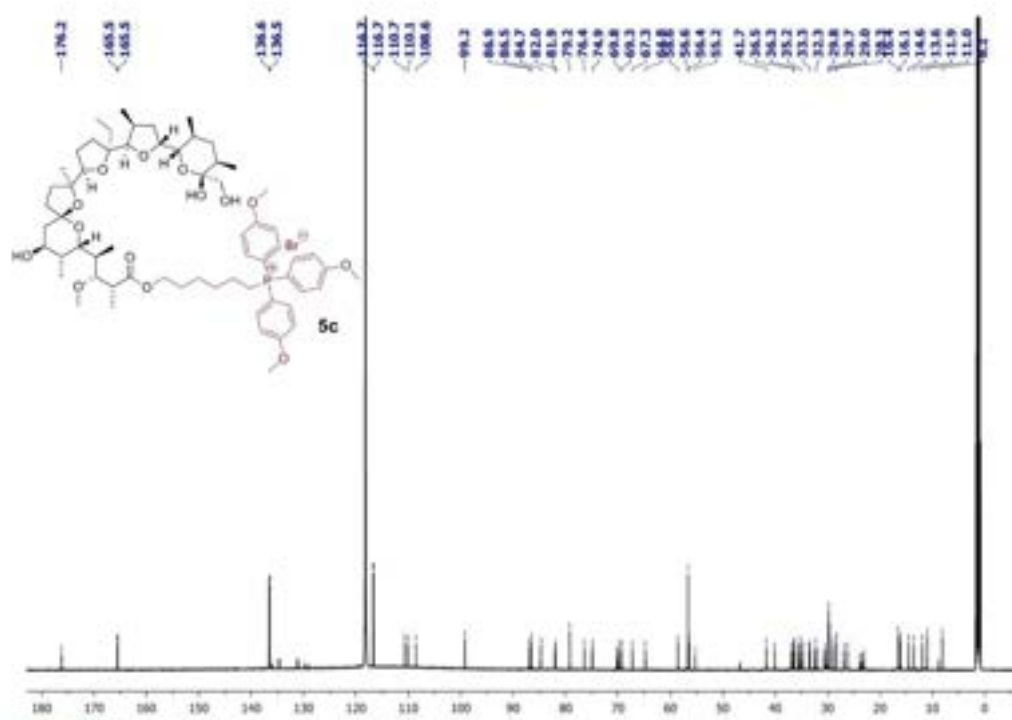


Figure S33. The ^{13}C NMR spectrum of **5c** in acetonitrile- d_3 .

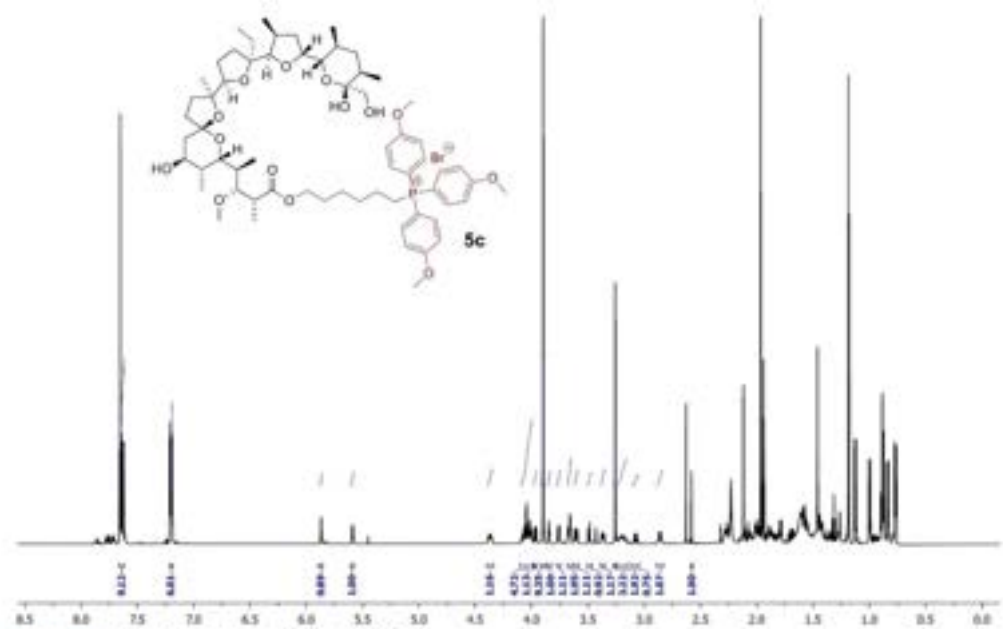
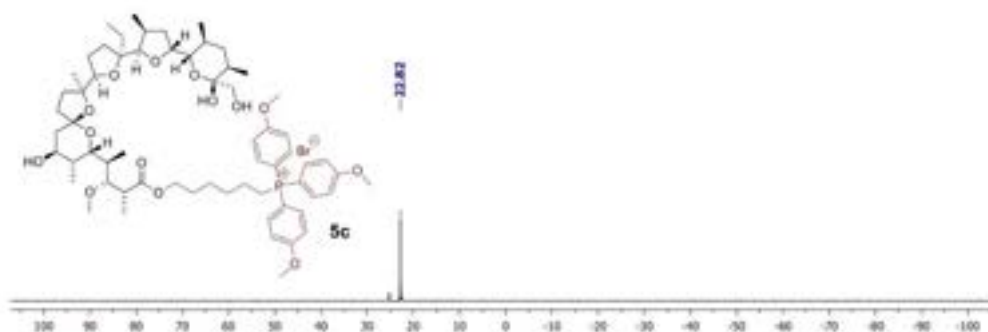
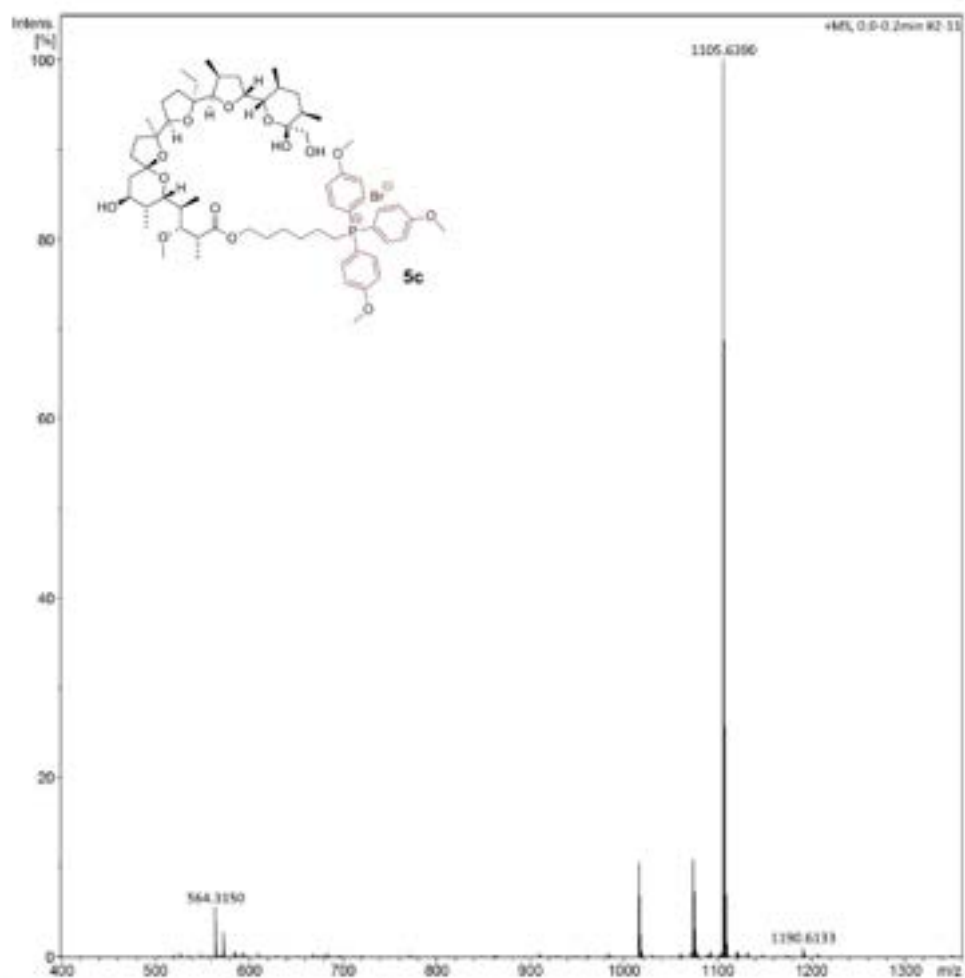


Figure S34. The ^1H NMR spectrum of **5c** in acetonitrile- d_3 .

Figure S35. The ^{31}P NMR spectrum of **5c** in acetonitrile- d_3 .Figure S36. The ESI high resolution mass spectrum of **5c**.

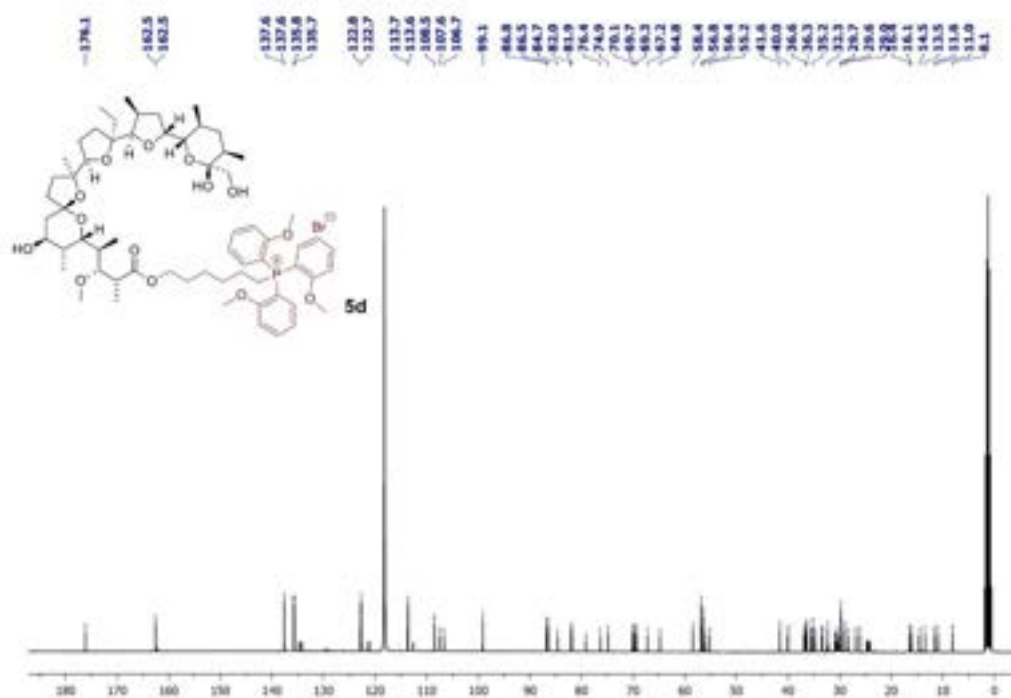


Figure S37. The ¹³C NMR spectrum of **5d** in acetonitrile-d₃.

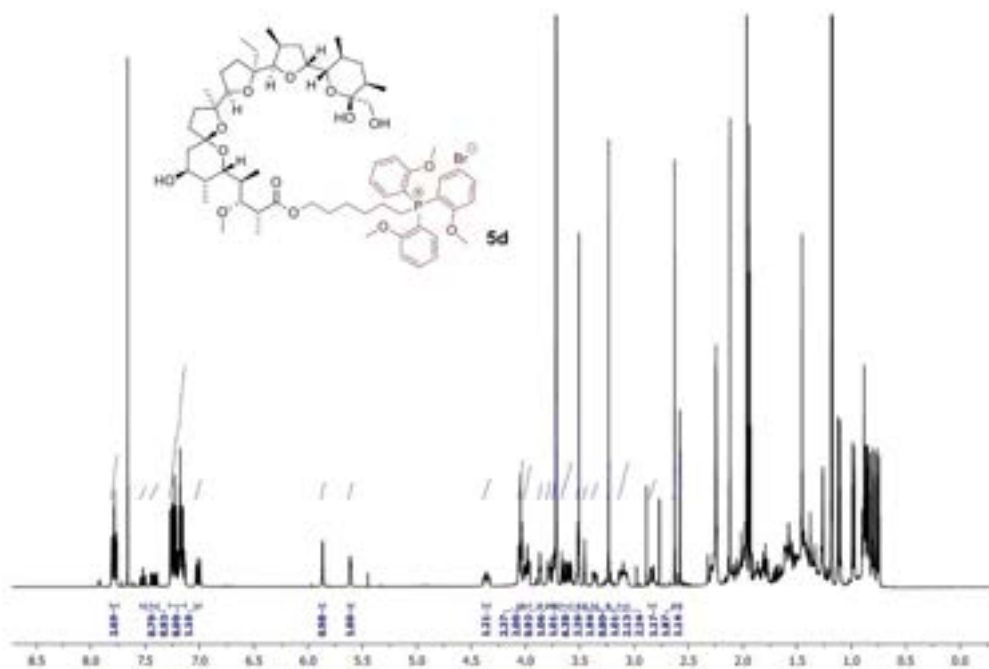
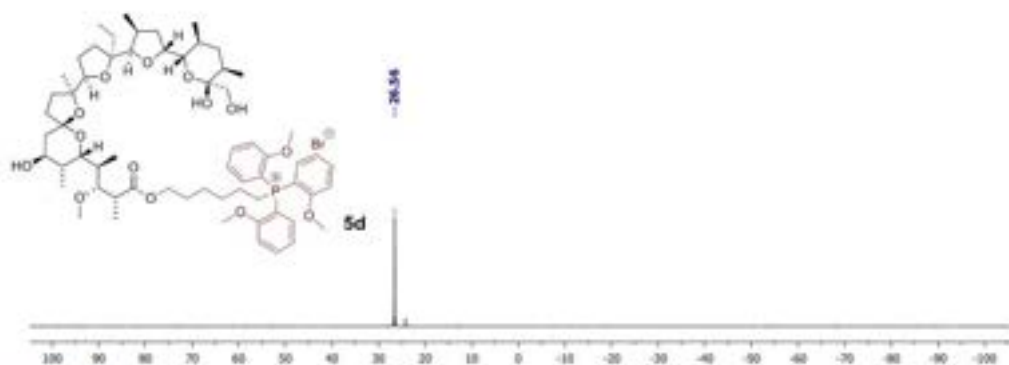
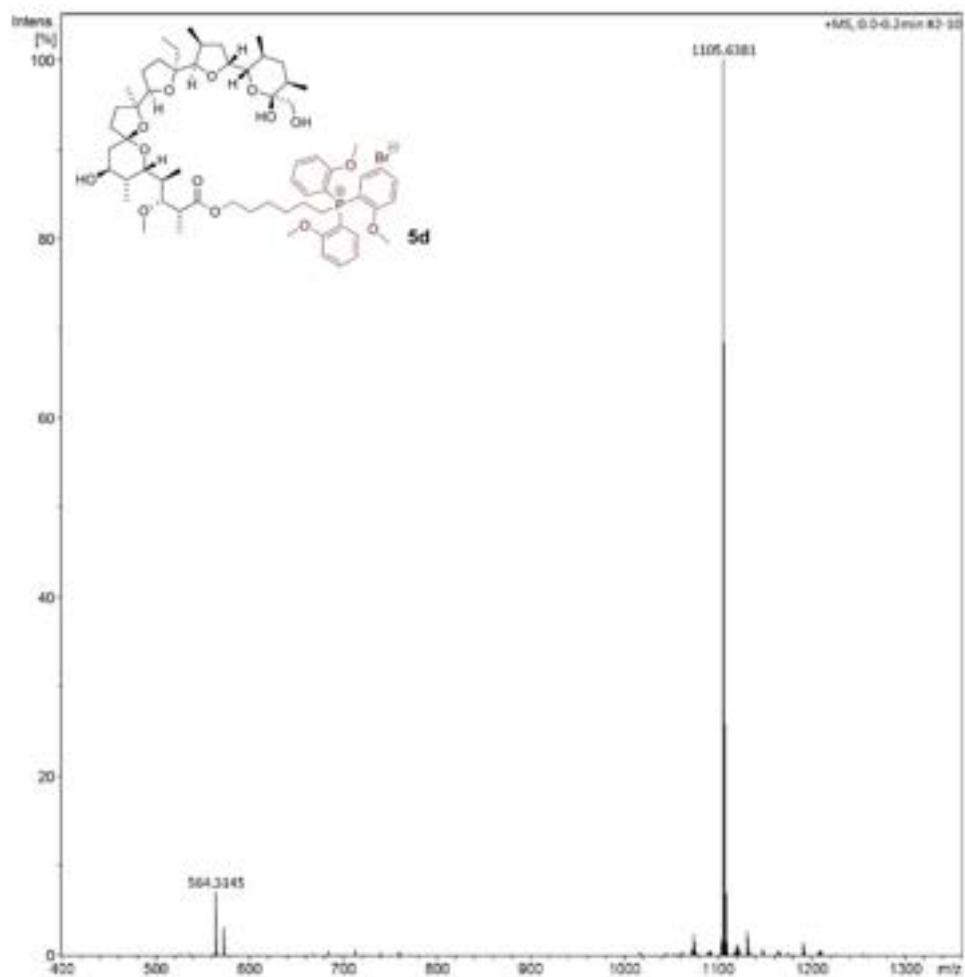


Figure S38. The ¹H NMR spectrum of **5d** in acetonitrile-d₃.

Figure S39. The ^{31}P NMR spectrum of **5d** in acetonitrile- d_3 .Figure S40. The ESI high resolution mass spectrum of **5d**.

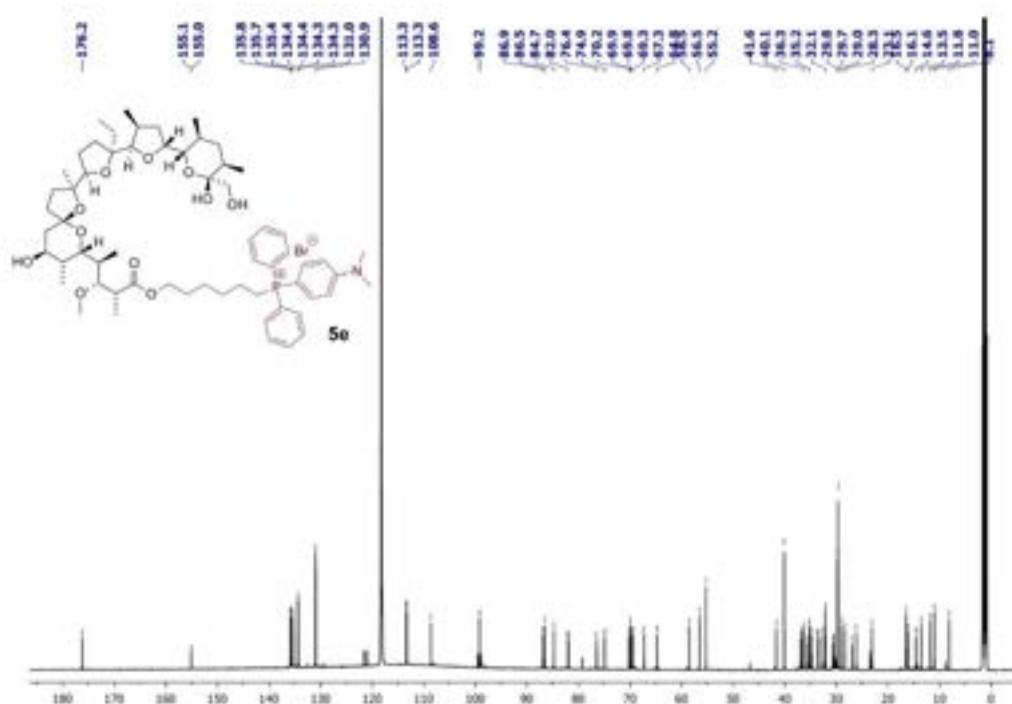


Figure S41. The ^{13}C NMR spectrum of **5e** in acetonitrile- d_3 .

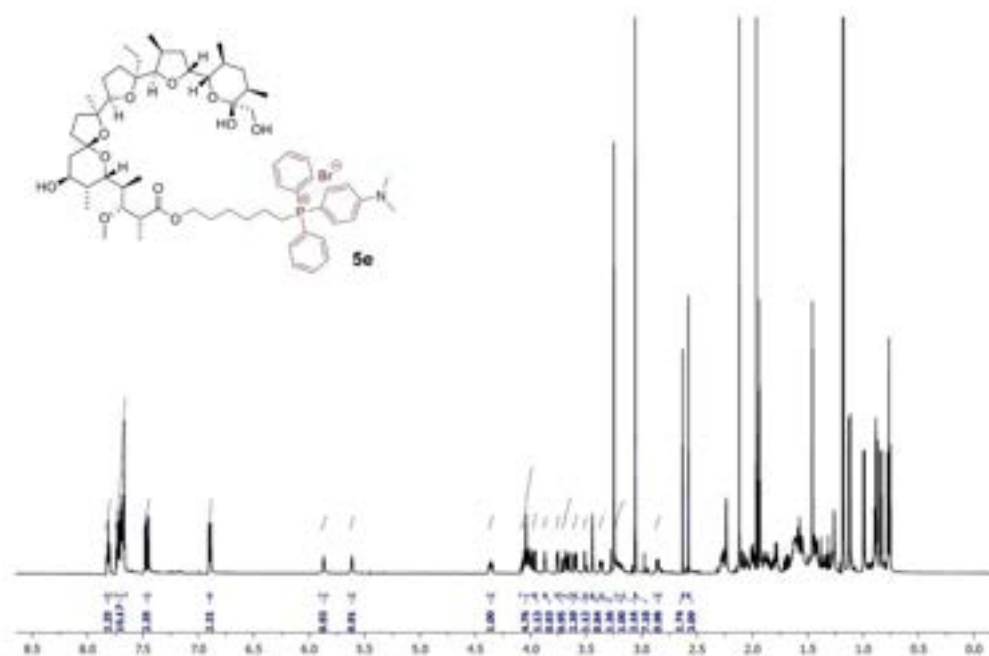


Figure S42. The ^1H NMR spectrum of **5e** in acetonitrile- d_3 .

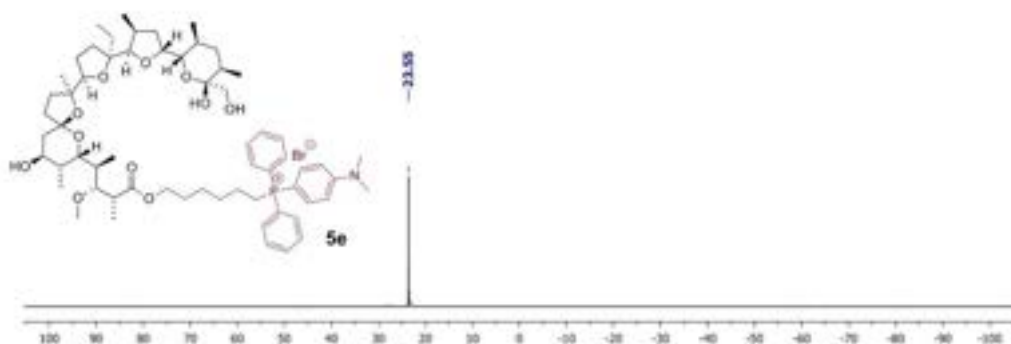


Figure S43. The ^{31}P NMR spectrum of **5e** in acetonitrile- d_3 .

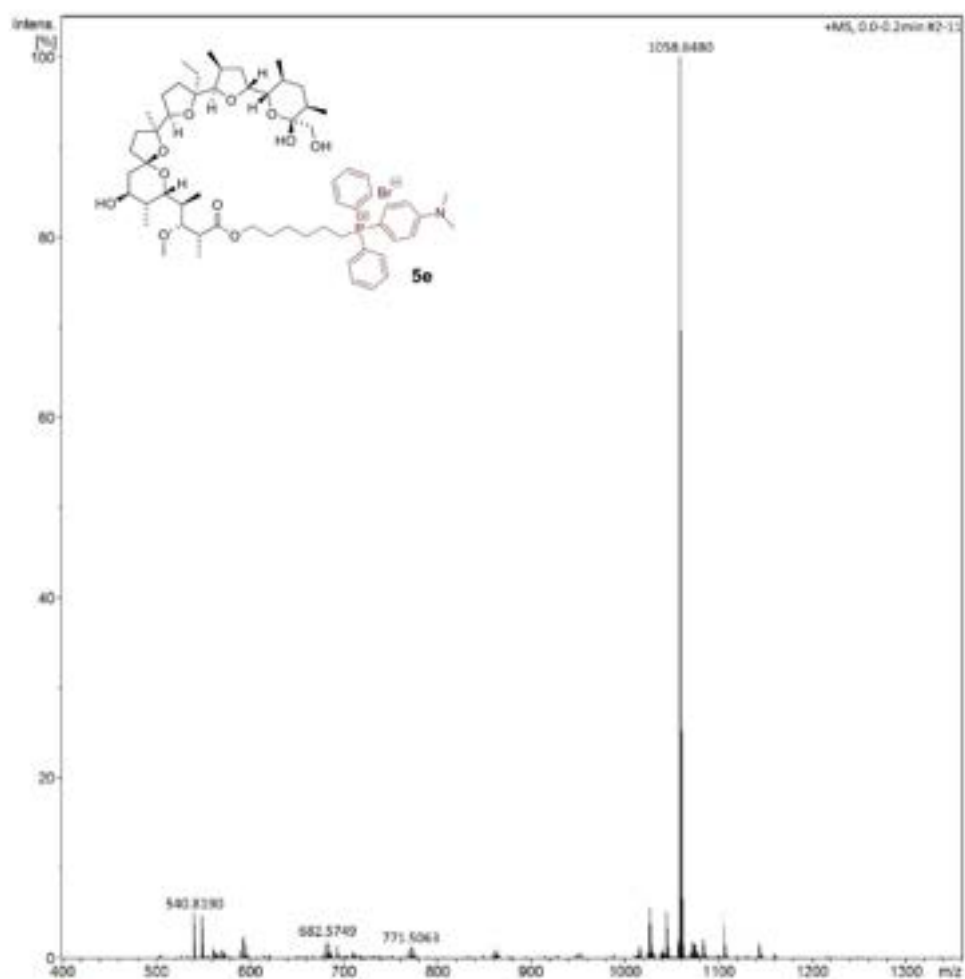
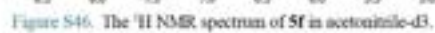
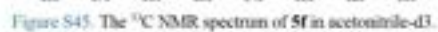
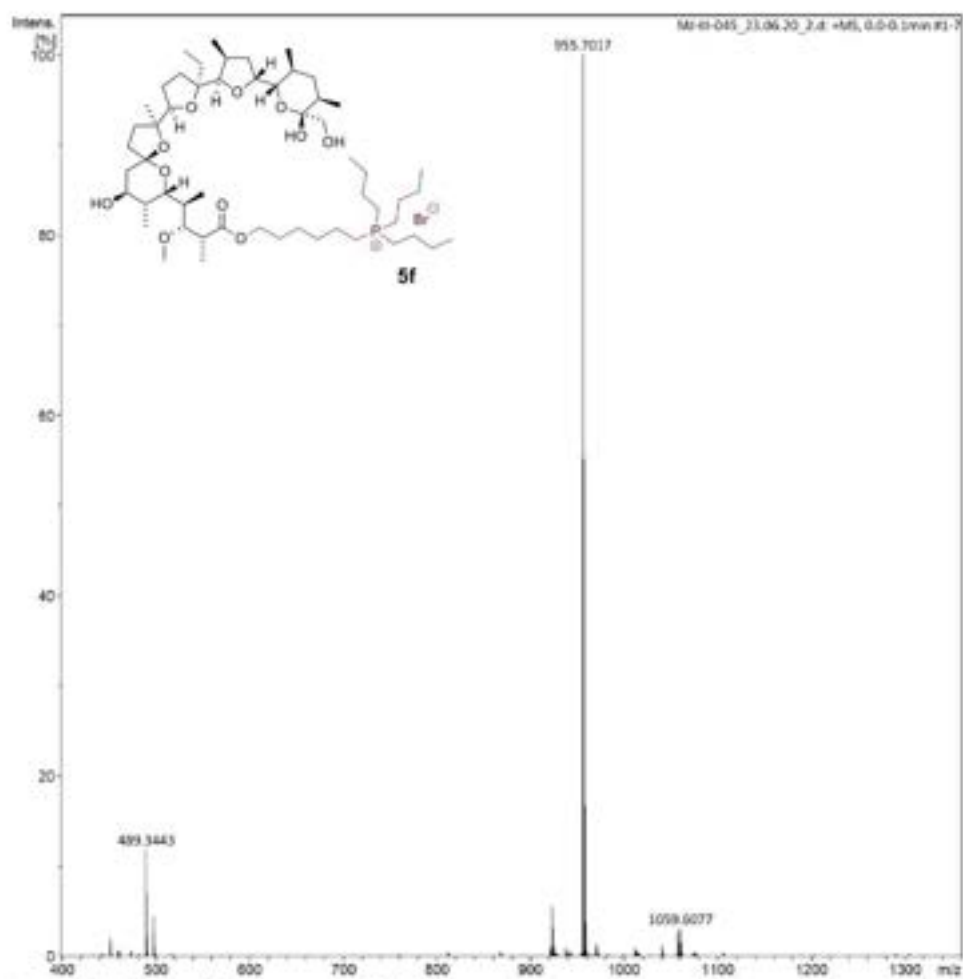
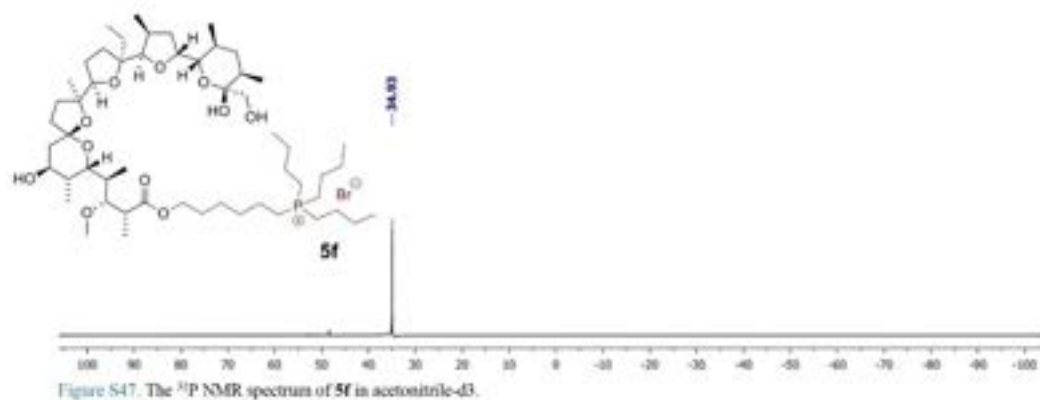


Figure S44. The ESI high resolution mass spectrum of **5e**.





11.6. Publikacja VI

Michał Sulik, Robert Graniczny, Jan Janczak, Dagmara Kłopotowska,
Joanna Wietrzyk, Adam Huczyński

*From Pseudocyclic to Macrocyclic Ionophores: Strategies toward the Synthesis of Cyclic
Monensin Derivatives*

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Opis wkładu doktoranta w powstanie publikacji: Jestem odpowiedzialny za opracowanie metodologii syntezy oraz oczyszczania azydowej oraz aminowej pochodnej monenzyny. Pełniłem rolę opiekuna pracy laboratoryjnej lic. Roberta Granicznego, który przeprowadził syntezę cyklicznych pochodnych monenzyny. Odpowiadałem za koordynowanie badań w tym kontakt z innymi naukowcami. Przeprowadziłem też analizę otrzymanych wyników, w tym tych z badań biologicznych. Odpowiadałem za napisanie manuskryptu.

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From Pseudocyclic to Macrocyclic Ionophores: Strategies toward the Synthesis of Cyclic Monensin Derivatives

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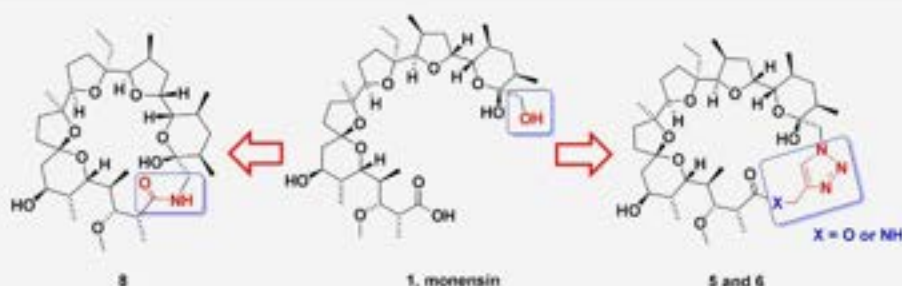
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ABSTRACT: There has been a long search for a simple preparation of new cyclic analogues of ionophore antibiotics. We report a simple and general synthesis of three new cyclic derivatives of polyether ionophore, monensin A (MON). The application of the Huisgen 1,3-dipolar cycloaddition of azides and terminal alkynes to macrocyclization results in a concise, synthetic route to monensin lacton or lactam in only 4 steps. Additionally, macrolactamization by a simple amidation reaction using HATU, a commonly used conjugating agent, gives 72% yields and utilizes neither high dilution techniques nor template effects in the cyclization step. This in turn enables ready access to a range of unnatural MON analogues, showing the ability to form complexes with monovalent and divalent metal cations.

INTRODUCTION

Among the numerous drugs used in modern medicine, many of them belong to the broad group of macrocyclic compounds.^{1,2} There are many examples of natural macrocycles, including erythromycin—a macrolide antibiotic used for the treatment of a number of bacterial infections, ivermectin—a macrocyclic lactone used in the treatment of many parasitic diseases, and valinomycin—dodecadepsipeptide being a representative of natural, cyclic ionophores (Figure 1).^{3–5}

In 1967, Pressman et al. proved that valinomycin was capable of coordinating potassium cations and transporting them across biological membranes.⁶ This discovery is considered to be the beginning of chemistry of ionophores which has been a very intensively studied group of compounds ever since. To date, hundreds of ionophores have been described.^{9,10} Their ability to complex metal cations makes them useful in analytical chemistry as components of ion-selective electrodes, and in organic synthesis as a phase transfer catalysts.^{11,12}

Moreover, due to the ability to transport metal cations across biological membranes, these compounds exhibit a wide spectrum of biological activities, including antibacterial, antiparasitic, and anticancer ones.^{13,14} Among the ionophores that are currently of greatest interest in medicinal chemistry,

attention should be paid to the group of noncyclic carboxylic ionophores. These compounds, whose main representative is monensin A (MON), demonstrate the ability to imitate cyclic ionophores due to the presence of intramolecular hydrogen bonds in their structure (Figure 2).^{15,16}

The pseudocyclic structure facilitates MON coordination of metal cations (with the highest selectivity toward the sodium cation) and transport them across cell membranes, which leads to the disturbance in the intracellular pH and ion concentration gradient, and subsequently to mitochondrial damage, vacuolization, and cell death (Figure 2).^{15,16}

To date, many MON derivatives have been synthesized by the chemical modification of its C1 carboxyl group or the hydroxyl group attached to the C26 carbon atom.^{17–23} However, an interesting and still undeveloped research direction is the cyclization of the MON molecule, which

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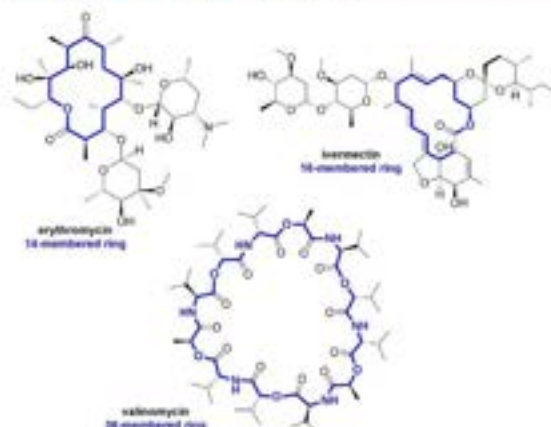


Figure 1. Chemical structures of erythromycin, ivermectin, and valinomycin.

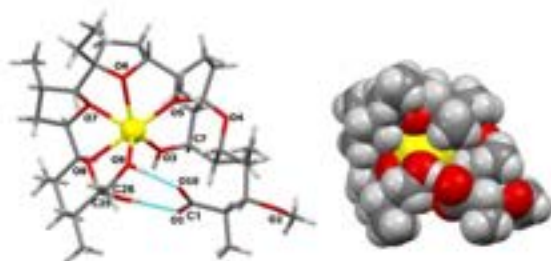


Figure 2. Wireframe representation and space-filling presentation of the crystal structure of monensin A sodium salt (This figure was reproduced using data downloaded from the Cambridge Crystallographic Data Centre CCDC: 620458).

should allow the transformation of its pseudocyclic skeleton into a macrocyclic structure, leading to a change in its structural properties, including the number of rotatable bonds and polar surface area.⁶ Derivatives of this type have already been obtained applying the Corey–Nicolaou macrolactonization reaction using 2,2'-dipyridyl disulfide and triphenylphosphine on unmodified MON or its amides with chiral amino acids.^{15,21,24–26}

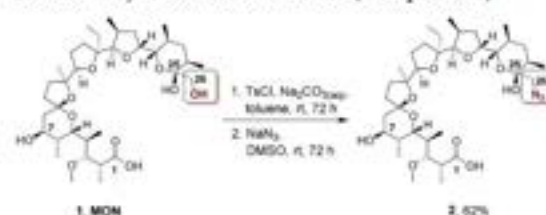
It is worth noting that historically MON is one of the first natural compounds ever to be macrocyclized.

Noteworthy, all macrocyclic MON derivatives synthesized so far belong to the group of lactones. It should be noted that under physiological conditions, esters can undergo hydrolysis due to the presence of esterases.²⁷ Therefore, we decided to synthesize more stable macrocyclic MON derivatives by applying an amide bond and/or a 1,2,3-triazole ring.

RESULTS AND DISCUSSION

To the best of our knowledge, despite many modifications performed on the C26 hydroxyl group of MON, its conversion into an azide group has not been established yet. After conducting a variety of chemical reactions, we present the first scalable, efficient, and simple method for obtaining C26 azide of MON (Scheme 1). The first step involves the tosylation of the C26-hydroxyl group of MON, and then the resulting tosylate is directly subjected to the nucleophilic substitution reaction with the excess of sodium azide (Scheme 1). The

Scheme 1. Synthesis of MON Azide (Compound 2)¹²



¹²Reaction conditions and yield are indicated

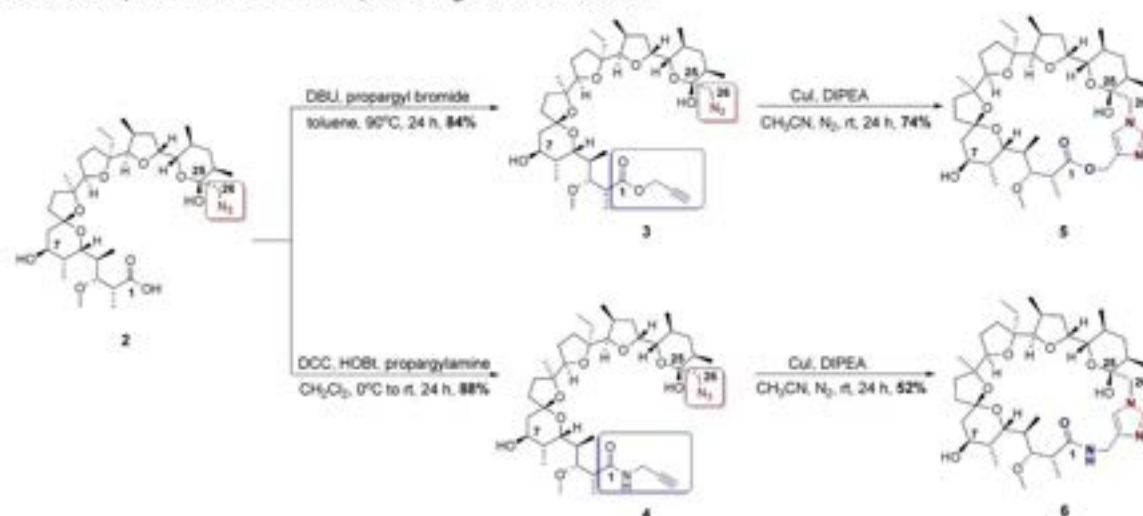
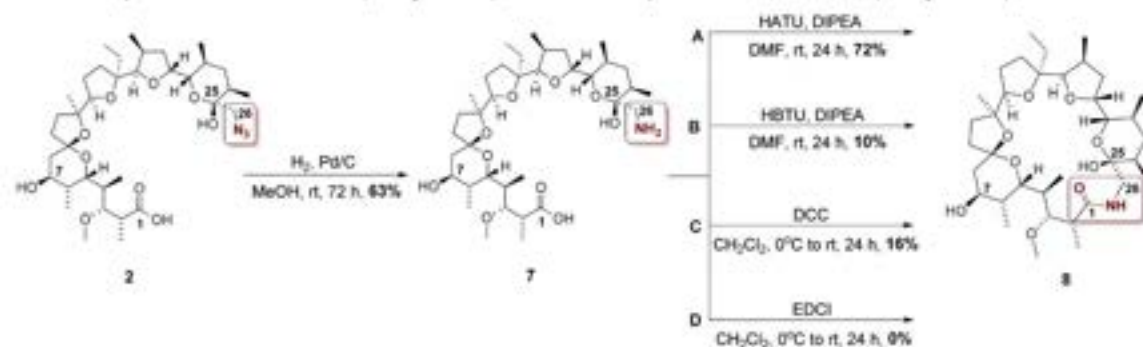
obtained C26-azide of MON (compound 2) was purified chromatographically. Having access to the azide functionality (compound 2), an application of Nobel prize-honored distinction Meldal and Sharpless click reaction in the synthesis of new macrocyclic MON derivatives was a natural step. The 1,2,3-triazole linker is a very attractive bioisostere in medicinal chemistry, which both structurally and electronically mimics the amide bond.^{28,29}

In the context of chemical modification of ionophores by click chemistry, the most suitable and efficient method is the application of Meldal CuAAC reaction, which involves the use of copper(I) iodide.^{17,30} To conduct the intramolecular click reaction, which will enable the formation of the macrocyclic system, it was necessary to introduce a triple bond into the structure of compound 2. We decided to synthesize both, the propargyl amide and ester of MON azide (Scheme 2). Enzymatic stability may have a crucial effect on the biological, and structural properties of the obtained macrocycles, thus this distinction will allow us to conclude whether there is a correlation between the structure, and the activity of obtained analogues (structure–activity relationship, SAR).

The obtained precursors (ester 3 and amide 4) were dissolved in acetonitrile, then DIPEA and copper(I) iodide were added. The reactions were carried out in an inert gas atmosphere to avoid oxidation of copper(I) iodide. Both click reactions had to be performed using a large amount of solvent (high dilution) to reduce the risk of an intermolecular Meldal reaction leading to polymerization. When the reaction was conducted with a high substrate concentration, a precipitate was formed. It was insoluble in most organic and inorganic solvents. The use of high dilution conditions allowed us to eliminate the formation of this polymeric byproduct. Both products were purified chromatographically, with a yield of 52% (for 6) and 74% (for 5), respectively (Scheme 2).

In the next step we decided to perform macrocyclization by introducing an amide bond. However, the method of synthesis of the C26 amine of MON has not been described so far. In order to obtain the amine, we decided to carry out the reduction of the azide group of 2. It is worth noting that this compound does not have multiple bonds in its structure, nor other functional groups sensitive to reduction in the presence of hydrogen, so this reductive reagent can be successfully used (Scheme 3). The resulting amine (compound 7) was purified chromatographically and used as a substrate in the macrocyclization reaction.

Compound 7, which has both a carboxyl and an amino group in its structure, can be treated as an amino acid. Therefore, the reagents used for peptide synthesis can be applied for the synthesis of the macrocyclic lactam of MON. For this purpose we selected the four most commonly used

Scheme 2. Synthesis of MON Macrocycles Using the Click Reaction^a^aReaction conditions and yields are indicatedScheme 3. Synthesis of MON Amine (Compound 7) and Its Macrocyclic Lactam Derivative (Compound 8)^a^aReaction conditions and yields are indicated

conjugating agents: HATU, HBTU, DCC, and EDCI (Scheme 3). As can be noted, the most effective method of macrolactamization was the use of HATU on the starting amino acid 7 (Scheme 3). The yield of this reaction (72%) was several times higher than in the case of other reagents used. Interestingly, the formation of the desired product 8 was not observed in the reaction with EDCI (Scheme 3).

The structure and purity of the obtained MON derivatives were determined by spectroscopic (NMR, FT-IR) and spectrometric (ESI-HRMS and ESI-MS) methods. In addition, compounds 2, 5, and 8 were obtained in crystalline form, which enabled scXRD analysis. In the ¹³C NMR spectra of MON macrocycles, the signals of the highest analytical significance were assigned to the carbonyl group of the ester/amide moiety at position C1. These signals appeared in the range of 175.5–178.3 ppm. Moreover, for compounds 5 and 6 we observed the appearance of a signal from the quaternary carbon atom of the triazole ring, in the range of 142.9–144.9 ppm. In the ¹H NMR spectra of lactams 6 and 8, we observed a diffuse signal from the amide group in the range of 6.50–7.41 ppm. In addition, for click derivatives

(compounds 5 and 6), a characteristic, intensive singlet appeared, originating from the hydrogen atom of the triazole ring in the range of 7.84–7.90 ppm. Furthermore, for compounds of pseudocyclic nature (compounds 2, 3, 4, and 7), the diagnostic signals are two characteristic doublets originating from the methylene group at the C26 position. We observed them in the range of 3.05–3.08 ppm, and 3.15–3.48 ppm, respectively. In the FT-IR spectra, the most distinctive signals originate from the stretching vibrations of the azide group (ν_{N_3}), which for the azide derivatives 3 and 4 appeared at 2102 cm⁻¹. Importantly, for the click reaction products, the signals at this value disappeared, which confirms that the reaction occurred in the expected direction. Moreover, for esters (compounds 3, and 5) the characteristic signal from the stretching vibrations of the ester carbonyl group appears in the narrow range of 1741–1744 cm⁻¹. The signal originating from the stretching vibrations of the amide carbonyl group (compounds 4, 6, and 8) appears in the range of 1641–1657 cm⁻¹. The detailed synthesis procedures, as well as NMR, ESI-MS, and FT-IR spectra of the obtained macrocyclic

derivatives, are included in the Supporting Information (Figures S1–S31).

Confirmation of the structure of the obtained derivatives and explanation of their structural properties is possible on the basis of the X-ray diffraction analysis. Thus, three of the obtained derivatives—MON azide (compound 2), macrocyclic lactone (compound 5), and macrocyclic lactam (compound 8) were characterized using the X-ray single crystal diffraction method. Therefore, we were able to compare the structural properties of the pseudocyclic MON derivative with those of macrocyclic derivatives. Detailed crystal data, refinement parameters and experimental details for all molecules are presented in Table S1 and Figures S32–S37 (Supporting Information). Single crystals of compound 2 were grown by crystallization in acetonitrile solution. The azide crystallized in the noncentrosymmetric space group $P2_1$ of the monoclinic system, as a solvate with the water molecule. The asymmetric unit of the compound 2–water complex is illustrated in Figure 3.

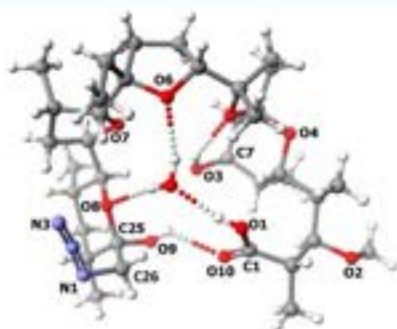


Figure 3. Crystal structure of the azide 2–water complex.

The molecule of compound 2 in the crystal exhibits pseudocyclic conformation, with the azide substituent directed outside the polar pocket of the molecule. The structure is stabilized by several hydrogen bonds, whose exact geometry is described in the Supporting Information (Table S2). An example is the hydrogen bond O9–H9A···O10 formed between a carboxyl group and a hydroxyl group attached to the C25 carbon atom (Figure 3). Noteworthy, the water molecule stabilizes the pseudocyclic structure of compound 2 by forming additional hydrogen bonds with the oxygen atoms O6 and O8 (as donor) and the oxygen atom O1 (as acceptor) (Figure 3). The atom numbering and additional structural aspects are presented in the Supporting Information Figure S32. Single crystals of compound 5 were grown by crystallization in acetonitrile solution with the addition of NaClO_4 (1.25 equiv. according to compound 5). The lactone crystallized in the noncentrosymmetric space group $P2_1$ of the monoclinic system, as a complex with a sodium cation and a perchlorate as a counterion, and as a solvate with the acetonitrile molecule. The asymmetric unit of the compound 5–sodium perchlorate complex is illustrated in Figure 4a.

The scXRD analysis confirmed the cyclic structure of the compound. It is worth noting that the nitrogen atoms of the triazole ring are directed outside the polar pocket of the molecule and do not participate in coordinating the sodium cation, which may be a natural consequence of the conformation of the azido group in compound 2. The

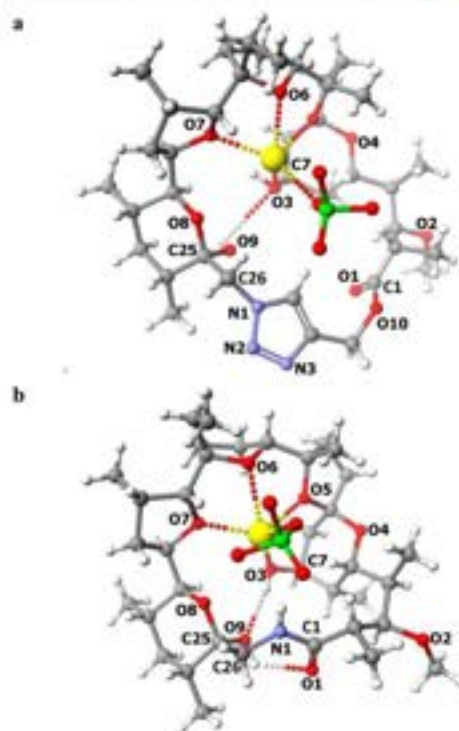


Figure 4. Crystal structure of: (a) sodium perchlorate–monensin lactone 5 complex, (b) sodium perchlorate–monensin lactam 8 complex.

structure is stabilized by a few hydrogen bonds, mainly O3–H3···O9 formed between the hydroxyl group attached to C25 carbon atom and the hydroxyl group attached to C7 carbon atom (Figure 4a). The hydroxyl group O9–H9 additionally forms hydrogen bonds with the perchlorate ion. The exact geometry of hydrogen bonds is described in the Supporting Information (Table S3). In addition, the perchlorate ion complements the coordination space of the sodium cation, thus stabilizing the resulting complex.

A significant change in structural properties is observed in compound 8, which is a macrocyclic lactam of MON. Single crystals of compound 8 were grown by crystallization in acetonitrile solution with the addition of NaClO_4 (1.25 equiv. according to compound 8). The lactam crystallized in the noncentrosymmetric space group $P2_12_12_1$ of the orthorhombic system, as a complex with a sodium cation and a perchlorate as a counterion. The asymmetric unit of compound 8–sodium perchlorate complex is illustrated in Figure 4b.

The molecule of compound 8 belongs to the group of macrocyclic compounds. The structure is additionally stabilized by hydrogen bonds, however, their number is much smaller than in the case of the pseudocyclic derivative 2. The exact geometry of hydrogen bonds is described in the Supporting Information (Table S4). Except for the O3–H3···O9 hydrogen bond formed between the hydroxyl group attached to C25 carbon atom and the hydroxyl group attached to C7 carbon atom, all the other hydrogen bonds are formed by the amide group being the donor. Interestingly, no hydrogen bonds are formed with the perchlorate ion, acting

Table 1. Antiproliferative Activity (IC_{50}) (μM) and the Calculated Values of the Selectivity (SI) and Resistance (RI) Indices of MON, Its Macrocylic Derivatives, and Reference Drugs

compound	cancer cells									normal cells
	A549		MCF-7		LoVo		LoVo/DX		RI ^b	BALB/3T3
	IC_{50}	SI ^a	IC_{50}	SI ^a	IC_{50}	SI ^a	IC_{50}	SI ^a		
monensin, 1	0.36 ± 0.14	6.1	0.53 ± 0.03	4.2	0.36 ± 0.11	6.1	0.17 ± 0.04	13.0	0.5	2.21 ± 0.35
5	7.32 ± 0.59	1.9	6.32 ± 0.79	2.2	4.46 ± 0.48	3.0	2.19 ± 0.57	6.2	0.5	13.6 ± 5.9
6	15.5 ± 2.9	1.5	12.2 ± 1.6	2.0	12.0 ± 2.9	2.0	4.14 ± 1.02	5.8	0.3	23.9 ± 2.7
8	11.2 ± 1.2	2.1	12.6 ± 3.2	1.9	7.36 ± 0.29	3.3	5.21 ± 1.15	4.6	0.7	24.0 ± 1.3
cisplatin	2.32 ± 0.90	1.3	2.42 ± 0.76	1.2	2.53 ± 0.79	1.2	2.4 ± 1.3	1.3	0.9	3.01 ± 1.75
doxorubicin	0.15 ± 0.06	0.3	0.16 ± 0.08	0.3	0.11 ± 0.03	0.5	5.9 ± 2.6	<0.1	53.6	0.05 ± 0.02

^aSelectivity index (SI) was calculated as the ratio of the IC_{50} value for the normal cell line BALB/3T3 to the IC_{50} value of a respective cancer cell line. A SI greater than 1.0 identifies compounds with antiproliferative activity toward cancer cells greater than toxicity against normal cells, while the compounds with SI values greater than 3.0 are considered highly selective.^{17,20,33–35} ^bResistance index (RI) was calculated as the ratio of the IC_{50} value for the LoVo/DX cell line to the IC_{50} value of the LoVo cell line. According to the RI value, the cell lines can be drug-sensitive (RI value in the range of 0–2), moderately drug-sensitive (RI value in the range of 2–10), and strongly drug-resistant (RI value above 10).^{17,20,34}

as the counterion, however, it stabilizes the structure by complementing the coordination space of the sodium cation.

In the FT-IR spectra of compound **8**, we observe a significant decrease in the wavenumber value for the stretching vibrations originating from the amide group after coordination with $NaClO_4$ (1650 cm^{-1} for **8** versus 1641 cm^{-1} for **8** with $NaClO_4$). Since the amide group is not involved in coordinating the sodium cation, it should be concluded that the presence of the molecule in the form of a complex additionally facilitates the formation of numerous hydrogen bonds by the amide group, which leads to the decrease in the wavenumber (Figures S30–S31). The atom numbering and additional structural aspects for compounds **5** and **8** are presented in the Supporting Information Figures S33–S34.

In addition to examining the structural properties of the obtained compounds, their ability to form complexes with metal cations is also important. Thanks to this property, ionophores can be widely used in analytical chemistry and organic catalysis, but also in medicinal chemistry, as compounds displaying potent biological activity. As mentioned above, ionophores form complexes with metal cations and transport them across the cellular lipid membranes, thus killing the cells by dissipating their membrane potential. MON exhibits a high affinity to the sodium cation, but this does not necessarily apply to its macrocyclic derivatives. The coordination space of these compounds is different from that of MON, which may cause a significant change in selectivity in coordinating metal cations. For this purpose, coordination assays were performed for MON macrocyclic derivatives **5**, **6**, and **8**.

We studied the ability to form complexes of cyclic MON derivatives with mono- (Li^+ , Na^+ , K^+ , Rb^+ , and Cs^+) and divalent (Mg^{2+} , Ca^{2+} , Sr^{2+} , and Ba^{2+}) cations using electrospray ionization mass spectrometry (ESI-MS) because it permitted analysis of host–guest complexes and other noncovalent complexes formed in solution (Supporting Information, Figures S15–S23).³¹ The ESI-MS spectra prove the formation of the respective $[5 + M]^+$, $[5 + M + H_2O]^+$, $[6 + M]^+$, $[8 + M]^+$, complexes, where $M = Li, Na, K, Rb,$ and Cs .

On the other hand, the signals in the ESI-MS spectra of the complexes formed between **5**, **6**, or **8** and appropriate divalent metal perchlorates $M(ClO_4)_2$, where $M = Mg, Ca, Sr$ and Ba , demonstrate the presence of the $[5 + MClO_4]^+$, $[5 + MClO_4 + H_2O]^+$, $[6 + MClO_4]^+$, $[8 + MClO_4]^+$, and $[8 - H + MClO_4]^+$ ion pair complexes probably formed due to the ion pairing

between divalent metal–cation and perchlorate anion that occurs in acetonitrile solution and gas phase. Moreover, the interaction of Na^+ cation with perchlorate anion has also been observed in the crystalline complexes of **5** and **8** (Figure 5). Therefore, scXRD and ESI-MS studies show that cyclic derivatives of MON should be recognized as ion pair receptors.³²

Furthermore, to assess how macrocyclization affects the biological activity of MON analogues, we evaluated the antiproliferative activity of the obtained derivatives. Various cell lines were selected for the study, including human lung carcinoma (A549), human breast adenocarcinoma (MCF-7), human colon adenocarcinoma (LoVo), its doxorubicin-resistant subline (LoVo/DX), and normal murine embryonic fibroblasts (BALB/3T3). Moreover, the antiproliferative activities of two commonly used anticancer drugs, cisplatin and doxorubicin, were also determined. The results are shown in Table 1. Detailed information concerning biological assay can be found in the Supporting Information.

First, the high antiproliferative activity of unmodified MON is worth highlighting. This ionophore proved to be effective against all tested cancer cell lines with IC_{50} values $<0.6\text{ }\mu M$ (compound **1**, Table 1). These results strongly support the thesis that MON is one of the most antiproliferative active ionophores, displaying higher biological activity than e.g. salinomycin or lasalocid.¹⁷ With IC_{50} value of $2.21\text{ }\mu M$ against normal BALB/3T3 cell line, the values of selectivity indices (SI) for MON were within the range of 4.2–6.1 for nonresistant cell lines, and 13.0 for LoVo/DX cell line. This indicates that MON effectively overcomes drug resistance of the LoVo/DX cell line, with the resistance index (RI) value of 0.5 (Table 1).

Second, macrocyclic MON derivatives exhibit lower antiproliferative activity than the unmodified ionophore, which supports previous literature reports,^{15,21,24} however, these compounds display much lower toxicity toward the normal BALB/3T3 cell line than MON itself (Table 1). The derivative with the highest biological activity in this series is compound **5**, which is a macrocyclic lactone of MON obtained by the click reaction (Table 1). Its several-fold higher activity than that of the structurally similar compound **6** (macrocyclic lactam obtained by the click reaction) suggests that the enzymatic stability of the linkers used is of key importance in the design of ionophore-based macrocyclic compounds. As mentioned above, the relatively low physiological stability of

the ester bond compared to the amide bond means that this compound may undergo hydrolysis in the intracellular environment.²⁷ Therefore, the increased antiproliferative activity of compound **5** may be a consequence of the cleavage of ester bond by cellular esterases, which leads to the reformation of the pseudocyclic scaffold. Interestingly, compound **6** is more effective in overcoming drug resistance of the LoVo/DX cell line than its ester analogue **5** ($RI = 0.3$ for **6** versus $RI = 0.5$ for **5**, Table 1), and the remaining tested compounds including **MON**. Compound **8** is characterized by the lowest toxicity toward normal BALB/3T3 cell line of all tested compounds, which is why the selectivity index toward the A549 and LoVo cell lines is the highest among the macrocyclic **MON** derivatives. However, this compound shows a lower ability to overcome drug resistance of the LoVo/DX cell line than the other **MON** derivatives (Table 1).

Lastly, despite the high therapeutic potential of cisplatin and doxorubicin, particular attention should be paid to their immense toxicity toward the BALB/3T3 cell line. All obtained macrocyclic **MON** derivatives are characterized by lower toxicity and higher selectivity indices than the reference drugs, which is their indisputable advantage (Table 1). Moreover, compound **5** showed even higher antiproliferative activity against the LoVo/DX cell line than cisplatin ($IC_{50} = 2.19 \mu M$ versus $IC_{50} = 2.40 \mu M$, respectively, Table 1). Taking into account the resistance index, the ability of the obtained compounds to overcome drug resistance of the LoVo/DX cancer cell line is higher than that of reference drugs used (Table 1).

CONCLUSIONS

Herein we present a new, efficient and scalable method for obtaining C26-azide and amine of **MON**, which is the main representative of ionophore antibiotics. This discovery opens a number of synthetic pathways that can be used to obtain new, highly bioactive analogues of this compound. In this work we decided to transform the pseudocyclic scaffold of **MON** into macrocyclic products via an amidation (macrolactamization) and click reaction using Meldal protocol. All three macrocyclic derivatives demonstrate the ability to form complexes with monovalent (Li^+ , Na^+ , K^+ , Rb^+ , and Cs^+) and divalent (Mg^{2+} , Ca^{2+} , Sr^{2+} , and Ba^{2+}) metal cations, as demonstrated by ESI-MS spectrometry. Furthermore, scXRD analysis showed that cyclic derivatives of **MON** should be recognized as ion pair receptors, as they exhibit interactions not only with cations, but also perchlorate anion which was used for crystallization and spectrometric analyses. Finally, the analysis of biological activities of all obtained analogues was performed, which showed that macrocyclic **MON** derivatives exhibit antiproliferative activity at the micromolar level toward all tested cancer cell lines. These compounds display lower activity, but also lower cytotoxicity than the unmodified ionophore, which may suggest deteriorated bioavailability of such macrocycles. Moreover, the type of linker used had a key impact on the biological activity of the obtained macrocycles, which is a good starting point for scientists who want to develop new bioactive agents based on the skeleton of pseudocyclic compounds, such as ionophore antibiotics.

EXPERIMENTAL SECTION

General Procedures. All reagents were purchased from two sources—Merck or Trimex Chemicals S.A., and used without further purification. Deuterated solvents for NMR analysis (CD_2Cl_2 , $CDCl_3$,

and CD_3CN) were stored over 3 Å molecular sieves for several days. Reaction mixtures were stirred using Teflon-coated magnetic stir bars and were monitored by thin layer chromatography (TLC) using aluminum-backed plates (Merck 60 F₂₅₄). TLC plates were visualized by UV-light (254 nm), followed by treatment with phosphomolybdic acid (PMA) (5% in absolute ethanol) and gentle heating. Products of the reactions were purified using CombiFlash Rf+ Lumen Flash Chromatography System (Teledyne Isco) with integrated ELS and UV detectors. All solvents used in flash chromatography were of HPLC grade (Merck) and were used as received. Solvents were removed using a rotary evaporator.

NMR spectra were recorded on a Bruker AvanceNEO 600 (^1H NMR at 600 MHz and ^{13}C NMR at 151 MHz) magnetic resonance spectrometer. ^1H NMR spectra are reported in chemical shifts downfield from TMS using the respective residual solvent peak as internal standard ($CDCl_3$, δ 7.26 ppm; CD_2Cl_2 , δ 5.32 ppm; CD_3CN , δ 1.94 ppm). ^1H NMR spectra are described as follows: chemical shift (δ , ppm), multiplicity (s = singlet, d = doublet, t = triplet, p = quintet, dd = doublet of doublets, dt = doublet of triplets, td = triplet of doublets, dq = doublets of quartets, qd = quartet of doublets, ddd = doublet of doublets of doublets, dqd = doublet of quartets of doublets, m = multiplet), coupling constant(s) in Hz, and integration. Significant peaks are reported within the overlapping ~ 2.00 – 1.20 ppm region of the ^1H NMR spectra. ^{13}C NMR spectra are reported in chemical shifts downfield from TMS using the respective residual solvent peak as internal standard ($CDCl_3$, δ 77.16 ppm; CD_2Cl_2 , δ 53.84 ppm; CD_3CN , δ 1.32 ppm, 118.26 ppm). Line broadening parameters were 0.5 or 1.0 Hz, while the error of chemical shift value was 0.1 ppm.

Electrospray ionization high-resolution mass spectra (ESI-HRMS) were recorded on a QTOF (Bruker micrOTOF-q) mass spectrometer in the positive ion detection mode. Samples were prepared in dry acetonitrile with the addition of $NaClO_4$. The mass range for ESI experiments was from $m/z = 400$ to $m/z = 1000$. To analyze the ability of compounds **5**, **6** and **8** to form complexes with metal cations, a electrospray ionization (ESI) measurements with positive mode detection was performed using PylonMass Spectrometer Model L. The complexes of compound **5**, **6** and **8** with cations were prepared by mixing acetonitrile solutions of these compounds and respective cation salts in the 1:15 mixture of acetonitrile. The concentration of studied compounds in final solutions used for the ESI measurement was $5 \times 10^{-5} \text{ mol dm}^{-3}$. The samples were infused directly to the ESI source. The major parameters were set as follows: Source: ESI + 3.5 kV 350 °C, Capillary: 150 V 300 °C Offset: 25 V Span: 0 V, full scan ranging 1000–2000 (m/z).

Synthesis. Isolation of **MON**. The sodium salt of **MON** was isolated from commercially available veterinary premix—Cosidin, as previously described.²¹ The obtained sodium salt of **MON** was then extracted with a solution of sulfuric acid ($pH = 1$), giving **MON** ready for further synthesis.

Synthesis of Monensin Azide (Compound 2). To a solution of **MON** (11.0 g, 16.39 mmol, 1.0 equiv) in toluene (120 mL) upon stirring, a solution of 0.1 M Na_2CO_3 was added (150 mL). After that, tosyl chloride (9.37 g, 49.18 mmol, 3.0 equiv) was added to the resulting mixture in one portion. The resulting solution was stirred at room temperature for the next 72 h. The reaction mixture was then transferred to a separatory funnel and extracted with 0.1 M Na_2CO_3 solution and brine. After that, the organic layer was concentrated under reduced pressure, and directed subsequently to the next stage of the process. The excess of sodium azide (3.19 g, 49.09 mmol, 3.0 equiv) was dissolved in 30 mL of DMSO (complete conversion of **MON** to tosylate was assumed). The solution of crude tosylate dissolved in 20 mL of DMSO was then added to the mixture. The resulting solution was stirred at room temperature for the next 72 h. The mixture was then diluted with large amount of water (over 300 mL) and extracted several times with methylene chloride. This step can be dangerous due to the possibility of an explosion as a result of the reaction between sodium azide and the chlorinated solvent, so the sequence of operations must be strictly followed.³³ Alternatively, compound **2** can be extracted from the aqueous phase into the ethyl

acetate phase. Purification on silica gel using the CombiFlash system (0 → 40% EtOAc/*n*-hexane) gave the pure product as a clear oil. The oil was diluted in *n*-pentane and evaporated to dryness three times to form an amorphous solid, with an isolated yield of 62% (7.10 g), a single spot by TLC; stains green with PMA; ^{13}C NMR (151 MHz, CDCl_3) δ 176.3, 107.7, 99.1, 86.0, 85.7, 84.6, 83.0, 81.8, 76.4, 75.6, 71.1, 67.0, 58.0, 56.1, 41.0, 38.5, 36.8, 36.0, 34.7, 34.3, 34.1, 33.4, 33.0, 32.6, 31.3, 30.6, 27.5, 27.3, 17.5, 16.1, 15.8, 15.1, 10.8, 10.6, 8.3 ppm; ^1H NMR (600 MHz, CDCl_3) δ 5.79 (s, 1H), 4.60 (s, 1H), 4.32 (ddd, $J = 10.4, 6.0, 2.9$ Hz, 1H), 4.10 (dd, $J = 11.3, 2.2$ Hz, 1H), 4.08 (d, $J = 4.0$ Hz, 1H), 4.07 (dd, $J = 10.2, 3.4$ Hz, 1H), 3.78 (s, 1H), 3.49 (d, $J = 13.1$ Hz, 1H), 3.46 (dd, $J = 10.5, 5.1$ Hz, 1H), 3.36 (s, 3H), 3.24 (dd, $J = 9.6, 2.5$ Hz, 1H), 3.07 (d, $J = 13.1$ Hz, 1H), 2.63 (dq, $J = 9.5, 6.7$ Hz, 1H), 2.28 (td, $J = 11.3, 6.3$ Hz, 1H), 2.24–2.18 (m, 2H), 2.15 (dq, $J = 13.7, 6.9, 2.5$ Hz, 1H), 2.04–1.93 (m, 3H), 1.91–1.80 (m, 2H), 1.74–1.31 (m, 13H), 1.48 (s, 3H), 1.24 (d, $J = 6.7$ Hz, 3H), 1.09 (d, $J = 6.9$ Hz, 3H), 0.90 (d, $J = 7.0$ Hz, 3H), 0.89 (m, 3H), 0.88 (d, $J = 7.2$ Hz, 3H), 0.86 (dd, $J = 6.6, 2.2$ Hz, 6H) ppm; MS (ESI) m/z : $[\text{M} + \text{Na}]^+$ Calcd for $\text{C}_{10}\text{H}_{19}\text{N}_3\text{NaO}_{10}$ 718.4249; Found 718.4242.

Synthesis of Propargyl Ester of MON Azide (Compound 3). Compound 2 (1.0 g, 1.44 mmol, 1.0 equiv) was dissolved in toluene (25 mL) and the solution was heated to 90 °C on a heating mantle. After 15 min DBU (372 mg, 2.44 mmol, 1.7 equiv) was added dropwise to the reaction mixture, and after another 20 min, propargyl bromide (513 mg, 4.31 mmol, 3.0 equiv) was added dropwise. The solution changed its color to brownish after a few minutes. After 24 h, the reaction mixture was concentrated on a rotary evaporator. Purification on silica gel using the CombiFlash system (0 → 20% EtOAc/*n*-hexane) gave the pure ester 3 as a clear oil. After twice evaporation to dryness with *n*-pentane, the product remained in an oily form (886 mg, 84% yield), a single spot by TLC; stains green with PMA; ^{13}C NMR (151 MHz, CD_3CN) δ 175.2, 108.4, 99.0, 87.9, 86.9, 86.4, 84.1, 82.3, 79.1, 77.9, 77.6, 76.5, 71.9, 68.4, 58.7, 57.5, 52.8, 41.4, 39.8, 37.9, 37.4, 36.9, 35.6, 35.2, 35.0, 34.7, 34.1, 32.7, 32.2, 31.0, 29.1, 26.7, 17.9, 16.4, 16.3, 12.8, 12.4, 11.3, 8.6 ppm; ^1H NMR (600 MHz, CD_3CN) δ 4.75 (dd, $J = 15.8, 2.5$ Hz, 1H), 4.69 (dd, $J = 15.8, 2.5$ Hz, 1H), 4.51 (s, 1H), 4.23 (ddd, $J = 8.7, 6.3, 2.9$ Hz, 2H), 3.99 (dd, $J = 9.1, 2.4$ Hz, 1H), 3.94 (d, $J = 4.2$ Hz, 1H), 3.72–3.68 (m, 2H), 3.60 (dd, $J = 10.3, 5.2$ Hz, 1H), 3.51 (m, 1H), 3.33 (s, 3H), 3.28 (d, $J = 12.6$ Hz, 1H), 3.06 (d, $J = 12.6$ Hz, 1H), 2.87 (t, $J = 2.5$ Hz, 1H), 2.67 (qd, $J = 6.9, 5.5$ Hz, 1H), 2.31–2.17 (m, 3H), 2.14 (ddd, $J = 11.8, 9.2, 2.3$ Hz, 1H), 2.04–1.27 (m, 17H), 1.37 (s, 3H), 1.17 (d, $J = 6.9$ Hz, 3H), 0.98 (d, $J = 6.9$ Hz, 3H), 0.93 (d, $J = 7.0$ Hz, 3H), 0.92–0.88 (m, 9H), 0.82 (d, $J = 6.8$ Hz, 3H) ppm; MS (ESI) m/z : $[\text{M} + \text{Na}]^+$ Calcd for $\text{C}_{10}\text{H}_{19}\text{N}_3\text{NaO}_{10}$ 756.4406; Found 756.4396.

Synthesis of Propargyl Amide of MON Azide (Compound 4). Upon stirring to a solution of 2 (1.0 g, 1.44 mmol, 1.0 equiv) in CH_2Cl_2 (25 mL), cooled in an ice bath, DCC (356 mg, 1.72 mmol, 1.2 equiv) was added in one portion. After 20 min HOBT (97 mg, 0.72 mmol, 0.5 equiv) was added. After another 10 min, propargylamine (198 mg, 3.69 mmol, 2.5 equiv) was added dropwise to the reaction mixture. After 24 h, the resulting DCU precipitate was filtered off and the reaction mixture was concentrated under reduced pressure. Purification on silica gel using the CombiFlash system (0 → 30% EtOAc/*n*-hexane) gave the pure product as a clear oil. After twice evaporation to dryness with *n*-pentane, the product remained in an oily form (927 mg, 88% yield), a single spot by TLC; stains green with PMA; ^{13}C NMR (151 MHz, CD_3CN) δ 175.1, 108.3, 99.1, 87.6, 86.8, 86.4, 84.1, 82.6, 81.7, 77.9, 77.5, 72.3, 71.9, 68.3, 58.6, 57.3, 42.7, 39.8, 38.0, 37.4, 36.4, 35.7, 35.1, 35.0, 34.6, 34.0, 32.9, 32.2, 31.0, 29.2, 29.1, 26.8, 17.9, 16.4, 16.3, 14.7, 12.4, 11.2, 8.60 ppm; ^1H NMR (600 MHz, CD_3CN) δ 6.82 (s, 1H), 4.70 (s, 1H), 4.24 (ddd, $J = 9.5, 6.3, 3.2$ Hz, 1H), 4.17 (d, $J = 8.7$ Hz, 1H), 4.00–3.91 (m, 4H), 3.73 (dd, $J = 10.2, 3.5$ Hz, 1H), 3.68–3.65 (m, 1H), 3.58 (dd, $J = 10.3, 5.1$ Hz, 1H), 3.38–3.31 (m, 2H), 3.34 (s, 3H), 3.06 (d, $J = 12.6$ Hz, 1H), 2.57 (s, 1H), 2.47–2.41 (m, 1H), 2.31–2.24 (m, 3H), 2.21 (dd, $J = 11.2, 6.3$ Hz, 1H), 2.16–2.11 (m, 1H), 2.04–1.26 (m, 16H), 1.38 (s, 3H), 1.12 (d, $J = 6.9$ Hz, 3H), 0.99 (d, $J = 6.9$ Hz,

3H), 0.93 (d, $J = 7.0$ Hz, 3H), 0.90 (t, $J = 7.4$ Hz, 3H), 0.89 (d, $J = 6.3$ Hz, 3H), 0.88 (d, $J = 7.1$ Hz, 3H), 0.83 (d, $J = 6.8$ Hz, 3H) ppm; MS (ESI) m/z : $[\text{M} + \text{Na}]^+$ Calcd for $\text{C}_{10}\text{H}_{19}\text{N}_3\text{NaO}_9$ 755.4566; Found 755.4554.

Synthesis of Macrocylic Lactone of MON Obtained by Click Reaction (Compound 5). Under a nitrogen atmosphere, to a solution of 3 (500 mg, 0.68 mmol, 1.0 equiv) in anhydrous CH_3CN , DIPEA (264 mg, 2.04 mmol, 3.0 equiv) was introduced, followed by the addition of catalytic CuI (13 mg, 0.068 mmol, 0.1 equiv) in one portion. The reaction mixture was stirred at room temperature for 24 h. After the consumption of the substrate (TLC and ESI-MS control), the organic solvent was removed on a rotary evaporator. The oily residue was dissolved in a small amount of CH_2Cl_2 and extracted a few times with 10% aq. EDTA solution. Organic phases were concentrated under reduced pressure. Purification on silica gel using the CombiFlash system (0 → 40% EtOAc/*n*-hexane) gave the pure product as a clear oil. The oil was diluted in *n*-pentane and evaporated to dryness three times to form an amorphous solid, with an isolated yield of 74% (370 mg), a single spot by TLC; stains green with PMA; ^{13}C NMR (151 MHz, CD_3Cl_2) δ 175.5, 142.9, 125.0, 108.1, 96.9, 86.5, 86.3, 85.8, 85.2, 79.9, 78.0, 76.3, 71.5, 67.7, 59.4, 58.2, 57.8, 41.2, 38.7, 38.4, 37.5, 37.3, 35.6, 34.4, 33.8, 33.5, 32.5, 30.1, 29.7, 27.5, 23.1, 17.8, 16.7, 16.3, 14.3, 13.5, 11.3, 11.1, 8.2 ppm; ^1H NMR (600 MHz, CD_3Cl_2) δ 7.90 (s, 1H), 5.27 (d, $J = 13.1$ Hz, 1H), 5.15 (d, $J = 13.1$ Hz, 1H), 4.91 (s, 1H), 4.60 (d, $J = 14.0$ Hz, 1H), 4.38 (d, $J = 8.4$ Hz, 1H), 4.26 (d, $J = 13.9$ Hz, 1H), 4.24–4.20 (m, 1H), 3.88 (dd, $J = 10.7, 2.1$ Hz, 1H), 3.82 (d, $J = 5.5$ Hz, 1H), 3.67–3.62 (m, 2H), 3.57–3.53 (m, 1H), 3.47 (dd, $J = 4.1, 2.6$ Hz, 1H), 3.33 (s, 3H), 2.62 (qd, $J = 7.0, 4.5$ Hz, 1H), 2.35 (ddd, $J = 10.1, 7.2, 3.4$ Hz, 1H), 2.29 (s, 1H), 2.16–2.08 (m, 2H), 2.05 (ddd, $J = 11.5, 5.6, 2.7$ Hz, 1H), 1.97 (ddd, $J = 9.3, 8.8, 2.8$ Hz, 2H), 1.82 (dt, $J = 11.4, 8.9$ Hz, 1H), 1.76–1.31 (m, 10H), 1.45 (s, 3H), 1.26 (d, $J = 6.9$ Hz, 3H), 1.25 (s, 3H), 1.04 (d, $J = 7.2$ Hz, 3H), 0.99 (d, $J = 7.0$ Hz, 3H), 0.97 (d, $J = 6.7$ Hz, 3H), 0.91 (t, $J = 7.4$ Hz, 3H), 0.87–0.85 (m, 6H) ppm; MS (ESI) m/z : $[\text{M} + \text{Na}]^+$ Calcd for $\text{C}_{10}\text{H}_{19}\text{N}_3\text{NaO}_{10}$ 756.4406; Found 756.4394.

Synthesis of Macrocylic Lactam of MON Obtained by Click Reaction (Compound 6). Under a nitrogen atmosphere, to a solution of 4 (500 mg, 0.68 mmol, 1.0 equiv) in anhydrous CH_3CN , DIPEA (264 mg, 2.04 mmol, 3.0 equiv) was introduced, followed by the addition of catalytic CuI (13 mg, 0.068 mmol, 0.1 equiv) in one portion. The reaction mixture was stirred at room temperature for 24 h. After the consumption of the substrate (TLC and ESI-MS control), the organic solvent was removed on a rotary evaporator. The oily residue was dissolved in a small amount of CH_2Cl_2 and extracted a few times with 10% aq. EDTA solution. Organic phases were concentrated under reduced pressure. Purification on silica gel using the CombiFlash system (0 → 30% EtOAc/*n*-hexane) gave the pure product as a clear oil. The oil was diluted in *n*-pentane and evaporated to dryness three times to form an amorphous solid, with an isolated yield of 52% (260 mg), a single spot by TLC; stains green with PMA; ^{13}C NMR (151 MHz, CD_3Cl_2) δ 176.7, 144.9, 128.3, 106.7, 97.6, 87.2, 85.8, 85.2, 83.6, 79.4, 78.2, 76.1, 70.4, 68.7, 57.4, 57.3, 43.3, 39.5, 39.1, 37.8, 36.2, 35.5, 34.9, 34.6, 34.0, 33.7, 33.3, 32.5, 31.7, 30.9, 28.6, 27.9, 17.4, 16.5, 16.3, 16.3, 12.5, 10.7, 8.4 ppm; ^1H NMR (600 MHz, CD_3Cl_2) δ 7.84 (s, 1H), 6.52–6.48 (m, 1H), 4.58 (s, 1H), 4.56 (d, $J = 14.4$ Hz, 1H), 4.45–4.37 (m, 3H), 4.31 (dd, $J = 14.9, 5.9$ Hz, 1H), 4.23 (d, $J = 4.5$ Hz, 1H), 3.87–3.85 (m, 1H), 3.75 (dd, $J = 10.0, 2.2$ Hz, 1H), 3.63 (dd, $J = 10.6, 1.8$ Hz, 1H), 3.55 (dd, $J = 11.3, 4.1$ Hz, 1H), 3.40 (dd, $J = 8.0, 2.7$ Hz, 1H), 3.31 (s, 3H), 3.28 (s, 1H), 2.62 (p, $J = 7.0$ Hz, 1H), 2.46–2.37 (m, 3H), 2.24 (dd, $J = 11.8, 10.0$ Hz, 1H), 2.03–1.39 (m, 17H), 1.62 (s, 3H), 1.24 (d, $J = 7.0$ Hz, 3H), 1.02 (dd, $J = 14.1, 7.0$ Hz, 9H), 0.98 (t, $J = 7.5$ Hz, 3H), 0.90 (d, $J = 6.1$ Hz, 3H), 0.85 (d, $J = 7.1$ Hz, 3H) ppm; MS (ESI) m/z : $[\text{M} + \text{Na}]^+$ Calcd for $\text{C}_{10}\text{H}_{19}\text{N}_3\text{NaO}_9$ 755.4566; Found 755.4553.

Synthesis of Monensin Amine (Compound 7). Under a nitrogen atmosphere, to a solution of 2 (2.0 g, 2.87 mmol, 1.0 equiv) in methanol upon stirring, the catalytic amount of palladium on carbon (Pd/C) was added. Then, a hydrogen-filled balloon was connected to the system. The reaction was continued until the azide was completely

consumed (TLC and ESI-MS control), replacing the hydrogen balloon if necessary (typically 72 h). After this time, the reaction mixture was filtered through Celite to remove Pd/C, and concentrated under reduced pressure. Purification on silica gel using the CombiFlash system (0 → 30% MeOH/CH₂Cl₂) gave the pure product as a clear oil. The oil was diluted in *n*-pentane and evaporated to dryness three times to form an amorphous solid, with an isolated yield of 63% (1.22 g), a single spot by TLC; stains green with PMA; ¹³C{¹H} NMR (151 MHz, CD₂Cl₂) δ 181.1, 108.0, 95.8, 86.4, 85.5, 84.2, 82.5, 77.1, 75.2, 71.9, 68.0, 58.0, 47.4, 44.4, 38.7, 37.8, 37.3, 36.7, 35.1, 34.5, 34.3, 34.0, 32.8, 31.5, 31.1, 30.1, 28.1, 28.0, 17.6, 16.7, 16.2, 15.9, 11.2, 10.8, 8.6 ppm; ¹H NMR (600 MHz, CD₂Cl₂) δ 6.17 (s, 7H hydrate), 4.33–4.29 (m, 1H), 4.09 (d, *J* = 11.2 Hz, 1H), 3.94 (d, *J* = 3.5 Hz, 1H), 3.88 (dd, *J* = 10.6, 2.5 Hz, 1H), 3.80 (d, *J* = 2.6 Hz, 1H), 3.45 (dd, *J* = 10.9, 4.0 Hz, 1H), 3.35–3.30 (m, 2H), 3.33 (s, 3H), 3.16 (d, *J* = 8.9 Hz, 1H), 3.08 (d, *J* = 11.8 Hz, 1H), 2.70 (d, *J* = 11.9 Hz, 1H), 2.39 (td, *J* = 13.5, 6.7 Hz, 1H), 2.22–2.12 (m, 5H), 2.09–2.02 (m, 2H), 1.99–1.22 (m, 16H), 1.46 (s, 3H), 1.16 (d, *J* = 6.7 Hz, 3H), 1.04 (d, *J* = 6.8 Hz, 3H), 0.93 (t, *J* = 7.4 Hz, 3H), 0.90 (d, *J* = 7.0 Hz, 3H), 0.88 (d, *J* = 3.6 Hz, 3H), 0.87 (d, *J* = 2.8 Hz, 3H), 0.84 (d, *J* = 6.5 Hz, 3H) ppm; MS (ESI) *m/z*: [M + H]⁺ Calcd for C₃₀H₄₈NO₁₀ 670.4325 Found 670.4519; [M + Na]⁺ Calcd for C₃₀H₄₈NNaO₁₀ 692.4344 Found 692.4332.

Synthesis of Macrocyclic Lactam of MON (Compound 8). **Method A.** Upon stirring to a solution of 7 (1.0 g, 1.49 mmol, 1.0 equiv) in DMF (15 mL) DIPEA (1.16 g, 8.96 mmol, 6.0 equiv) was added in one portion. After 15 min HATU (0.738 g, 1.94 mmol, 1.3 equiv) was added to the mixture. The reaction was carried out for 24 h and then concentrated under reduced pressure, and extracted with 0.1 M Na₂CO₃ solution and water. Purification on silica gel using the CombiFlash system (0 → 100% EtOAc/*n*-hexane) gave the pure product as a clear oil. The oil was diluted in *n*-pentane and evaporated to dryness three times to form an amorphous solid, with an isolated yield of 72% (701 mg), a single spot by TLC; stains green with PMA.

Method B. Upon stirring to a solution of 7 (1.0 g, 1.49 mmol, 1.0 equiv) in DMF (15 mL) DIPEA (1.16 g, 8.96 mmol, 6.0 equiv) was added in one portion. After 15 min HBTU (0.736 g, 1.94 mmol, 1.3 equiv) was added to the mixture. The reaction was carried out for 24 h and then concentrated under reduced pressure, and extracted with 0.1 M Na₂CO₃ solution and water. Purification on silica gel using the CombiFlash system (0 → 100% EtOAc/*n*-hexane) gave the pure product as a clear oil. The oil was diluted in *n*-pentane and evaporated to dryness three times to form an amorphous solid, with an isolated yield of 10% (97 mg), a single spot by TLC; stains green with PMA.

Method C. Upon stirring to a solution of 7 (250 mg, 0.37 mmol, 1.0 equiv) in CH₂Cl₂ (15 mL) cooled in an ice bath, DCC (100 mg, 0.48 mmol, 1.3 equiv) was added in one portion. After 24 h, the resulting DCU precipitate was filtered off and the reaction mixture was concentrated under reduced pressure. Purification on silica gel using the CombiFlash system (0 → 100% EtOAc/*n*-hexane) gave the pure product as a clear oil. The oil was diluted in *n*-pentane and evaporated to dryness three times to form an amorphous solid, with an isolated yield of 16% (39 mg), a single spot by TLC; stains green with PMA.

Method D. Upon stirring to a solution of 7 (250 mg, 0.37 mmol, 1.0 equiv) in CH₂Cl₂ (15 mL) cooled in an ice bath, EDCI (75 mg, 0.48 mmol, 1.3 equiv) was added in one portion. After 24 h, no product formation was observed (TLC and ESI-MS control). Increasing the reaction time to 72 h did not increase its efficiency. Nevertheless, purification on silica gel using the CombiFlash system (0 → 100% EtOAc/*n*-hexane) was performed. The reaction product was not isolated.

¹³C{¹H} NMR (151 MHz, CD₃CN) δ 178.3, 109.4, 98.6, 87.6, 86.4, 86.1, 83.5, 78.1, 74.5, 72.4, 69.2, 58.6, 46.4, 41.9, 39.1, 38.8, 38.7, 37.9, 37.2, 36.7, 36.1, 35.0, 34.4, 33.6, 33.3, 32.0, 31.5, 28.4, 25.6, 17.9, 17.1, 15.9, 15.3, 11.1, 10.3, 8.8 ppm; ¹H NMR (600 MHz, CD₃CN) δ 7.41 (s, 1H), 5.12 (s, 1H), 4.65 (d, *J* = 10.2 Hz, 1H), 4.32–4.25 (m, 2H), 4.15 (d, *J* = 4.7 Hz, 1H), 4.10 (dd, *J* = 13.4, 10.3 Hz, 1H), 3.86 (dd, *J* = 9.8, 2.8 Hz, 1H), 3.72–3.68 (m, 1H), 3.66 (dd, *J* = 11.1, 4.6 Hz, 1H), 3.40 (s, 3H), 3.35 (t, *J* = 2.7 Hz, 1H), 2.84 (s,

3H), 2.69 (qd, *J* = 7.1, 2.7 Hz, 1H), 2.49 (dd, *J* = 13.5, 1.6 Hz, 1H), 2.37–2.31 (m, 1H), 2.23–2.16 (m, 2H), 2.09–2.00 (m, 2H), 1.96–1.91 (m, 2H), 1.81–1.27 (m, 11H), 1.45 (s, 3H), 1.08 (d, *J* = 7.2 Hz, 3H), 0.98 (d, *J* = 7.1 Hz, 3H), 0.97 (t, *J* = 7.4 Hz, 3H), 0.93 (d, *J* = 6.7 Hz, 3H), 0.89 (dd, *J* = 6.9, 0.8 Hz, 6H), 0.83 (d, *J* = 6.1 Hz, 3H) ppm; MS (ESI) *m/z*: [M + Na]⁺ Calcd for C₃₀H₄₈NNaO₁₀ 674.4239; Found 674.4228.

Anticancer Activity Studies. Stock solutions of all tested compounds in a concentration of 10 mg/mL were prepared ex tempore for each experiment by dissolving 1 mg of the respective compound in 100 μL of dimethyl sulfoxide (DMSO, Avantor Performance Materials Poland, Gliwice, Poland). The solvent for further dilutions was OR culture medium, which is a mixture of OptiMEM and Roswell Park Memorial Institute (RPMI) 1640 medium (1:1) (OptiMEM–GIBCO, Thermo Fisher Scientific, USA, RPMI with L-glutamine–IET, Wrocław, Poland), supplemented with 5% fetal bovine serum (FBS, HyClone, Cytiva, USA). The compounds were tested at 8 different concentrations from the range of 100–0.001 μg/mL. As a control, cisplatin and doxorubicin were used in the concentration range of 10–0.01 μg/mL (both drugs from Accord Healthcare, Warsaw, Poland), as well as DMSO solvent in concentrations corresponding to its concentration in the samples, from the range of 1–0.001%.

Cell Lines and Culturing Conditions. Four human cancer cell lines and one murine normal cell line were used to evaluate antiproliferative activity of monensin and its derivatives: human lung carcinoma (A549), human breast adenocarcinoma (MCF7), human colon adenocarcinoma cell lines sensitive and resistant to doxorubicin (LoVo) and (LoVo/DX), respectively, and normal murine embryonic fibroblast cell line (BALB/3T3) clone A31. All cell lines are available in the IET cell line bank (Wrocław, Poland). A549 cells were cultured in a mixture of OptiMEM and Roswell Park Memorial Institute (RPMI) 1640 medium (1:1) (OptiMEM–GIBCO, Thermo Fisher Scientific, USA, RPMI with L-glutamine–IET, Wrocław, Poland), supplemented with 10% fetal bovine serum (FBS, HyClone, Cytiva, USA). MCF7 cells were cultured in a mixture of Eagle's medium (IET, Wrocław, Poland), supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1% MEM endogenous amino acid solution (Sigma-Aldrich, Germany), and 8 μg/mL insulin from bovine pancreas (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). LoVo and LoVo/DX cells were cultured in OR medium, supplemented with 10% fetal bovine serum (FBS, HyClone, Cytiva, USA) and 1.0 mM of sodium pyruvate (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Additionally, the medium for LoVo/DX contained 0.1 μg/mL doxorubicin. BALB/3T3 fibroblasts were cultured in Dulbecco's medium, supplemented with 10% fetal bovine serum (HyClone) and 2 mM L-glutamine. All culture media contained antibiotics: 100 U/mL penicillin and 100 μg/mL streptomycin. All cell lines were cultured in humid atmosphere at 37 °C and 5% CO₂.

Cell Viability Assays and SRB. Antiproliferative assays were performed using five cell lines: A549, LoVo, LoVo/DX, MCF7, and BALB/3T3. The cells were plated in 384-well plates (Greiner Bio-One, Kremsmünster, Austria) in the number of: 1500 cells per well for the A549, LoVo/DX and MCF7 cell lines and 2000 cells per well for the LoVo and BALB/3T3 cell lines. After 24 h, solutions of the tested compounds were added. The *in vitro* cytotoxicity of the tested compounds was determined using the sulforhodamine B (SRB) assay. Cultured cells were exposed to 8 different concentrations of the tested compounds from the range of 0.1–100, 0.01–10, or 0.001–1 μg/mL (depending on the activity of the compound). After 72 h of incubation, the cells were fixed by adding cold 50% (w/v) trichloroacetic acid (TCA) (Avantor Performance Materials, Gliwice, Poland) and were incubated at room temperature for 1 h. Then, the wells were washed with distilled water, and stained with 0.4% (w/v) solution of sulforhodamine B (Sigma-Aldrich, Germany) in 1% (v/v) acetic acid (Avantor Performance Materials, Gliwice, Poland) for 0.5 h. After incubation time, the plates with stained cells were rinsed with 1% (v/v) acetic acid. The protein-bound dye was solubilized with 10 mM TRIS solution (Avantor Performance Materials, Gliwice, Poland) for 0.5 h. All steps were performed at room temperature using an

EL406 microplate washer (BioTek Instruments Inc., Winooski, Vermont, USA). Absorbance of each solution was read at Synergy H4 Hybrid Multi-Mode Microplate Reader (BioTek Instruments Inc., Winooski, Vermont, USA) at the 540 nm wavelength. In each experiment, samples containing specific concentrations of the respective compounds were applied in triplicate. The experiments were repeated four or five times. Inhibition of proliferation was determined using the following formula:

$$\% \text{inhibition} = \left(\left(\frac{A_p - A_m}{A_c - A_m} \right) \times 100 \right) - 100$$

A_m —absorbance of the control medium

A_c —absorbance of control cells

A_p —absorbance of cells treated with the tested compounds

The experimental results are presented in the form of $IC_{50} \pm SD$ (dose causing inhibition of proliferation of 50% of the cancer cell population $\pm$ standard deviation). The DMSO was inactive.

■ ASSOCIATED CONTENT

Data Availability Statement

The data underlying this study are available in the published article and its Supporting Information.

■ Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.joc.4c02715>.

Experimental details, materials and methods, NMR, FT-IR, and ESI-MS spectra, as well as the details of X-ray crystallography of the reported compounds (PDF)

FAIR data, including the primary NMR FID files, for compounds 2–8 (ZIP)

Accession Codes

Deposition Numbers 2391724–2391726 contain the supplementary crystallographic data for this paper. These data can be obtained free of charge via the joint Cambridge Crystallographic Data Centre (CCDC) and Fachinformationszentrum Karlsruhe Access Structures service.

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Notes

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■ ABBREVIATIONS

A549, human lung carcinoma; aq, aqueous solution; BALB/3T3, normal murine embryonic fibroblasts; CuAAC, Cu(I)-catalyzed azide–alkyne cycloaddition; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DCC, *N,N'*-dicyclohexylcarbodiimide; DIPEA, *N,N*-diisopropylethylamine; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; EDCI, 1-ethyl-3-(3-dimethylamino)propylcarbodiimide; equiv., equivalent; ESI-MS, electrospray ionization mass spectrometry; ESI-HRMS, electrospray ionization high resolution mass spectrometry; FT-IR, Fourier-transform infrared spectroscopy; HATU, hexafluorophosphate azabenzotriazole tetramethyl uranium; HBTU, hexafluorophosphate benzotriazole tetramethyl uranium; HOBt, hydroxybenzotriazole; IC_{50} , half maximal inhibitory concentration; LoVo, human colon adenocarcinoma; LoVo/DX, doxorubicin-resistant subline of LoVo; MCF-7, human breast adenocarcinoma; MON, monensin; NMR, nuclear magnetic resonance spectroscopy; RI, resistance index; rt, room temperature; SAR, structure–activity relationship; scXRD, single crystal X-ray diffraction; SI, selectivity index; TsCl, 4-toluenesulfonyl chloride

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Supplementary material

From pseudo-cyclic to macrocyclic ionophores: strategies towards the synthesis of cyclic monensin derivatives

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Spectroscopic and spectrometric analysis of newly synthesized compounds

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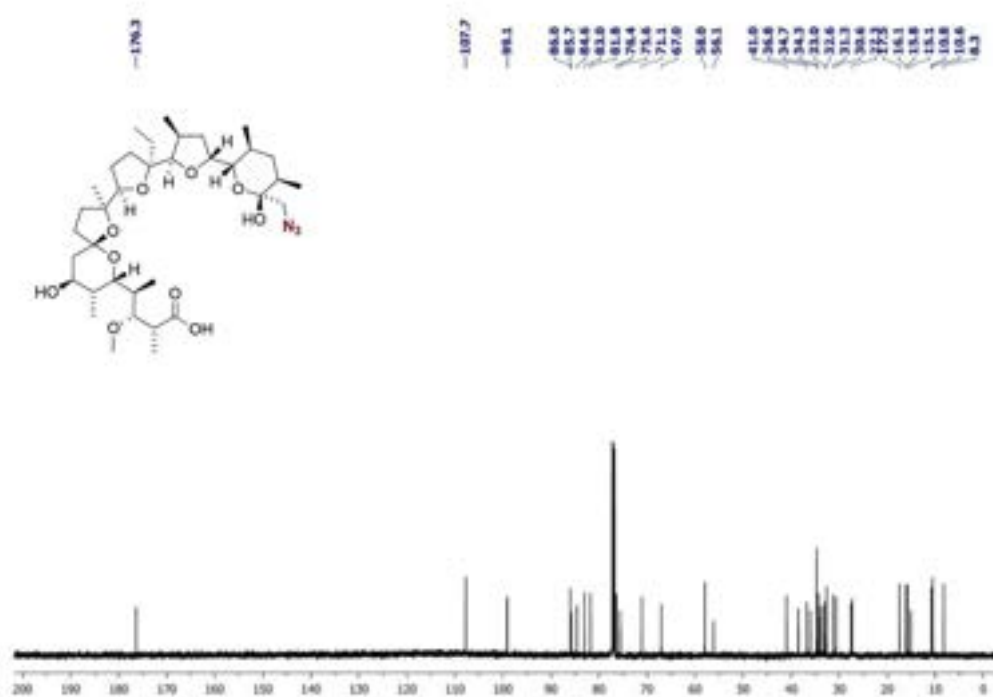


Figure S1. The ^{13}C (^1H) NMR (151 MHz) spectrum of **2** in chloroform- d .

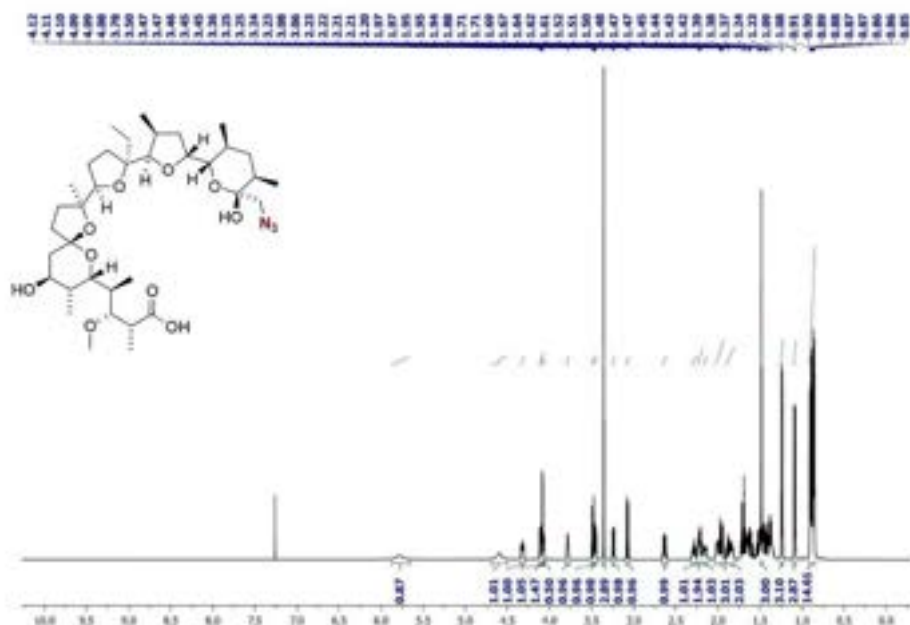


Figure S2. The ^1H NMR (600 MHz) spectrum of **2** in chloroform- d .

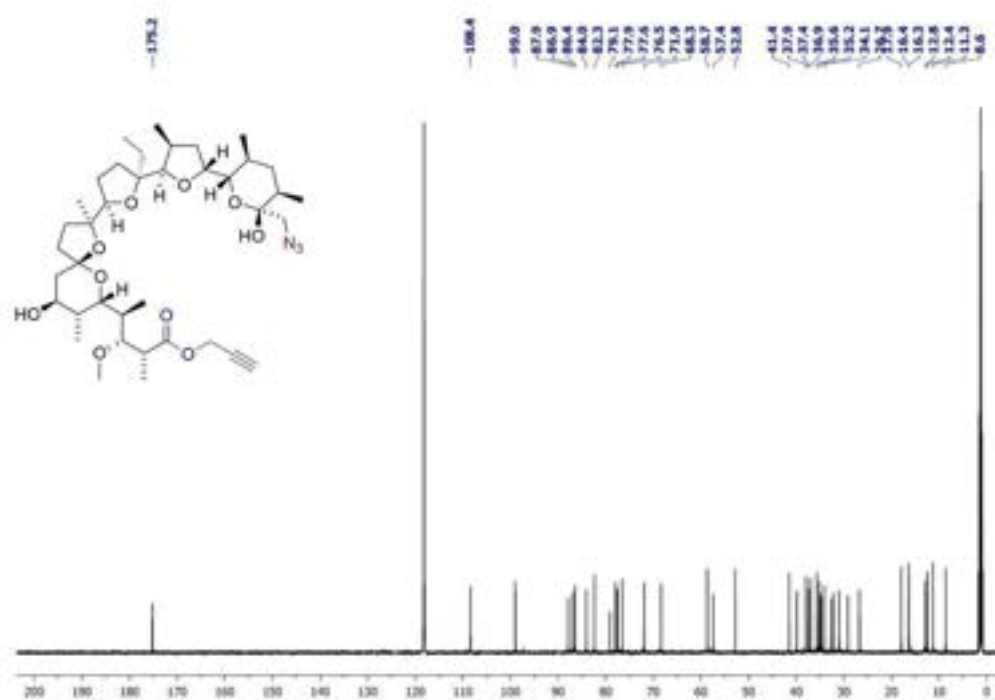


Figure S3. The ^{13}C NMR (151 MHz) spectrum of **3** in acetonitrile- d_3 .

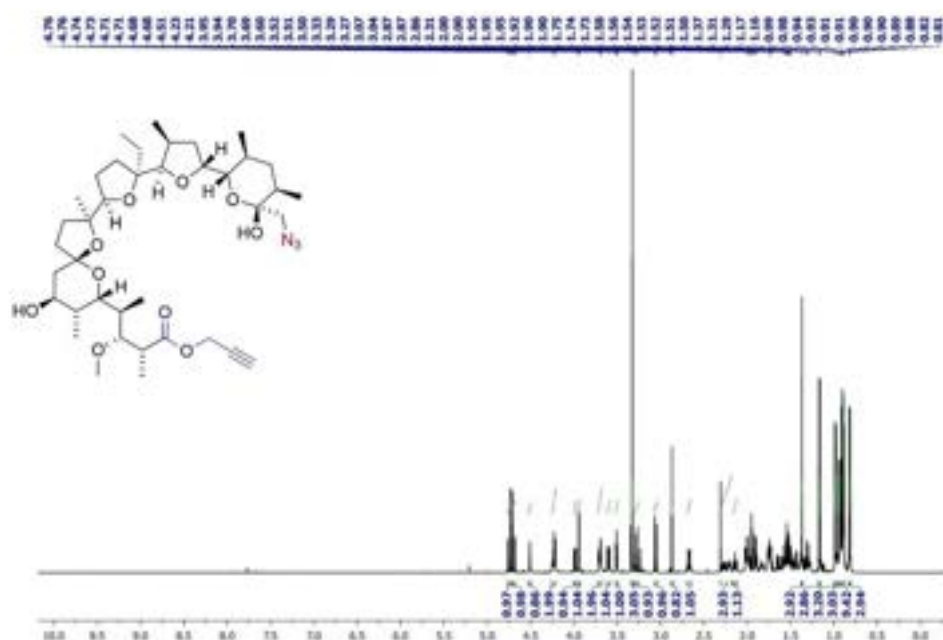


Figure S4. The ^1H NMR (600 MHz) spectrum of **3** in acetonitrile- d_3 .

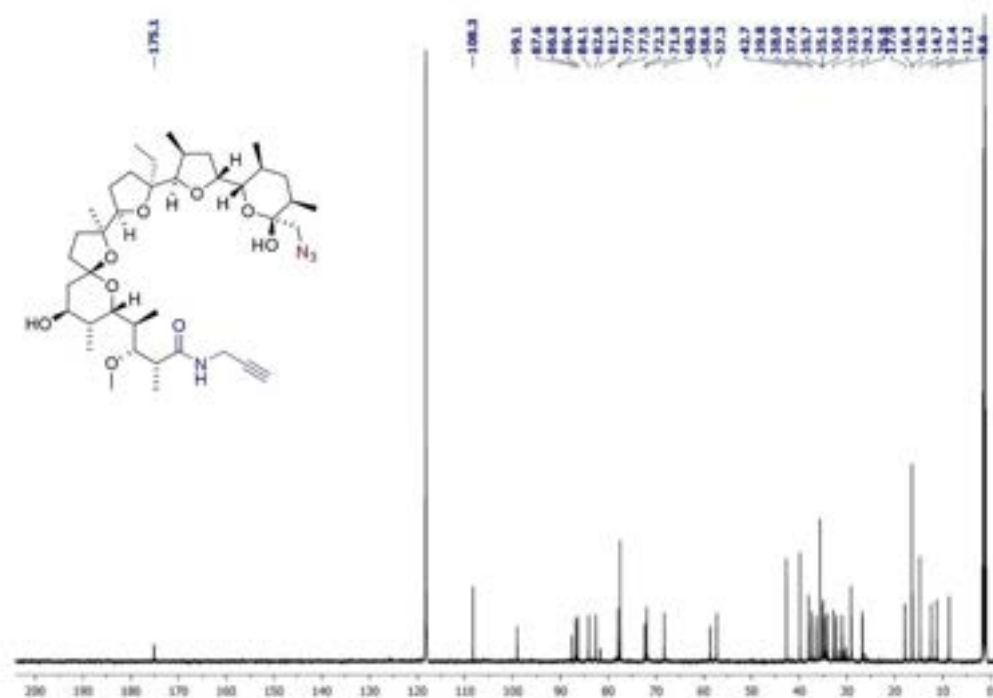


Figure S5. The $^{13}\text{C}\{^1\text{H}\}$ NMR (151 MHz) spectrum of **4** in acetonitrile- d_3 .

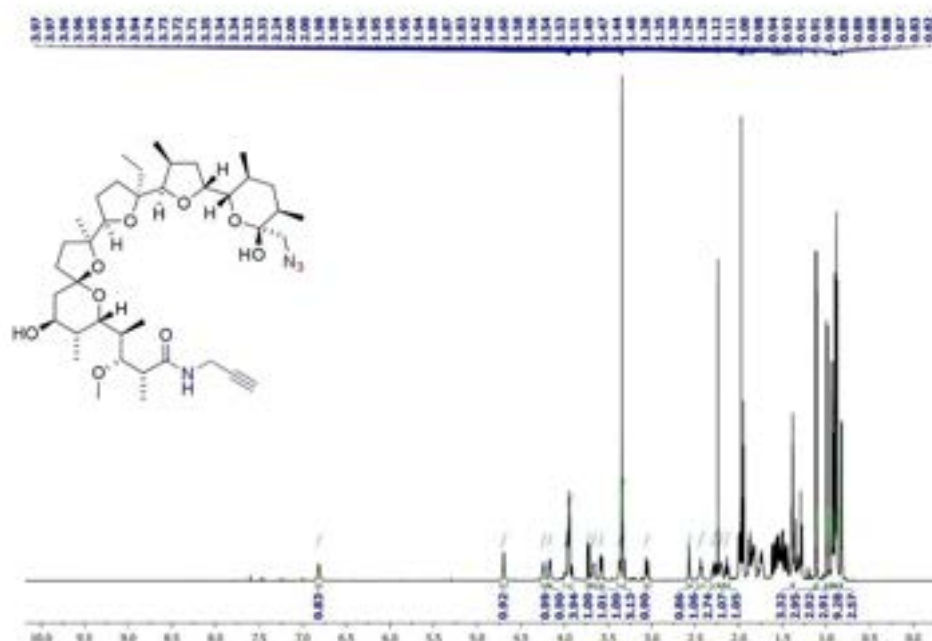


Figure S6. The ^1H NMR (600 MHz) spectrum of **4** in acetonitrile- d_3 .

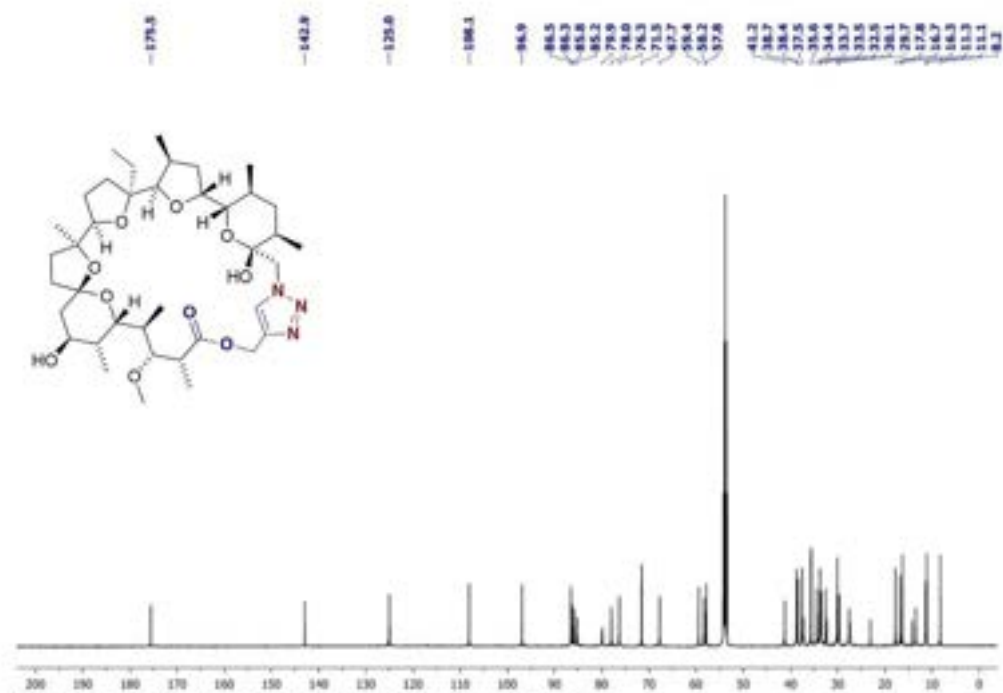


Figure S7. The ^{13}C (^1H) NMR (151 MHz) spectrum of **5** in dichloromethane- d_2 .

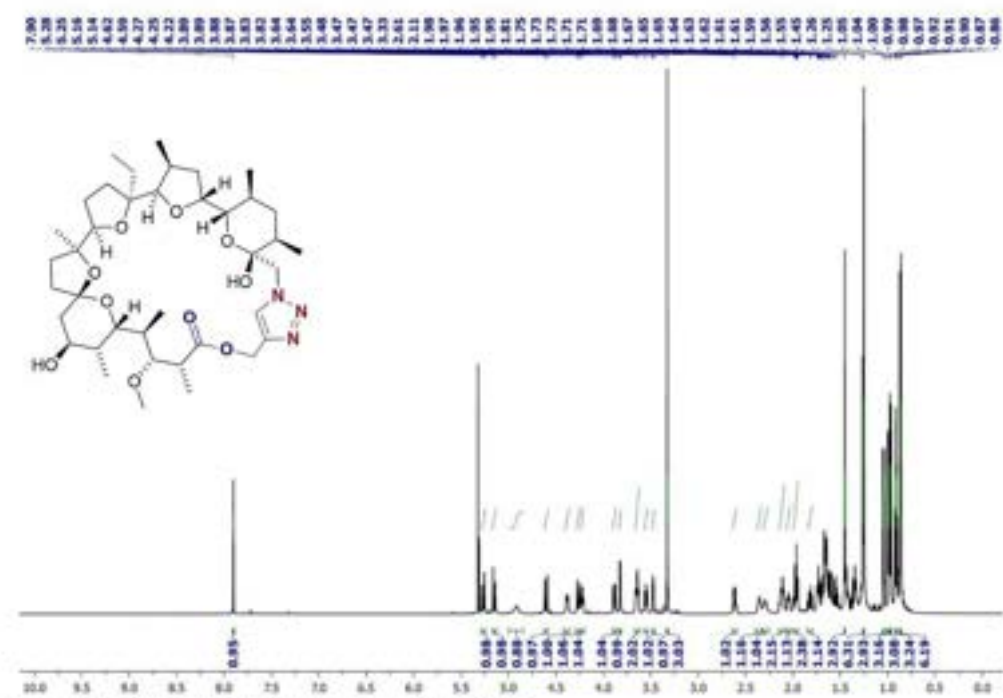


Figure S8. The ^1H NMR (600 MHz) spectrum of **5** in dichloromethane- d_2 .

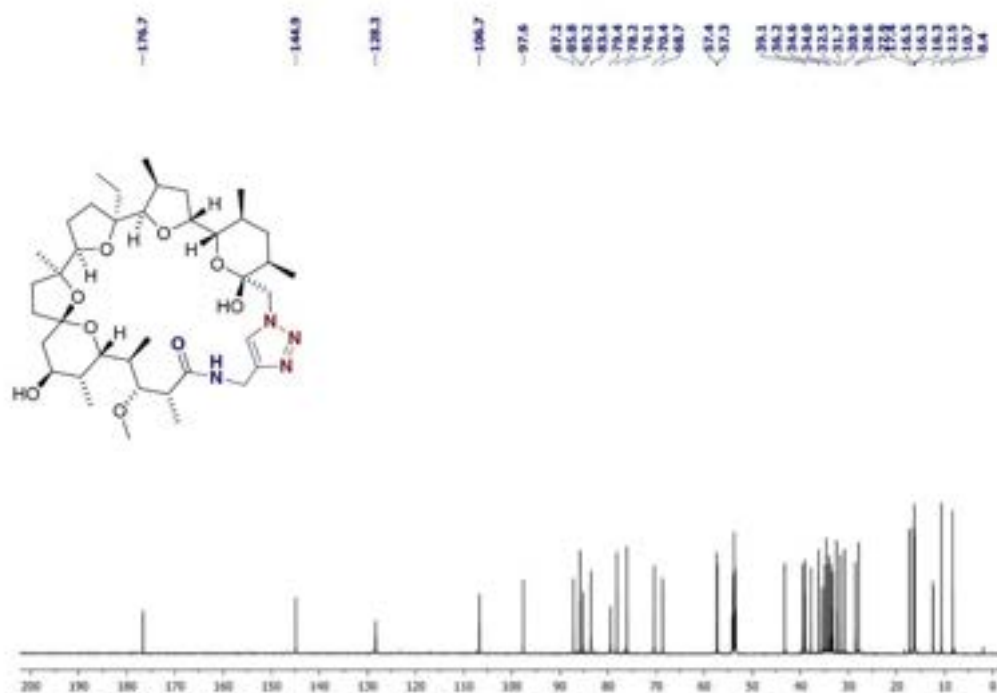


Figure S9. The ^{13}C (^1H) NMR (151 MHz) spectrum of **6** in dichloromethane- d_2 .

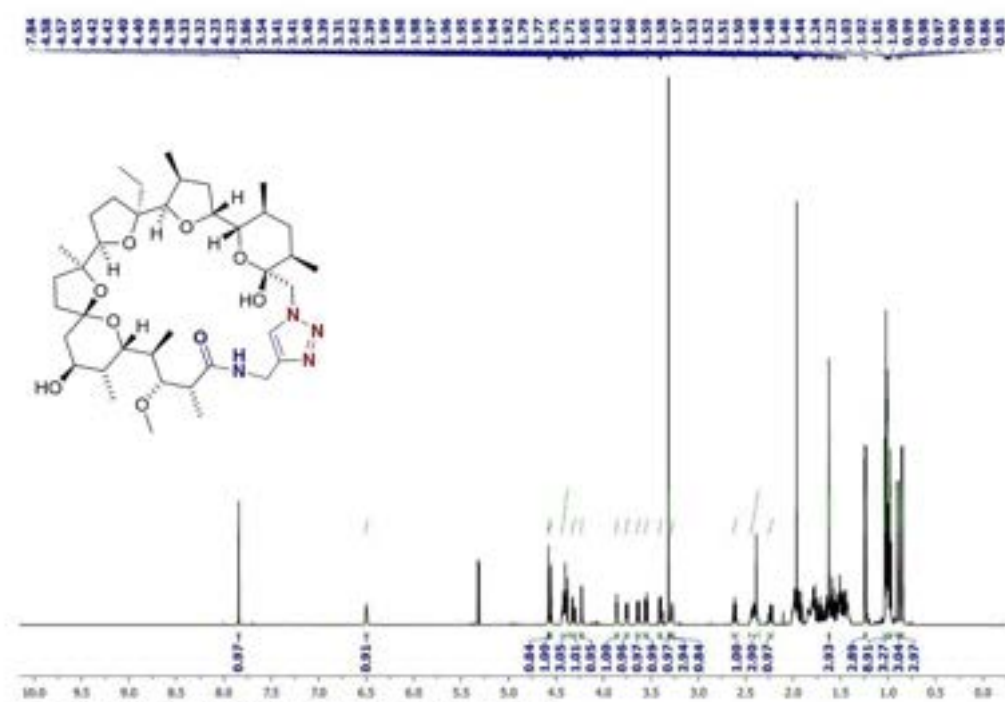


Figure S10. The ^1H NMR (600 MHz) spectrum of **6** in dichloromethane- d_2 .

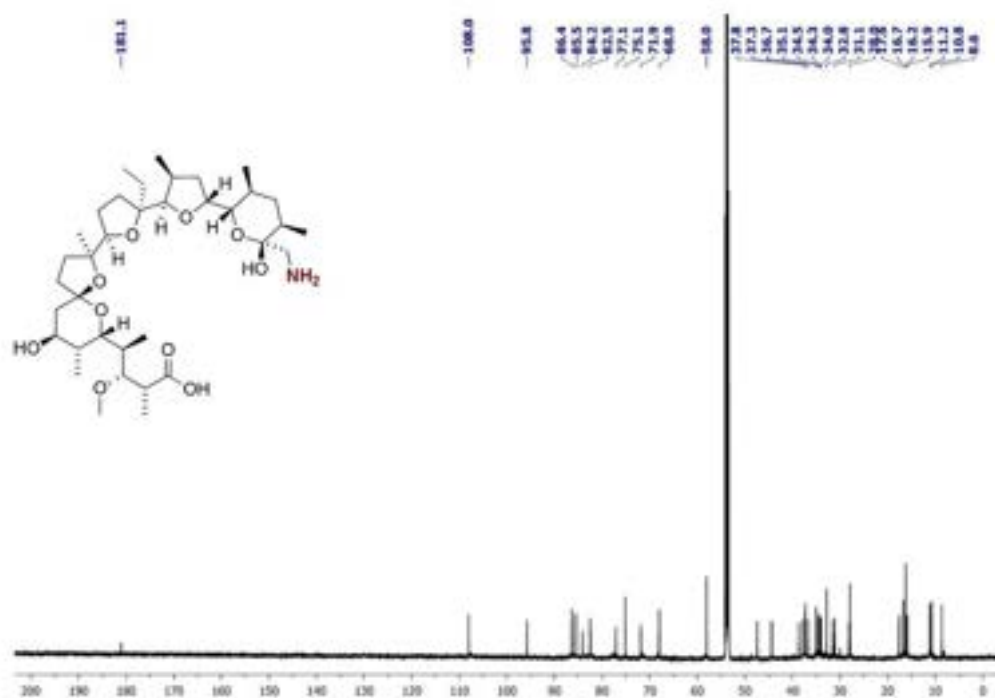


Figure S11. The $^{13}\text{C}\{^1\text{H}\}$ NMR (151 MHz) spectrum of 7 in dichloromethane- d_2 .

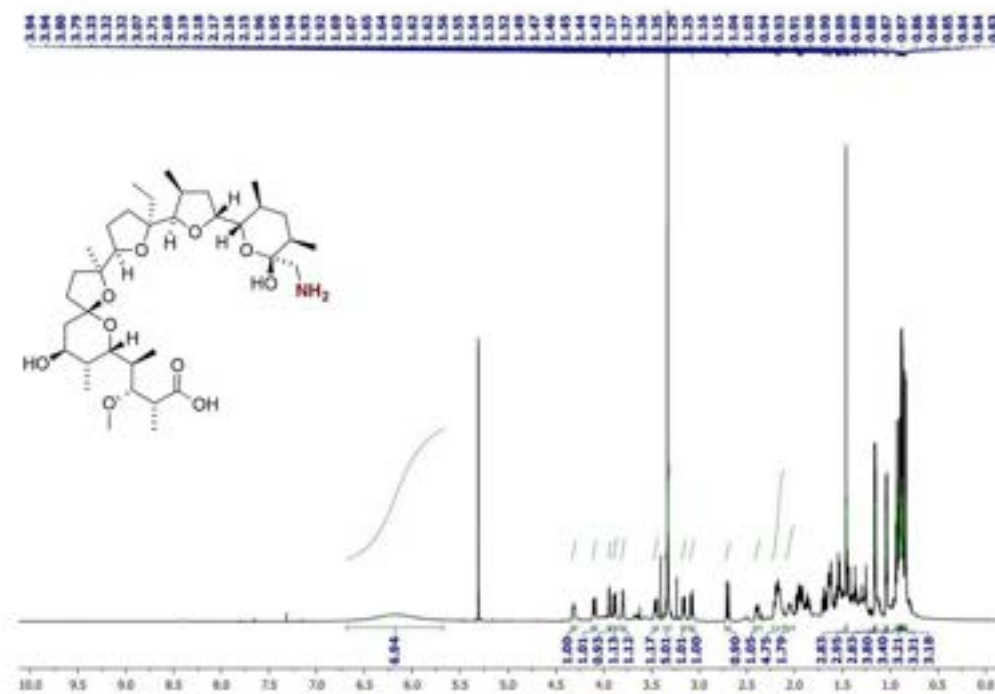
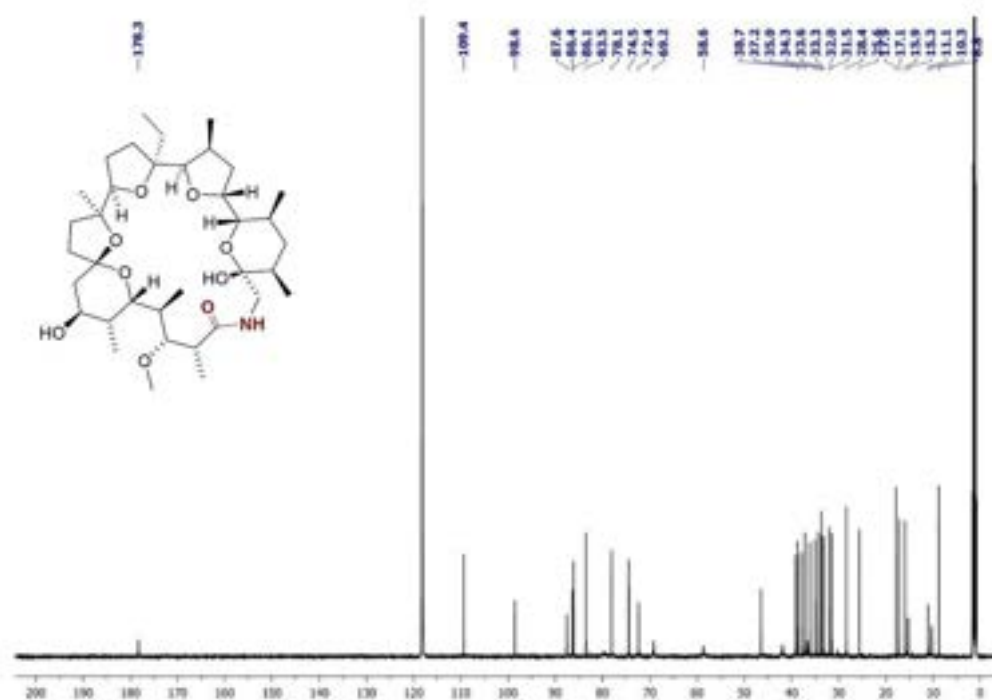
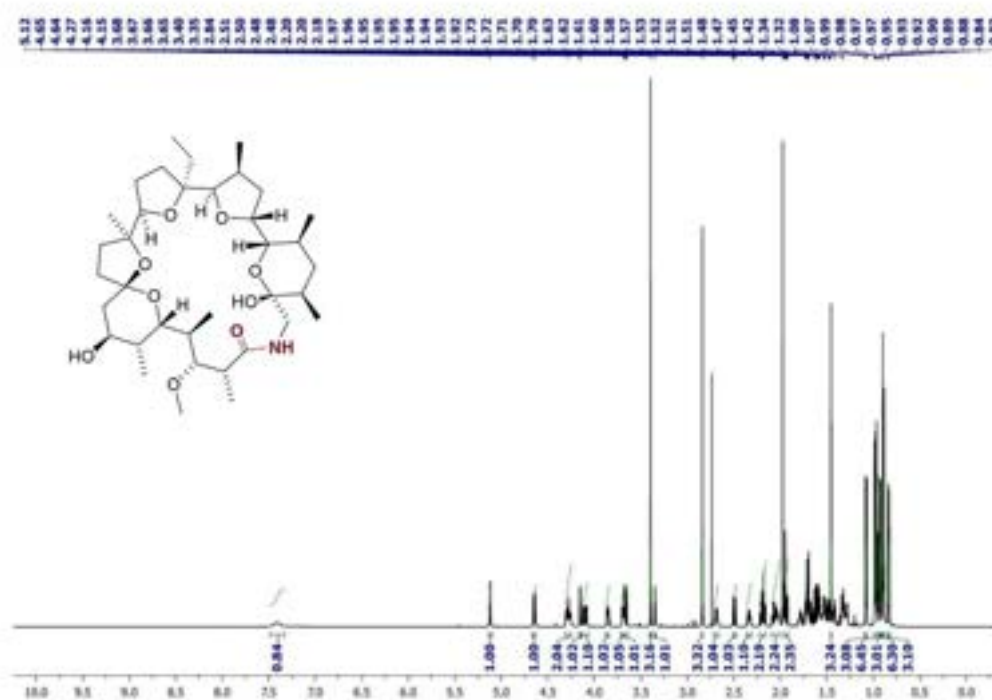


Figure S12. The ^1H NMR (600 MHz) spectrum of 7 in dichloromethane- d_2 .

Figure S13. The ^{13}C NMR (151 MHz) spectrum of **8** in acetonitrile- d_3 .Figure S14. The ^1H NMR (600 MHz) spectrum of **8** in acetonitrile- d_3 .

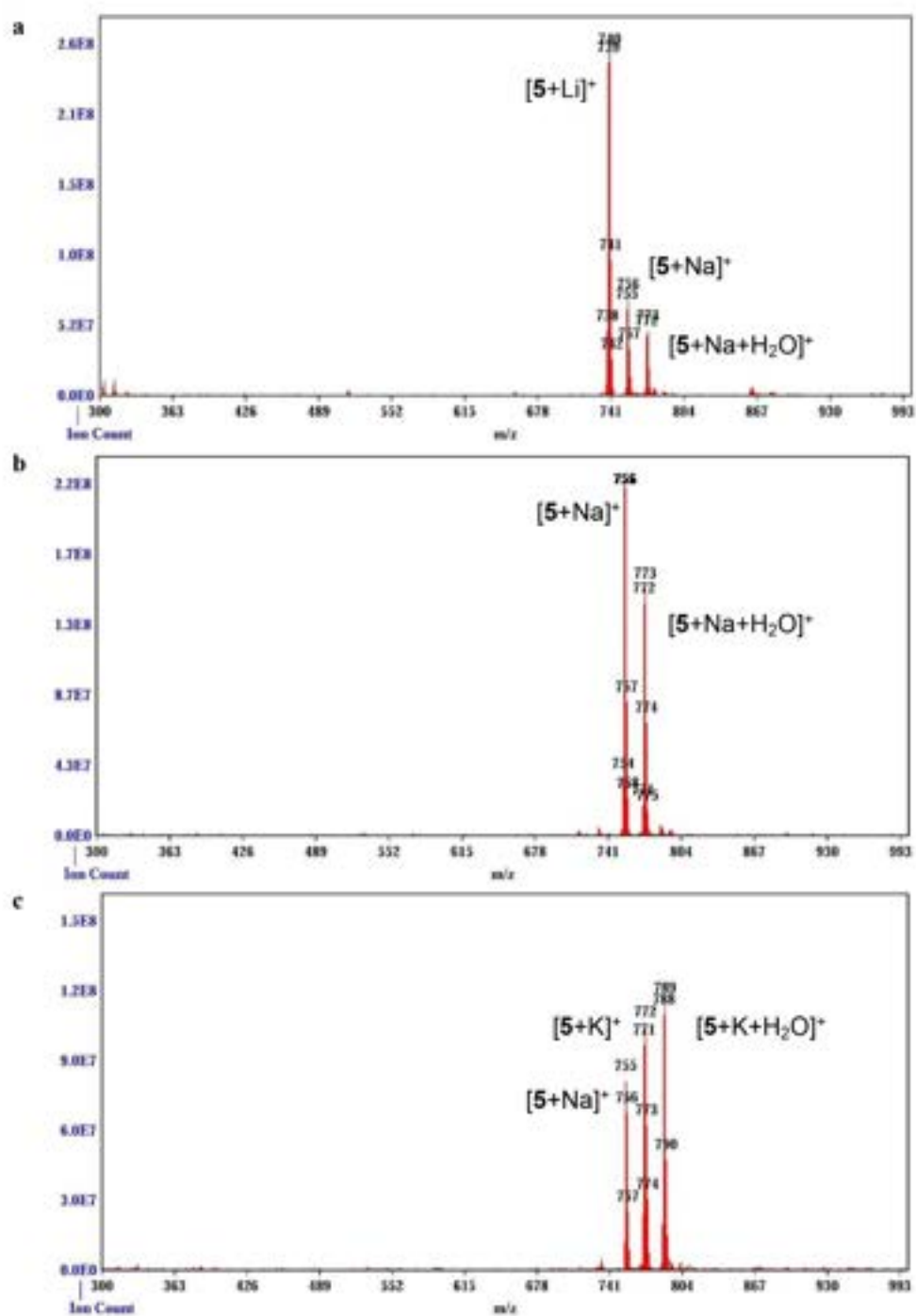


Figure S15. The ESI mass spectra of a mixture of **5** with (a) LiClO_4 , (b) NaClO_4 , and (c) KClO_4 .

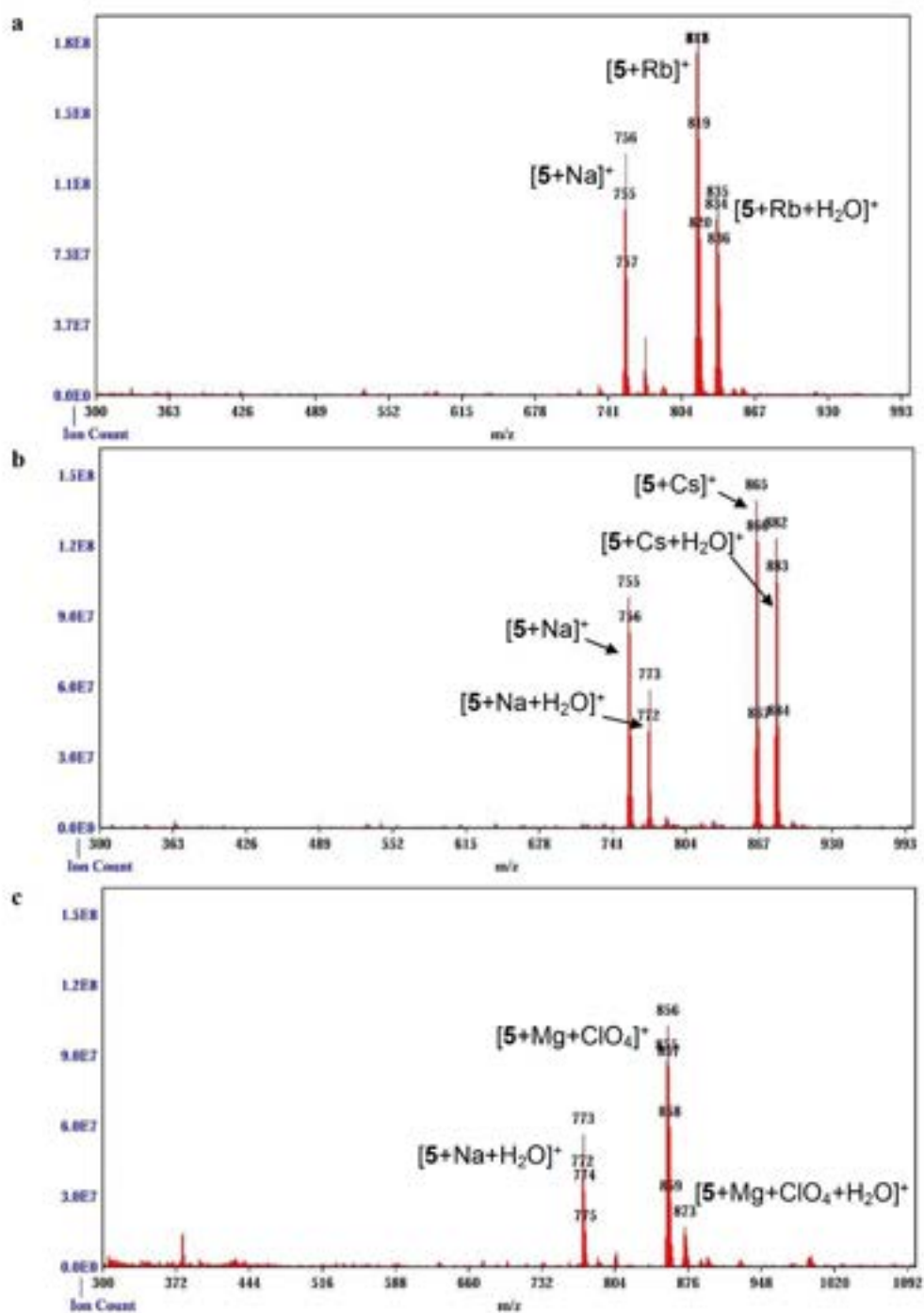


Figure S16. The ESI mass spectra of a mixture of **5** with (a) $RbClO_4$, (b) $CsClO_4$, and (c) $Mg(ClO_4)_2$.

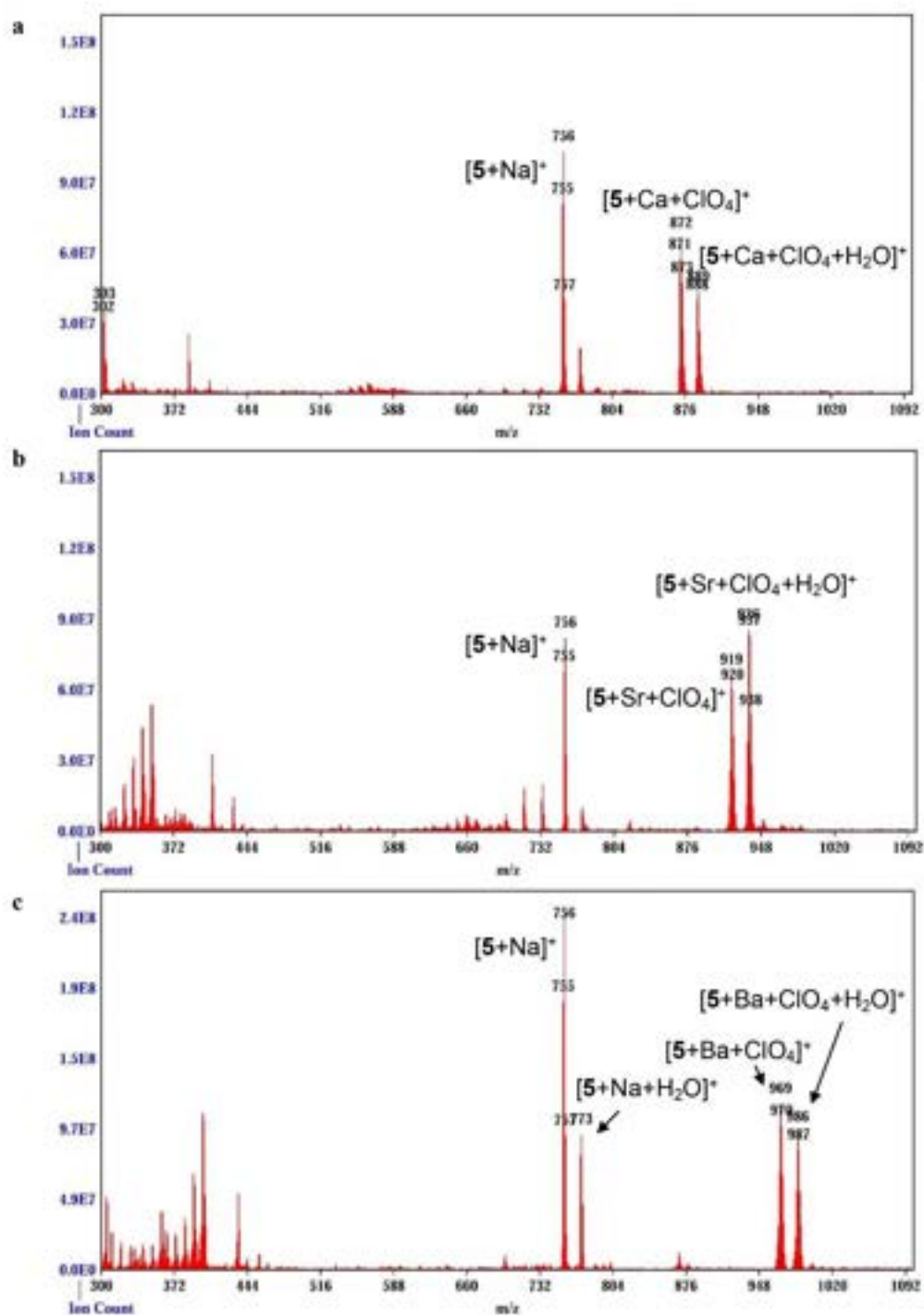
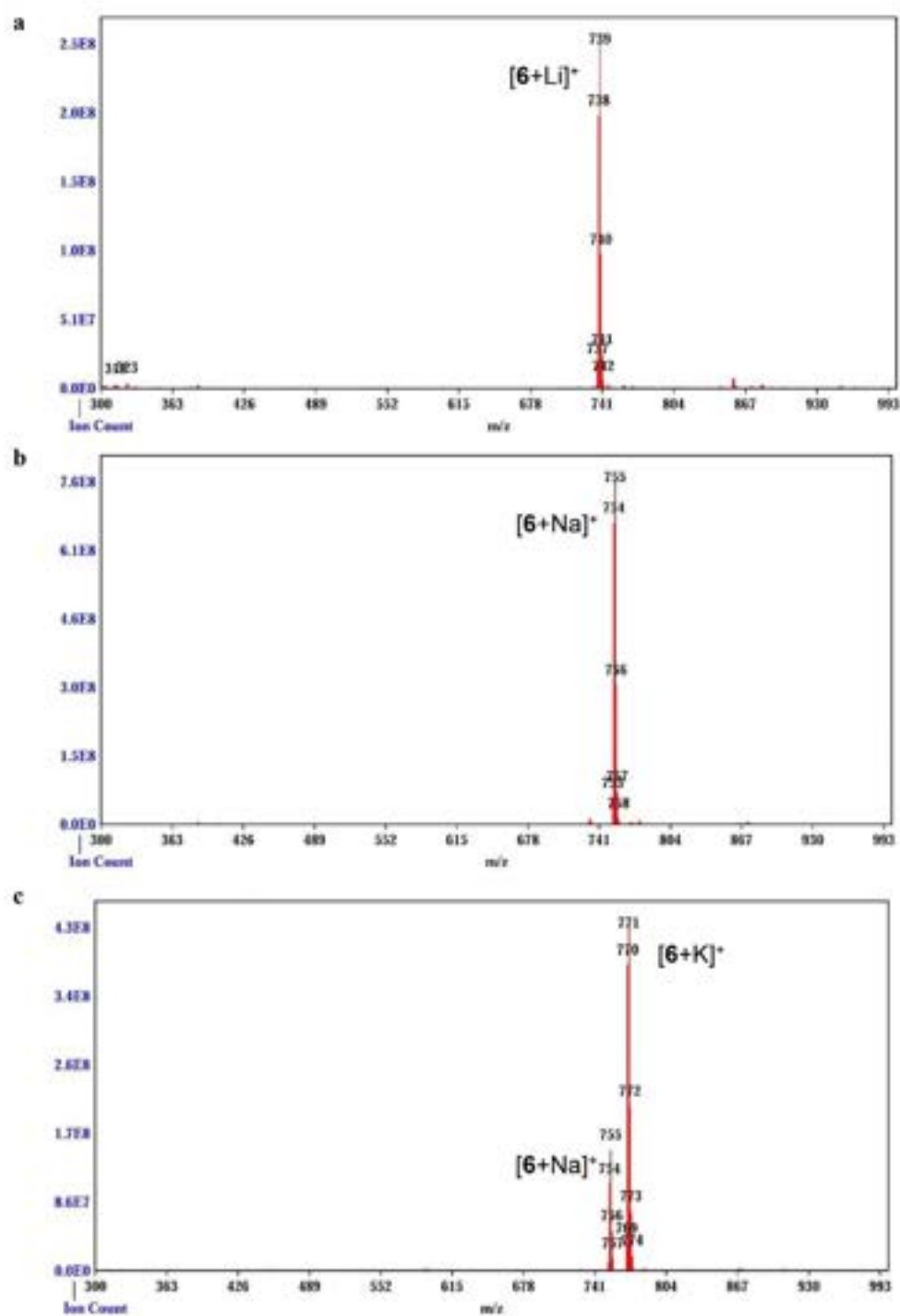


Figure S17. The ESI mass spectra of a mixture of 5 with (a) $Ca(ClO_4)_2$, (b) $Sr(ClO_4)_2$, and (c) $Ba(ClO_4)_2$.

Figure S18. The ESI mass spectra of a mixture of 6 with (a) $LiClO_4$, (b) $NaClO_4$, and (c) $KClO_4$.

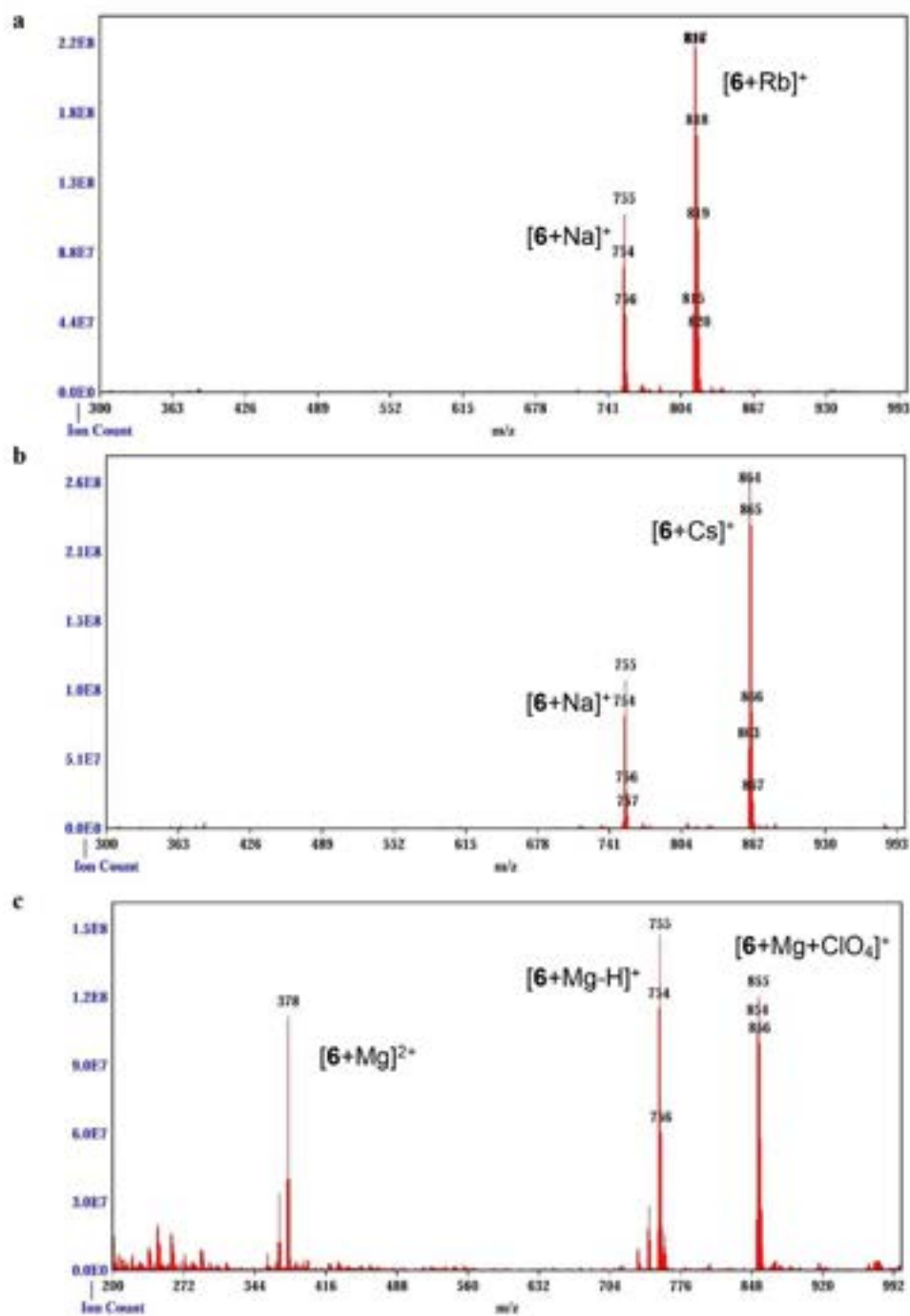


Figure S19. The ESI mass spectra of a mixture of 6 with (a) RbClO_4 , (b) CsClO_4 , and (c) $\text{Mg}(\text{ClO}_4)_2$.

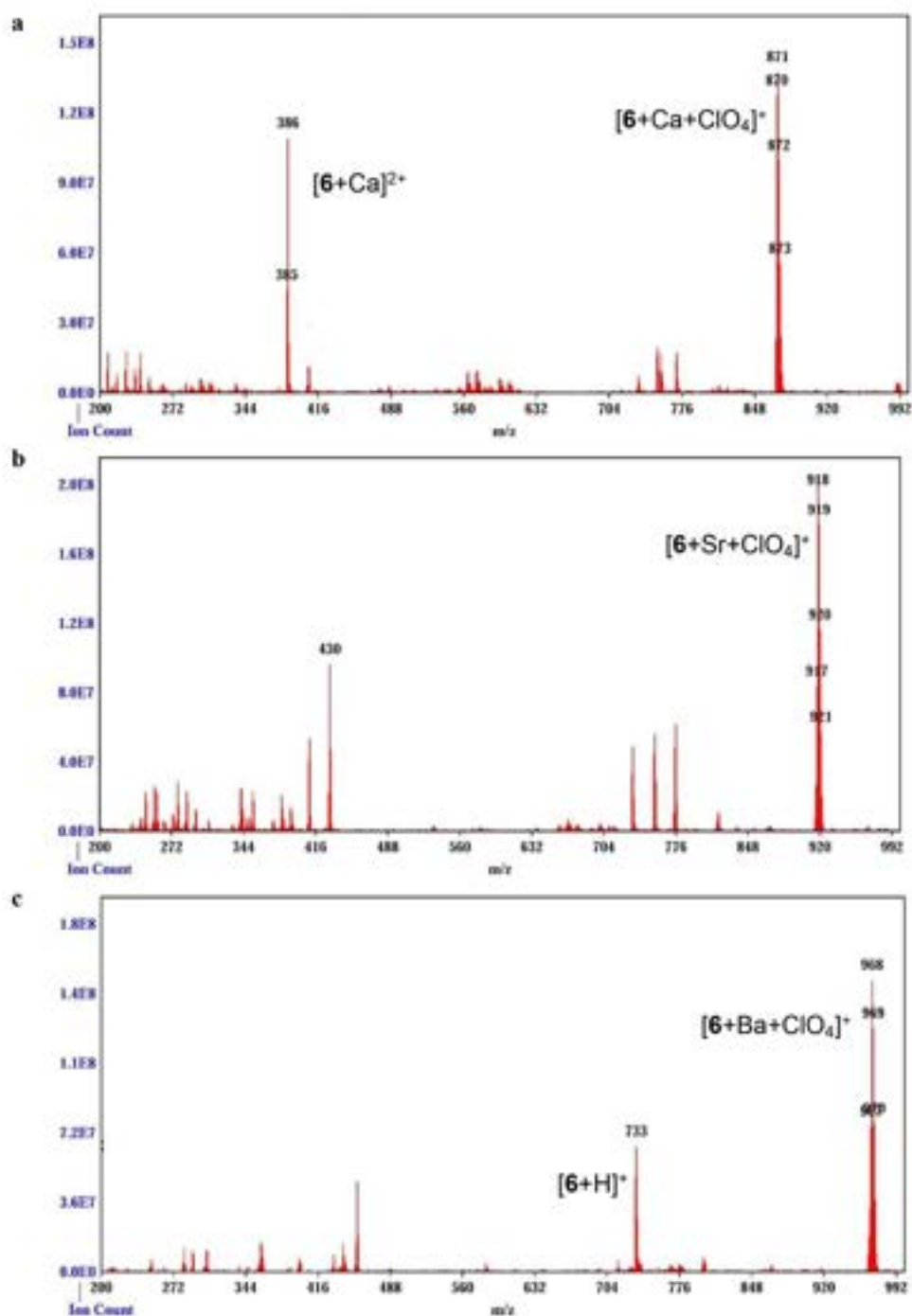


Figure S20. The ESI mass spectra of a mixture of **6** with (a) $Ca(ClO_4)_2$, (b) $Sr(ClO_4)_2$, and (c) $Ba(ClO_4)_2$.

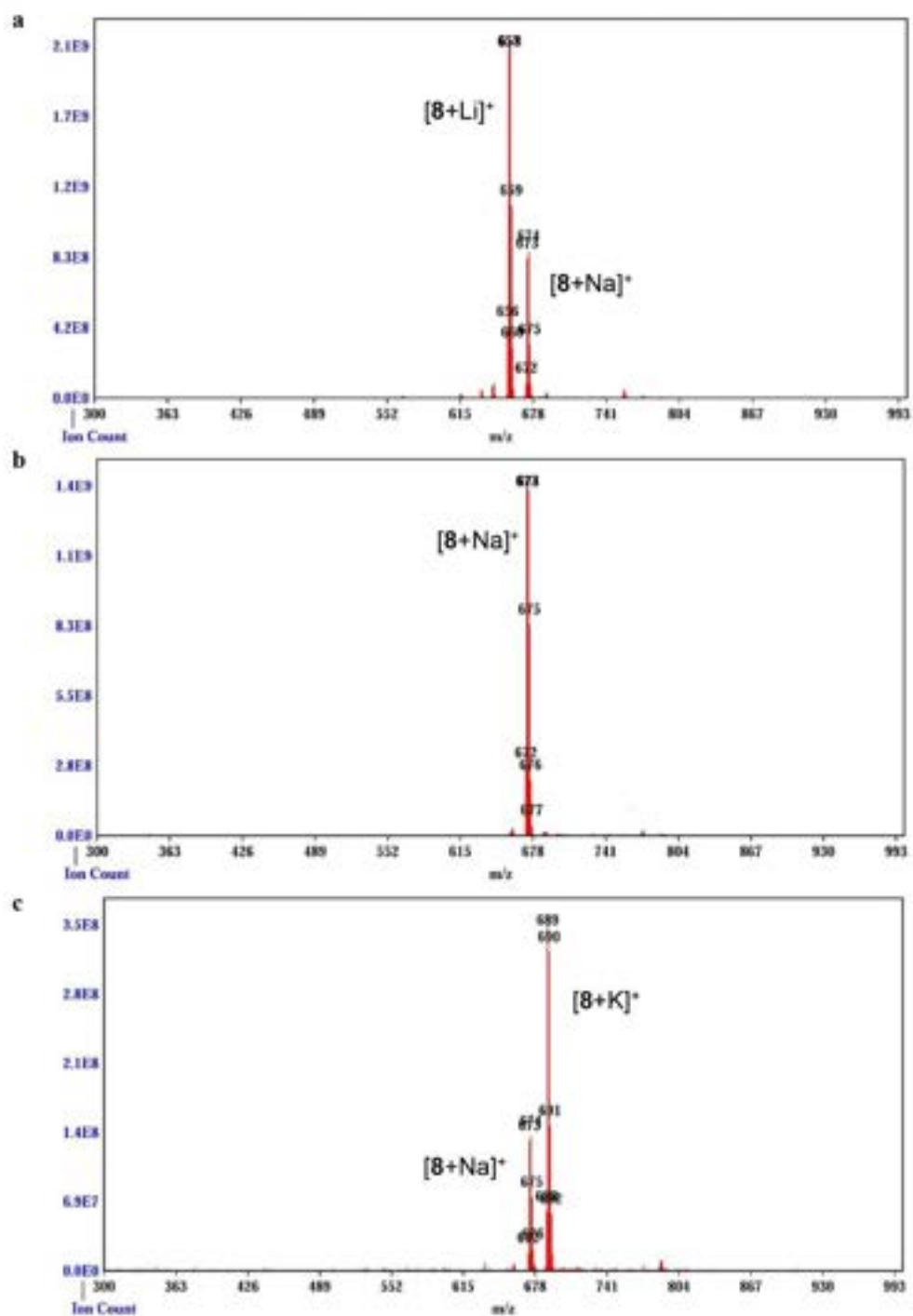


Figure S21. The ESI mass spectra of a mixture of **8** with (a) LiClO₄, (b) NaClO₄, and (c) KClO₄.

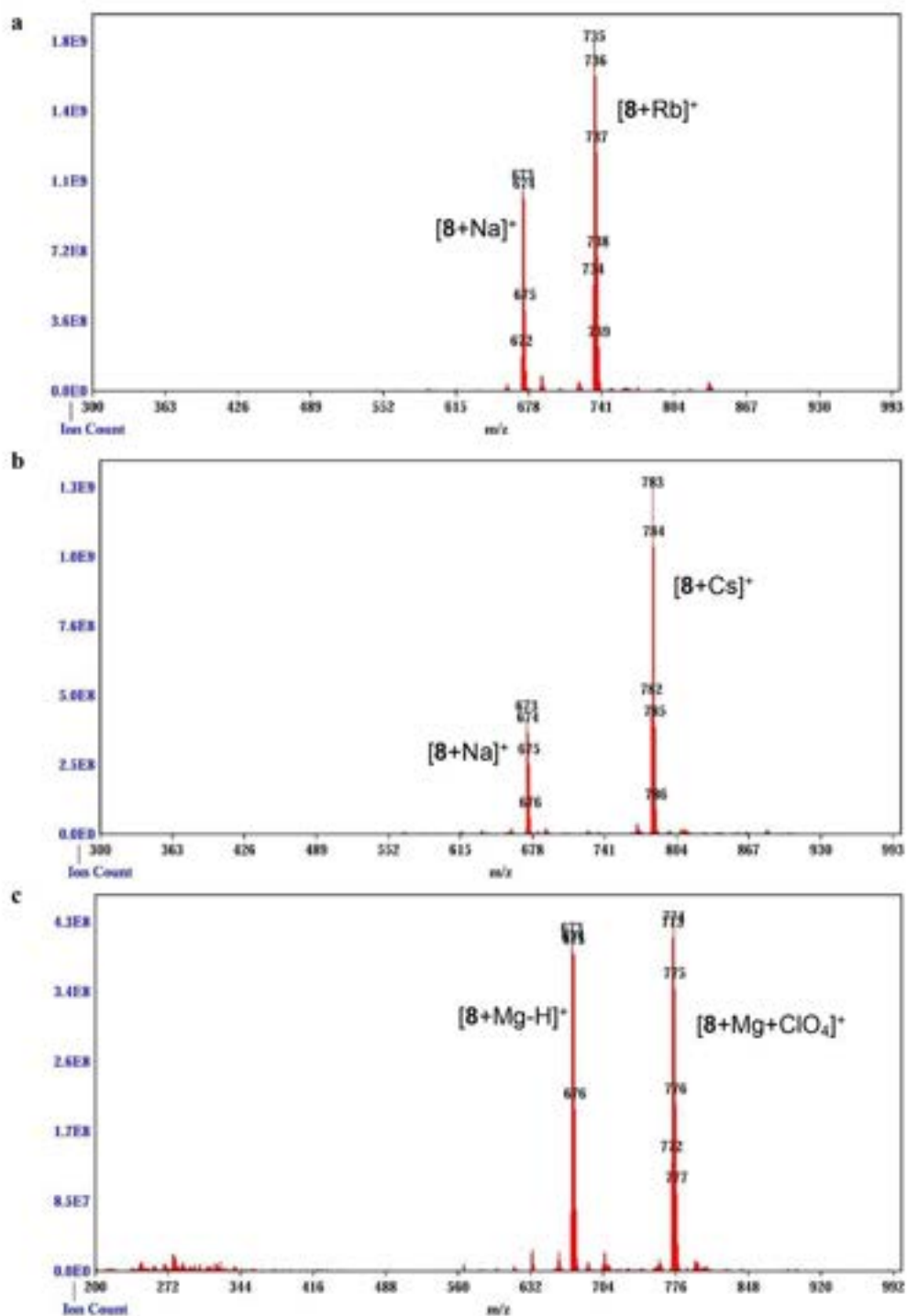
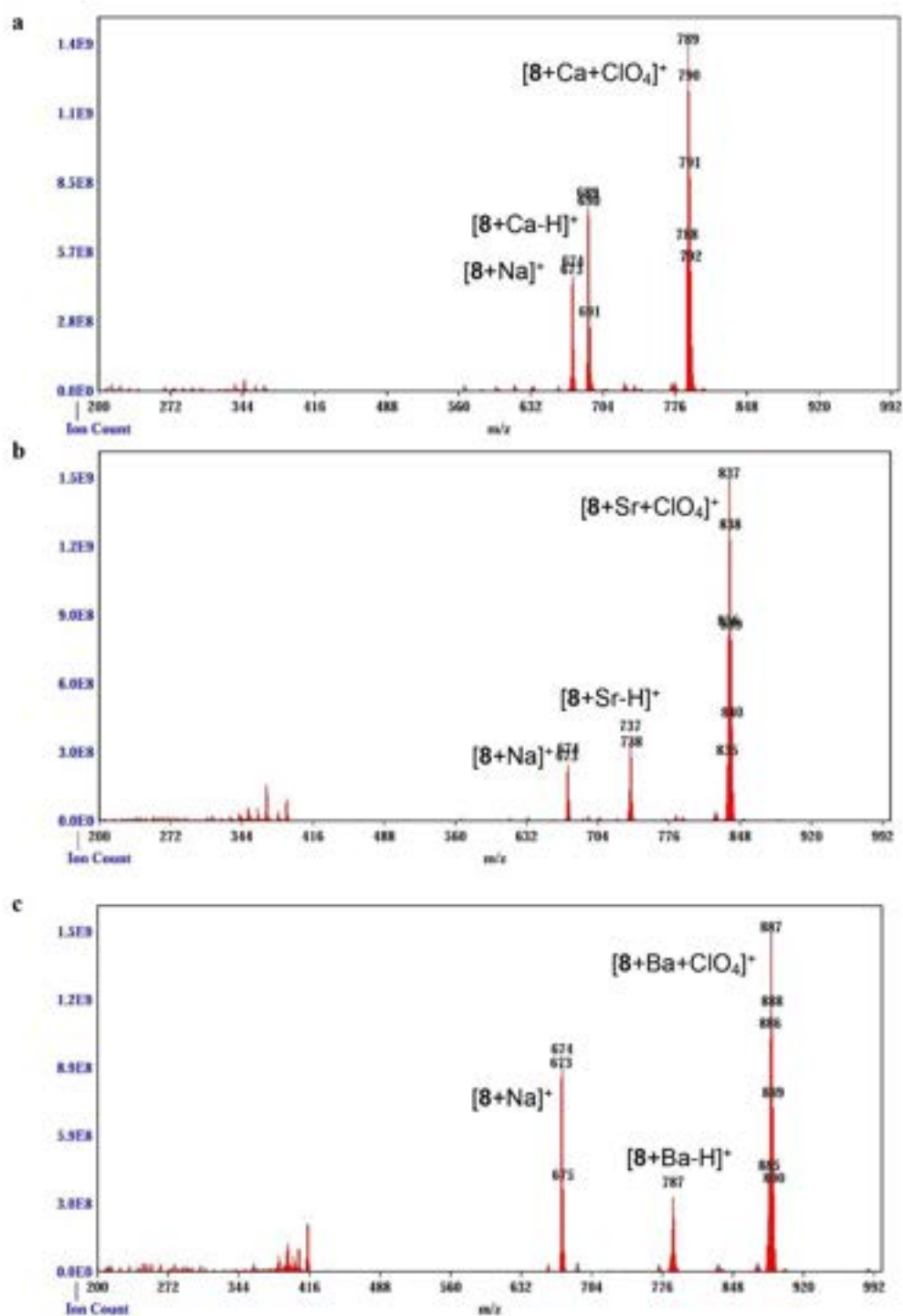
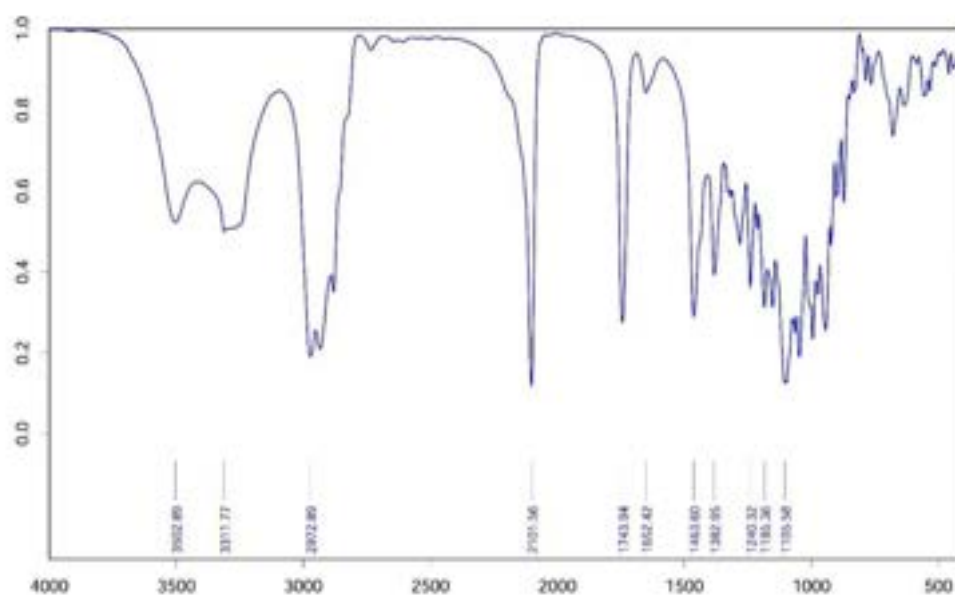
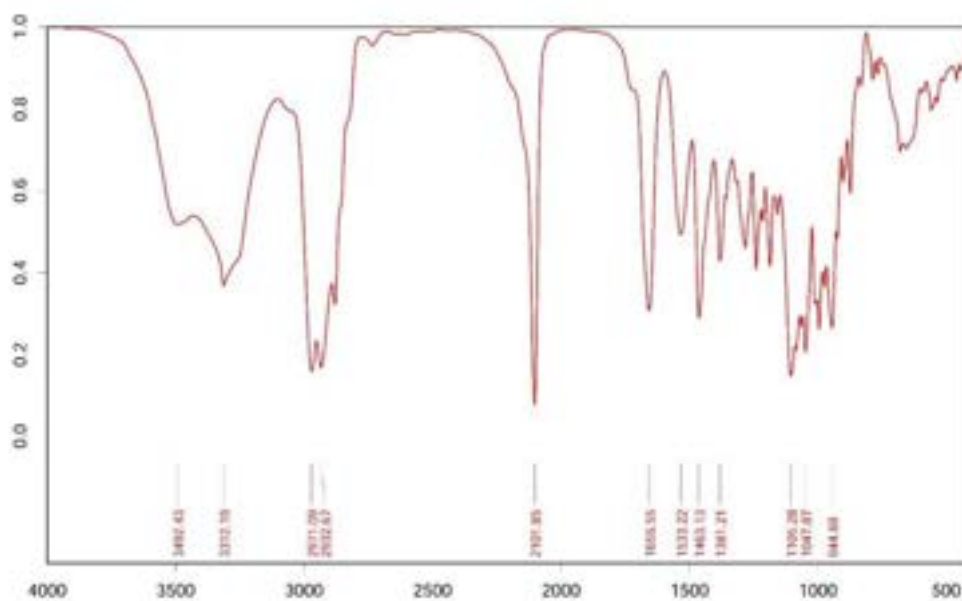


Figure S22. The ESI mass spectra of a mixture of **8** with (a) RbClO_4 , (b) CsClO_4 , and (c) $\text{Mg}(\text{ClO}_4)_2$.

Figure S23. The ESI mass spectra of a mixture of **8** with (a) $\text{Ca}(\text{ClO}_4)_2$, (b) $\text{Sr}(\text{ClO}_4)_2$, and (c) $\text{Ba}(\text{ClO}_4)_2$.

Figure S24. The FT-IR spectrum of **3** in the range of 4000-500 cm⁻¹.Figure S25. The FT-IR spectrum of **4** in the range of 4000-500 cm⁻¹.

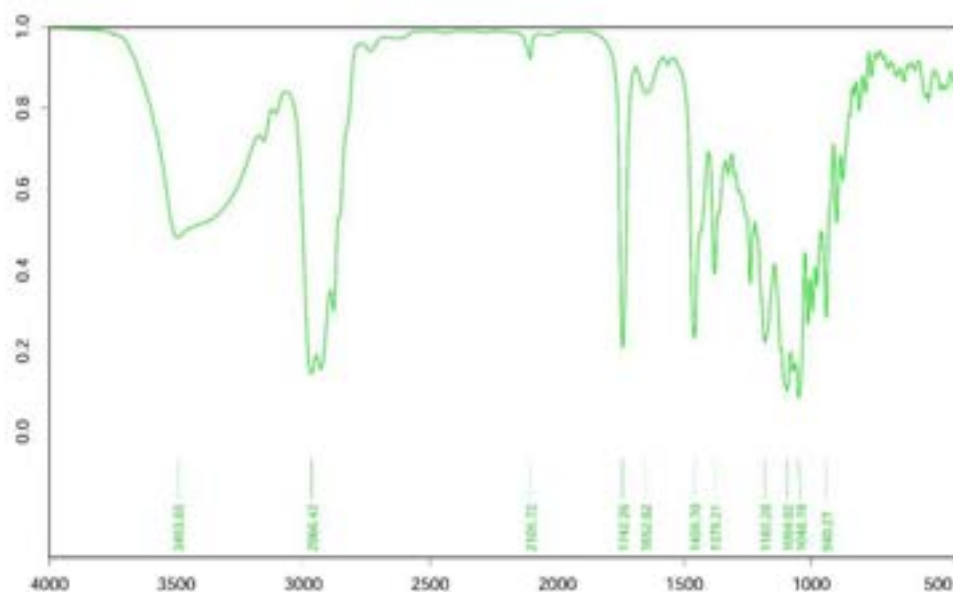


Figure S26. The FT-IR spectrum of **5** in the range of 4000-500 cm⁻¹.

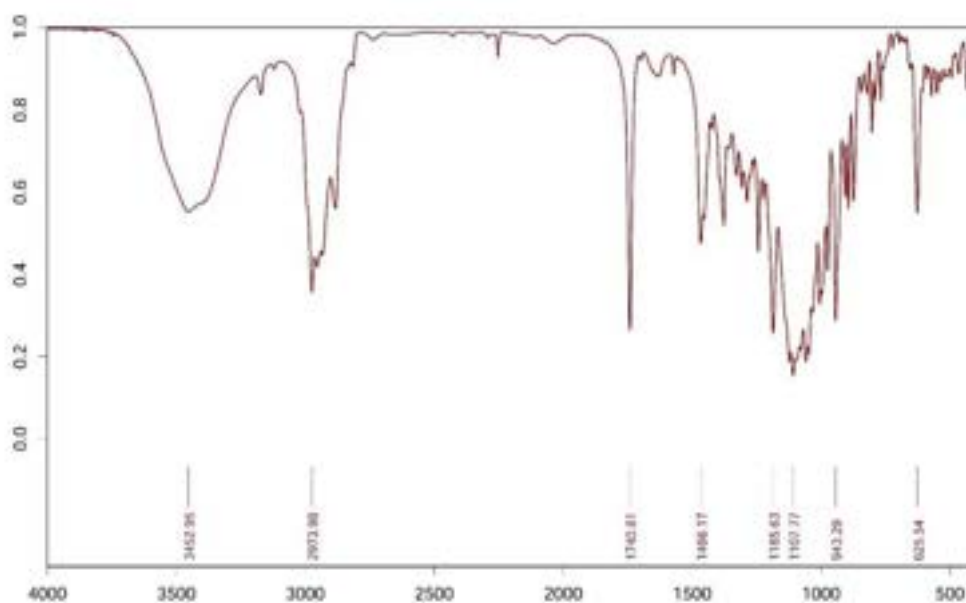
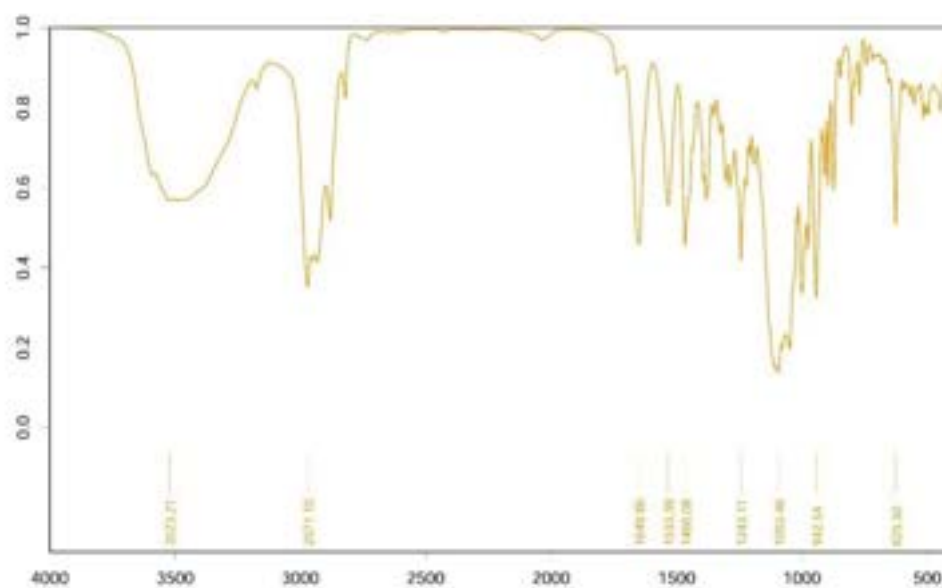
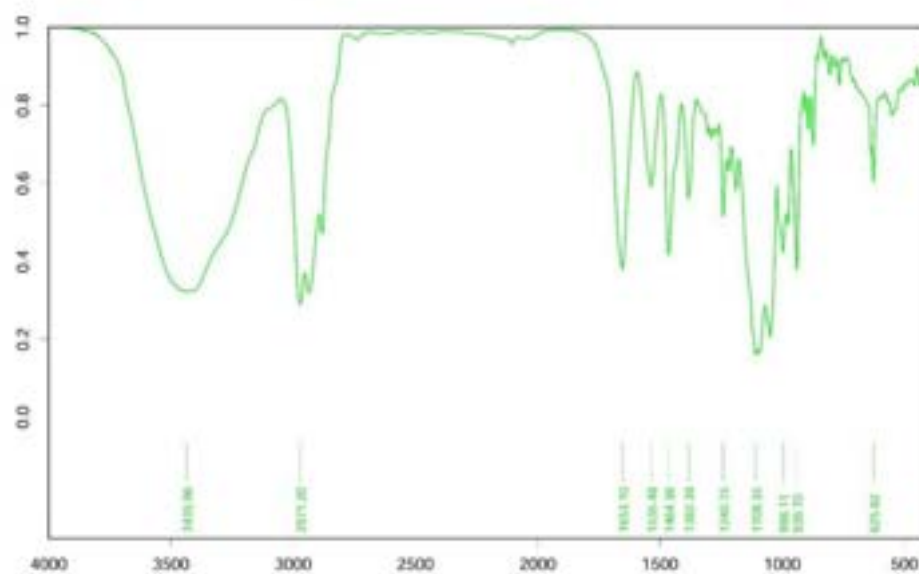


Figure S27. The FT-IR spectrum of **5** with the addition of NaClO₄ in the range of 4000-500 cm⁻¹.

Figure S28. The FT-IR spectrum of **6** in the range of 4000-500 cm⁻¹.Figure S29. The FT-IR spectrum of **6** with the addition of NaClO₄ in the range of 4000-500 cm⁻¹.

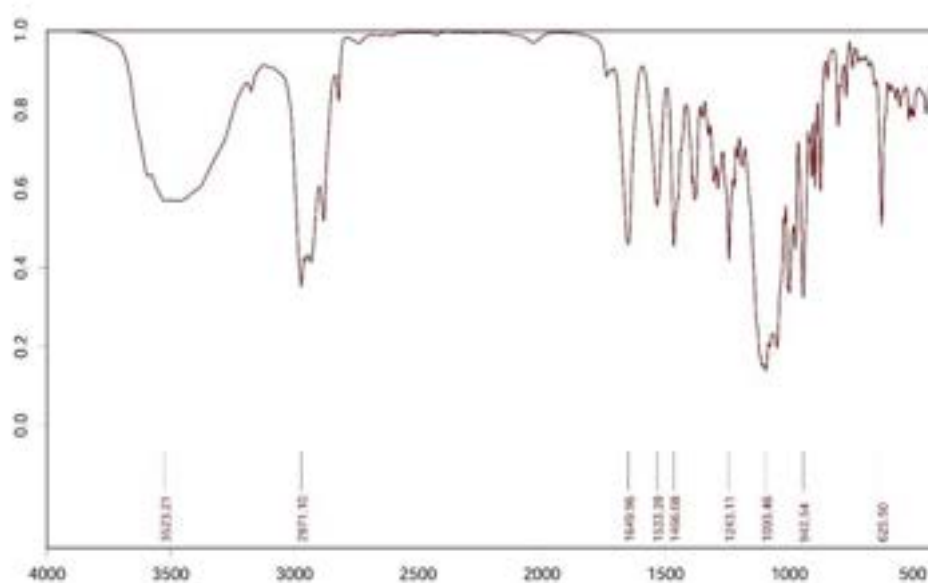


Figure S30. The FT-IR spectrum of **8** in the range of 4000-500 cm⁻¹.

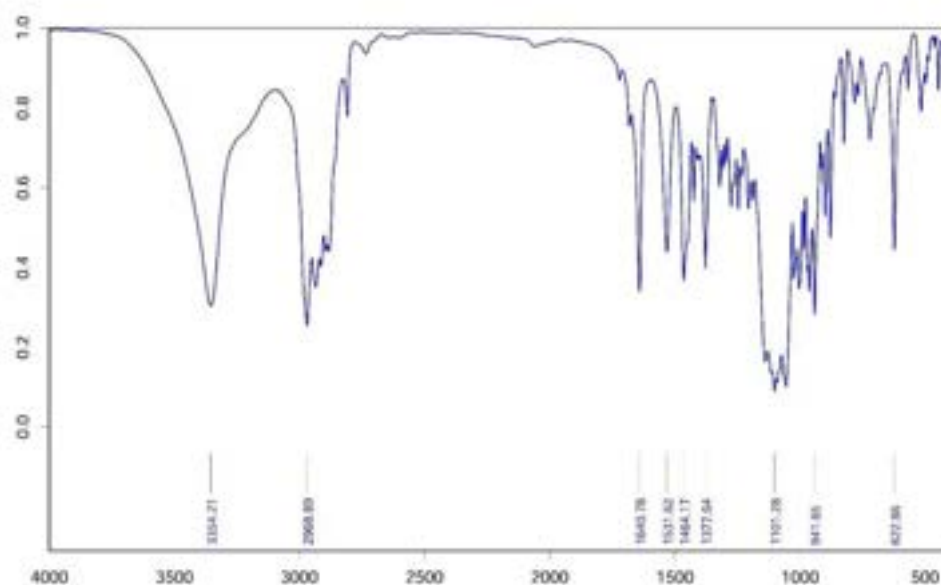


Figure S31. The FT-IR spectrum of **8** with the addition of NaClO₃ in the range of 4000-500 cm⁻¹.

X-ray measurements

The X-ray intensity data for compounds **2**, **5**, and **8** were collected using graphite monochromatic Mo K α radiation on a four-circle κ geometry Xcalibur diffractometer with Sapphire2 area CCD detector. Data collections were made using the CrysAlisPro 1.171.42.93a. Integration, scaling of the reflections, corrections for Lorenz and polarization effects and absorption corrections were performed using the CrysAlisPro 1.171.42.93a program.¹ The structures were solved by the direct methods using SHELXT-2014/7² and refined using SHELXL-2018/3 program.³ The hydrogen atoms joined to carbon atoms were introduced in their geometrical positions and treated as rigid. The H atoms involved in the hydrogen bonds were refined if they gave reasonable hydrogen bonds, otherwise they were constrained. The final difference Fourier maps showed no peaks of chemical significance. Details of the data collection parameters, crystallographic data and final agreement parameters are collected in Table S1. The hydrogen bonds are summarized in Table S2–S4. Visualizations of the structures were made with the Diamond 3.0.⁴

Table S1. Crystal data, collection data and structure refinement parameters for compounds **2**, **5**, and **8**.

	2	5	8
Formula	C ₃₆ H ₆₀ N ₃ O ₁₀ ·H ₂ O	C ₃₉ H ₆₇ ClN ₃ NaO ₁₄ ·C ₂ H ₅ N	C ₃₆ H ₆₇ ClNNaO ₁₃
Molecular weight	713.89	897.41	774.29
Temperature (K)	100	100	100
Crystal system	Monoclinic	Monoclinic	Orthorhombic
Space group	<i>P</i> 2 ₁	<i>P</i> 2 ₁	<i>P</i> 2 ₁ 2 ₁ 2 ₁
Unit cell dimensions	<i>a</i> (Å)	8.2504(2)	12.0123(4)
	<i>b</i> (Å)	17.1298(5)	14.9800(5)
	<i>c</i> (Å)	13.7271(3)	22.0196(6)
	β (°)	97.865(2)	—
<i>V</i> (Å ³)	1921.77(8)	2254.35(19)	3962.3(2)
<i>Z</i>	2	2	4
<i>F</i> (000)	776	960	1664
<i>D</i> _{calc} (g cm ⁻³)	1.234	1.322	1.298
θ range (°)	3.0–27.9°	3.0–26.9°	3.1–27.8°
μ (mm ⁻¹)	0.09 mm ⁻¹	0.16 mm ⁻¹	0.17 mm ⁻¹
Crystal size (mm)	0.31 × 0.26 × 0.21	0.31 × 0.28 × 0.23	0.28 × 0.24 × 0.19
<i>T</i> _{min} / <i>T</i> _{max}	0.978/1.000	0.923/1.000	0.982/1.000
Total / unique / obs reffs	42469/9508/7643	41081/10198/8912	20210/9341/6500
<i>R</i> _{int}	0.041	0.048	0.046

$R = [F^2 - 2\sigma(F^2)]^2$	0.047	0.094	0.068
$wR = [F^2 \text{ all refls}]^2$	0.097	0.230	0.154
S	1.00	1.00	1.01
Flack parameter	-0.1 (2)	0.07(6)	0.04(5)
$\Delta\rho_{\text{max}}, \Delta\rho_{\text{min}}$ (eÅ ⁻³)	0.28, -0.23	0.71, -0.47	0.76, -0.70

^a $R = \sum [|F_o| - |F_c|] / \sum F_o$, $wR = [\sum [w(F_o^2 - F_c^2)^2] / \sum wF_o^4]^{1/2}$; $w^{-1} = \sigma^2(F_o^2) + (aP)^2 + bP$ where $P = (F_o^2 + 2F_c^2)/3$, $a = 0.0391$ and $b = 0.5022$ for **2**, $a = 0.0803$ and $b = 11.2600$ for **5** and $a = 0.0503$ and $b = 3.7385$ for **8**.

Table S2. Hydrogen bond geometry for **2** (Å, °).

<i>D</i> —H... <i>A</i>	<i>D</i> —H	H... <i>A</i>	<i>D</i> ... <i>A</i>	<i>D</i> —H... <i>A</i>
O1—H1...O11	0.86 (3)	1.83 (4)	2.663 (3)	164 (3)
O3—H3A...O5	0.80 (4)	2.15 (4)	2.809 (3)	140 (3)
O3—H3A...O11	0.80 (4)	2.60 (4)	3.136 (3)	126 (3)
O9—H9A...O10	0.86 (4)	2.37 (4)	2.943 (3)	124 (3)
O9—H9A...O3	0.86 (4)	2.42 (4)	3.035 (3)	128 (3)
O11—H11C...O6	0.99 (4)	1.89 (4)	2.869 (3)	170 (3)
O11—H11D...O7	1.02 (4)	2.44 (3)	3.078 (3)	120 (2)
O11—H11D...O8	1.02 (4)	1.96 (4)	2.940 (3)	160 (3)

Table S3. Hydrogen bond geometry for **5** (Å, °).

<i>D</i> —H... <i>A</i>	<i>D</i> —H	H... <i>A</i>	<i>D</i> ... <i>A</i>	<i>D</i> —H... <i>A</i>
O3—H3A...O9	0.88 (2)	1.95 (5)	2.737 (8)	149 (6)
O9—H9...O11 ⁱ	0.84 (3)	2.22 (8)	2.844 (8)	131 (9)
O9—H9...O13 ⁱ	0.84 (3)	2.53 (9)	3.094 (9)	126 (9)

Symmetry code: (i) $x-1, y, z$.

Table S4. Hydrogen bond geometry for **8** (Å, °).

<i>D</i> —H... <i>A</i>	<i>D</i> —H	H... <i>A</i>	<i>D</i> ... <i>A</i>	<i>D</i> —H... <i>A</i>
N1—H1...O13a	0.86 (6)	2.60 (6)	3.303 (11)	140 (5)
N1—H1...O14a	0.86 (6)	2.08 (6)	2.859 (11)	151 (5)
N1—H1...O22b	0.86 (6)	2.32 (6)	3.152 (10)	163 (5)
N1—H1...O23b	0.86 (6)	2.35 (6)	3.018 (13)	135 (5)
O3—H3...O9	0.82 (6)	1.95 (6)	2.760 (5)	170 (6)

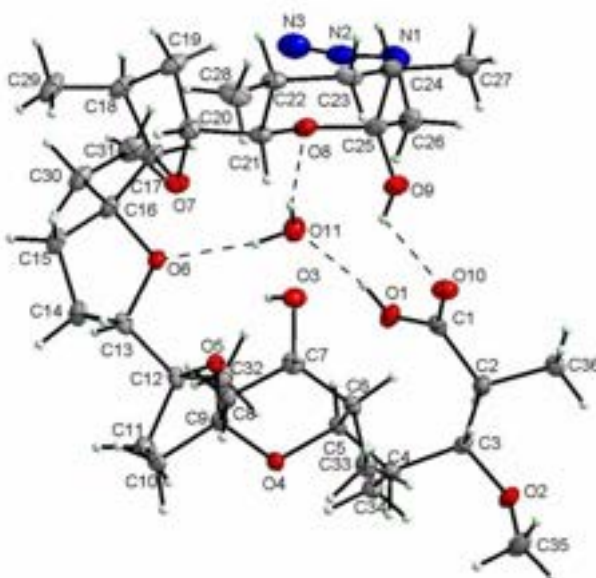


Figure S32. The crystal structure of the azide **2**. The anisotropic displacement parameters are presented at a probability level of 40%, and the H atoms are shown as circles with arbitrary radii.

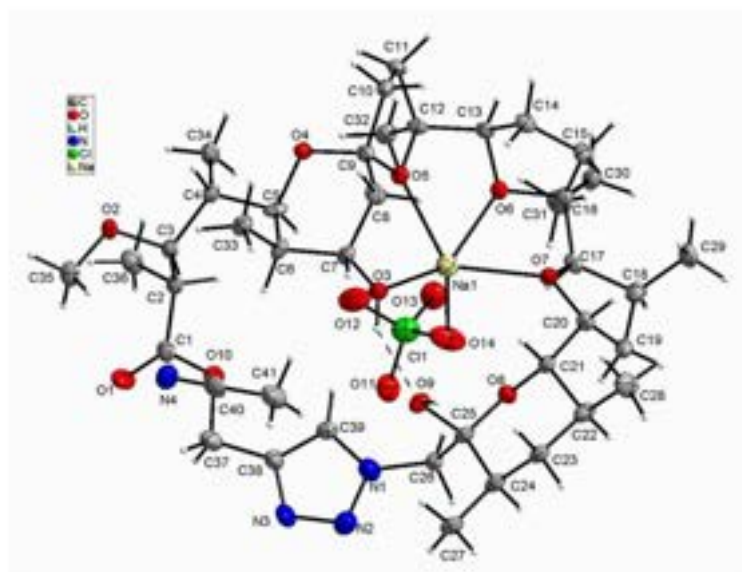


Figure S33. Crystal structure of the lactone **5**. The anisotropic displacement parameters are presented at a probability level of 40%, and the H atoms are shown as circles with arbitrary radii.

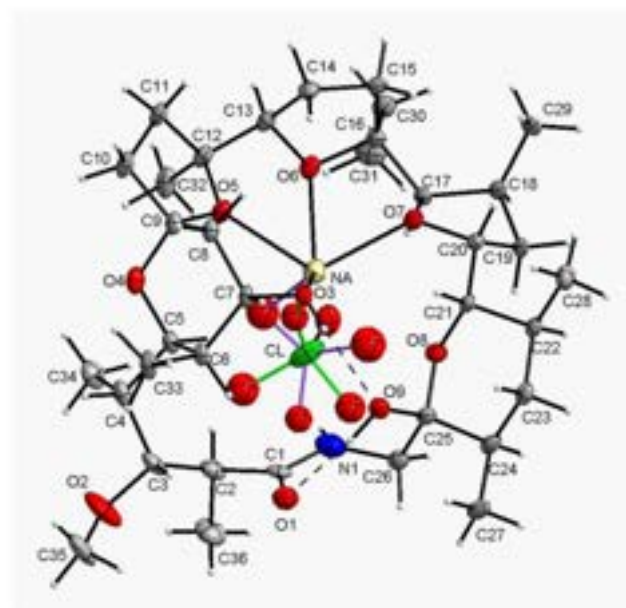


Figure S34. Crystal structure of the lactam **8**. In the structure, the perchlorate exist in two orientations, the superposition of which is shown in the picture. The anisotropic displacement parameters are presented at a probability level of 40% while the oxygen atoms of both orientations of perchlorate anion are shown at the 30% and the H atoms are shown as circles with arbitrary radii.

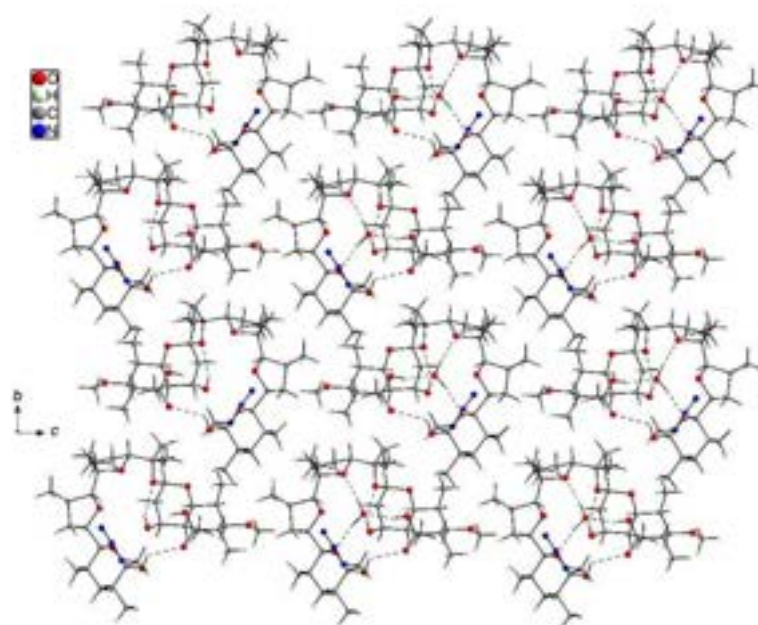


Figure S35. Packing along for **2**.

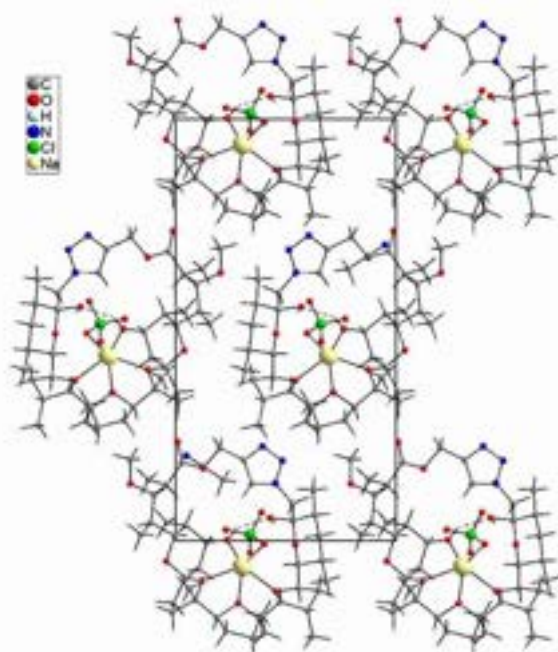


Figure S36. Packing along for **5**.

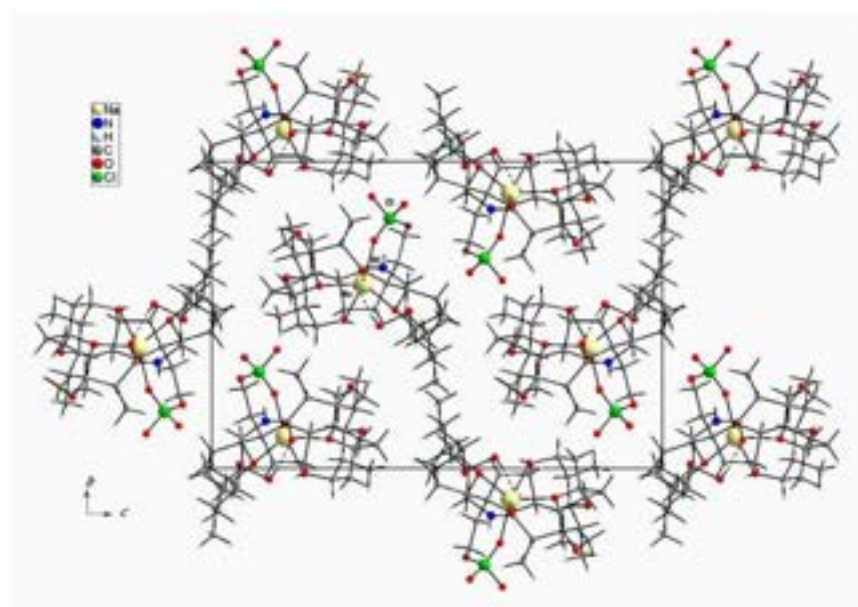


Figure S37. Packing along for **8**.

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- (4) K. Brandenburg, H. P. DIAMOND Version 3.0. Crystal Impact GbR, Bonn, Germany 2006.

11.7. Publikacja VII

Michał Sulik, Eyob A. Workneh, Sofia Santana, Bárbara Teixeira, Miguel Prudêncio,
Jan Janczak, Adam Huczyński

Chemical modification of monensin as a source of potent antiplasmodial agents

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Opis wkładu doktoranta w powstanie publikacji: Jestem w pełni odpowiedzialny za syntezę oraz oczyszczanie wszystkich pochodnych monenzyny opisanych w pracy. Odpowiadałem za koordynowanie badań w tym kontakt z innymi naukowcami. Przeprowadziłem też analizę otrzymanych wyników, w tym tych z badań biologicznych. Odpowiadałem za napisanie manuskryptu.



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Chemical modification of monensin as a source of potent antiplasmodial agents

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ABSTRACT

Malaria remains a significant public health issue and one of the leading causes of child mortality worldwide. Due to the growing problem of drug resistance, new modes of fighting the disease are searched for. In this context, ionophore antibiotics, natural compounds with high potential for combating parasitic diseases, deserve special attention. The primary representative of such compounds, monensin (MON), demonstrates exceptionally high antiplasmodial activity. In this work, the C26-amino derivative of MON was used as a convenient substrate for the synthesis of its acyl analogues, such as amides and urea. All derivatives exhibited strong activity against the hepatic stage of *Plasmodium berghei* infection *in vitro*, which exceeded that shown by the reference drug primaquine. The IC₅₀ value for MON O-phenyl urethane (8) was less than 1 nM.

1. Introduction

According to WHO data, in 2023, malaria caused the death of nearly 600,000 people, making it the deadliest parasitic disease in the modern world.¹ Malaria is caused by *Plasmodium* parasites transmitted in their sporozoite form by infected female *Anopheles* mosquitoes.^{1,2} After injection into their mammalian host during an infected mosquito bite, sporozoites asymptotically infect and replicate inside hepatocytes, generating thousands of hepatic merozoites. Following their release into the bloodstream, merozoites cyclically infect erythrocytes causing the symptoms of malaria. These include fever and headache, but may progress to cause severe disease, or even death.^{1,3,4} The most important malaria control strategies involve vector control, the introduction of the RTS,S/AS01, and the R21/Matrix-M malaria vaccines, and chemotherapies.^{1,5} Several medicines are currently available to treat malaria (e.g. chloroquine, primaquine, and artemisinin combination therapies), but due to the growing problem of drug resistance, the quest for new

antimalarial drug candidates is highly desirable.^{1,3,6}

Among many natural compounds with high biological activity, ionophore antibiotics have merited particular attention, as they exhibit a wide range of antiparasitic activities.⁷ The main representative of this group of compounds is monensin (MON, Fig. 1), which is successfully used in veterinary practice as an animal feed additive that prevents coccidiosis (Coxidin®).⁸ The high antimicrobial activity of MON results from its natural ability to coordinate metal cations and transfer them across biological membranes.^{9,9} This phenomenon leads to the disturbance of the intracellular pH and ion concentration gradient, which leads to mitochondrial damage, vacuolization, and eventually cell death.^{7,9} In addition to its antioecidal activity, MON has proven its potential in combating parasites causing diseases such as trypanosomiasis, leishmaniasis, toxoplasmosis, and malaria.^{7,10–14} Several studies aimed at the chemical modification of MON skeleton to obtain compounds with enhanced biological activity have been reported.^{15–17} They concerned the anticancer and antibacterial properties of this type of

Abbreviations: aq, aqueous solution; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; HRMS, high resolution mass spectrometry; Huh7, human hepatoma cell line; IC₅₀, half maximal inhibitory concentration; MON, monensin A; NMR, nuclear magnetic resonance spectroscopy; rt, room temperature; SAR, structure-activity relationship; TEA, triethylamine; TTP⁺, triphenylphosphonium cation; TiCl₄, 4-toluenesulfonyl chloride; WHO, World Health Organization.

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derivatives.^{16–18} Although reports on the antiparasitic activity of MON analogues are still limited, some studies have shown that rational modification of its structure can lead to the derivatives with higher antiparasitic potential than the unmodified ionophore.^{7,13,14,17–19} MON and some of its analogues have been shown to display potent activity against malaria parasites. Among them, C26-modified analogues, such as tosylate or carbonate, exhibited *in vitro* activity against *P. falciparum* with IC_{50} values of 1.52 and 1.08 nM, respectively, exceeding the activity of the starting compound (Fig. 1).^{7,13,14} Nevertheless, the chemical modification of MON to find new antiplasmodial derivatives remains a very promising, and still underexplored, research direction.

2. Results and discussion

2.1. Design strategy

As mentioned above, the antimicrobial activity of ionophore antibiotics results from their natural ability to coordinate metal cations and transport them across biological membranes.^{7–9} This process occurs via electroneutral transport with deprotonation of the carboxyl group or electrogenic transport without deprotonation of the carboxyl group, without or with a change in the transmembrane potential, respectively.^{20,21} This means that modification of group C26 of MON is a promising approach to obtain its bioactive analogues, as it does not hinder the carboxyl group from participating in the ion exchange mechanism. However, it has also been shown that ionophores with a modified carboxyl group can perform a third type of transport, known as biomimetic transport.^{26,27}

Chemical modification of C1 carboxyl group of MON is a well-known and frequently used method for the synthesis of numerous analogues of this ionophore.^{9,15} The molecule's C26 hydroxyl group has also often been modified, but no method has been reported for converting it into another functional group as a convenient lead structure. Thus, C26 derivatives of MON were limited to its esters, ethers, or O-urethanes.^{9,15} However, our team has recently developed the first scalable, efficient, and simple method for obtaining MON's C26 amine.²² The reaction consists of tosylation and subsequent acidification of C26 hydroxyl group. The obtained azide was then reduced to amine using hydrogen in the presence of palladium on carbon (compound 2, Scheme 1).²²

2.2. Chemistry

Having access to an amine precursor, it is possible to synthesize numerous analogues, such as amides or ureas. Therefore, in this paper, we present the synthesis and antimalarial activity of a narrow library of compounds, which illustrates the potential for developing different groups of new MON derivatives. We chose to synthesize aromatic analogues as representatives of each group of derivatives because previous

studies have shown their antimicrobial potential.¹⁷ Acylated derivatives of 2 include its benzoyl amide (compound 3) and phenyl urea (compound 4). In addition, an amide conjugate of MON with a triphenylphosphonium cation (TPP⁺, compound 5) was also obtained (Scheme 1). This chemical moiety is naturally directed to the mitochondria due to their highly negative membrane potential.^{23,24}

It is worth noting that several reports have suggested that Plasmodium mitochondria are important therapeutic targets.^{25,26} Furthermore, to extend the knowledge about the antimalarial activity of C1 derivatives of MON, its benzyl ester (compound 7) and ester conjugate with TPP⁺ (compound 6) were also synthesized (Scheme 1). For comparative purposes, the resynthesis of MON O-phenyl urethane (compound 8) was performed, as this compound showed high antimicrobial activity in previous studies.¹⁷

The purity and structure of the newly synthesized and resynthesized MON derivatives (2–8) were determined using spectroscopic (¹H NMR, ¹³C NMR, ³¹P NMR) and spectrometric (HRMS) methods (see Supplementary Information). In the ¹³C NMR spectra of MON C26-analogues (compounds 3, 4, 5, and 8), the signals of the highest analytical significance were assigned to the carboxyl group and appeared in the range of 177.4–184.2 ppm. For C1-esters of MON (compounds 6, and 7), the signals from the ester group appeared in the narrow range of 175.2–176.0 ppm. With respect to the newly introduced carbonyl group at position C26 of MON (compounds 3, 4, 5, and 8), respective signals from the amide/urethane/urea group appeared in the range of 154.7–174.1 ppm. The HRMS analysis confirmed the formation of the intended products, with [M + Na]⁺ as the main peak (100 % intensity) for compounds 3, 4, 7, and 8, and [M]⁺ for compounds 5, and 6. The NMR spectra of all obtained MON derivatives, are included in the Supplementary material (Figs. S1–S16).

X-ray diffraction analysis was employed to confirm and explain the structure of MON derivatives as well as to extract possible structure-activity relationships (SAR). Therefore, two of the C26 derivatives obtained – MON phenyl urea (compound 4) and MON amide conjugate with TPP⁺ (compound 5) as their sodium salts were characterized using the X-ray single crystal diffraction method. Detailed crystal data, refinement parameters and experimental details for both molecules are presented in Table S1 and Figs. S17–S18 (Supplementary material). Single crystals of compounds 4 and 5 were grown by crystallization in acetonitrile solution. The urea derivative crystallized in the non-centrosymmetric space group *R*3:1 of the trigonal system, as a solvate with water molecules, while the amide derivative crystallized in the non-centrosymmetric space group *P*2₁(2)₁2₁ of the orthorhombic system with bromide as counterions and as a solvate with water molecule. The asymmetric unit of both compounds are illustrated in Fig. 2.

Both molecules form complexes with the sodium cation, with the C26 substituent not involved in the coordination process. The pseudo-cyclic structure is stabilized by several hydrogen bonds, whose exact geometry

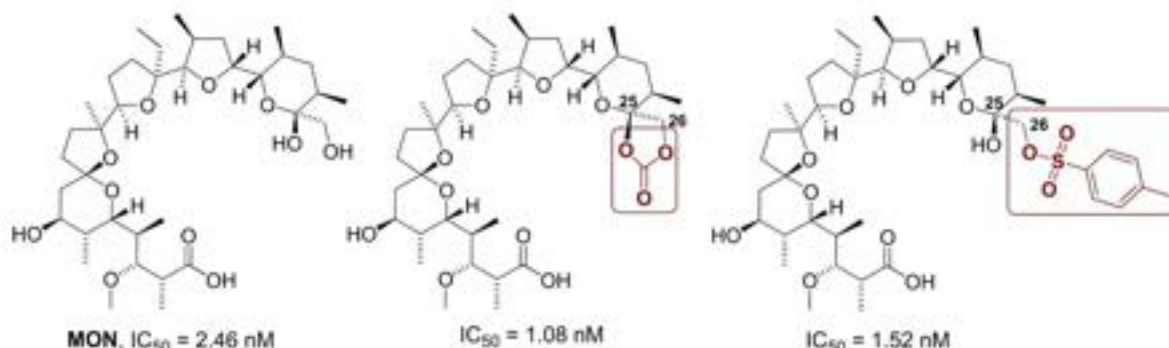
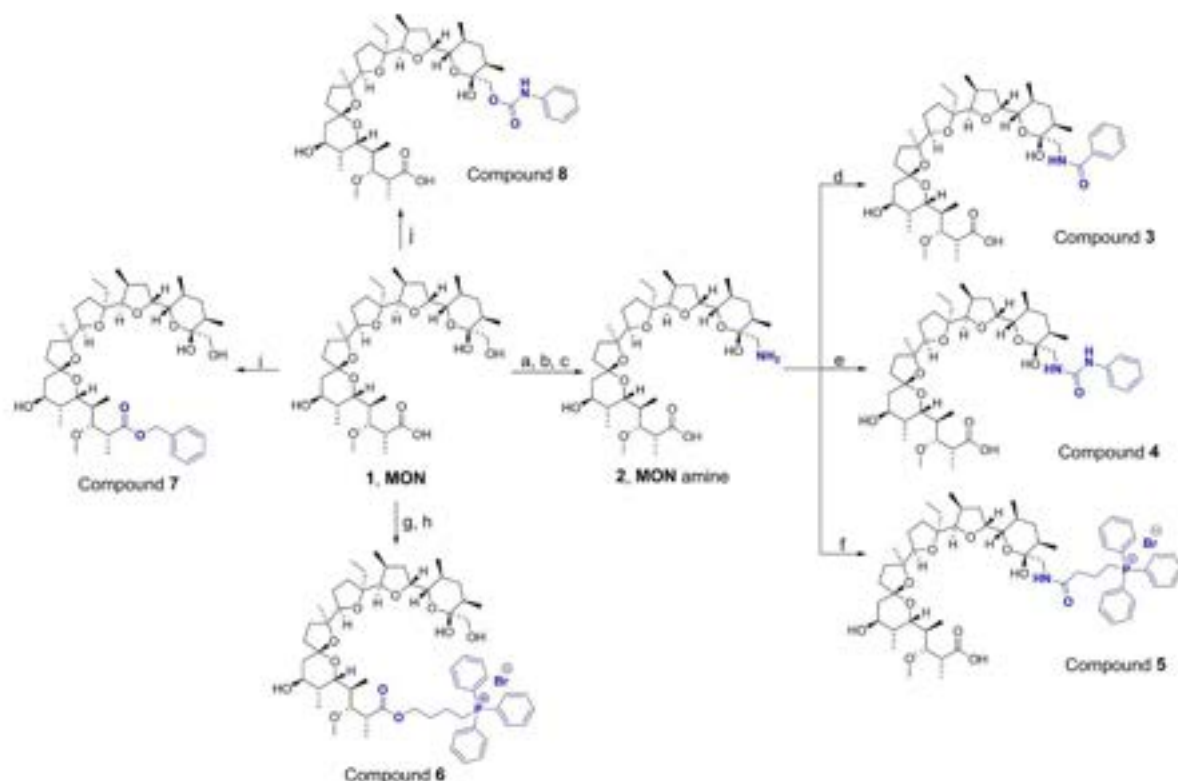


Fig. 1. *In vitro* activity of MON and its C26-derivatives against *P. falciparum*.



Scheme 1. Synthesis of MON aromatic derivatives. *Reagents and conditions:* (a) TlCl, Na₂CO₃/Na₂O, toluene, rt, 72 h; (b) NaN₃, DMSO, rt, 72 h; (c) H₂, Pd/C, MeOH, rt, 72 h; (d) benzoyl chloride, TEA, CH₂Cl₂, 0 °C to rt, 24 h; (e) phenyl isocyanate, toluene, rt, 72 h; (f) (3-carboxypropyl)triphenylphosphonium bromide, ethyl chloroformate, TEA, DMF, -5 °C to rt, 24 h; (g) 1,4-dibromobutane, DBU, toluene, 90 °C, 24 h; (h) triphenylphosphine, CH₂CN, reflux, 72 h; (i) benzyl bromide, DBU, toluene, 90 °C, 24 h; (j) phenyl isocyanate, toluene, rt, 14 days.

is described in the [Supplementary material](#) (Tables S2–S3). In the case of **4**, we can see that the urea moiety is involved in the formation of hydrogen bonds N1–H1–O1 and N2–H2N–O1. The structure is additionally stabilized by the hydrogen bonds formed by water molecules acting as donors ([Fig. 2a](#)). With respect to compound **5**, the only hydrogen bond formed between the atoms constituting its molecule is the O1–H1A–O10. The formation of the remaining hydrogen bonds involves the bromide anion as an acceptor or a water molecule as a donor ([Fig. 2b](#)).

A comparison of the crystal structure of urea **4** with the previously published crystal structures of urethane **8** leads to several interesting observations ([Fig. 3](#)).²⁷ Firstly, the nitrogen atom of the urethane group in compound **8**, similarly to the urea group of compound **4**, participates in the formation of a hydrogen bond stabilizing the pseudo-cyclic structure of the compound (N1–H1–O1).²⁷ Secondly, a formation of hydrogen bond O10–H10O–O2 is observed for both compounds.²⁷ Thirdly, although the hydroxyl group O4–H4O acts as the hydrogen bond donor for both compounds, in the case of urea **4**, the role of the acceptor is assumed by the oxygen atom O10 of the hydroxyl group, whereas in the structure of urethane **8**, the role of the acceptor is played by the oxygen atom O2 of the carboxyl group ([Fig. 3](#)).²⁷ Despite very similar structural features, both compounds crystallize in different space groups, with urea **4** crystallizing in space group R3:H of the trigonal system, and urethane **8** crystallizing in space group P1 of the triclinic system.²⁷

2.3. In vitro antiparasoidal activity

To evaluate the antiparasitic potential of MON derivatives, all six analogues (compounds **3–8**), the MON parent compound (compound **1**), and primaquine, employed as a reference, were screened *in vitro* for their antiparasoidal activity against the hepatic stage of *P. berghei* infection ([Fig. 4](#), [Table 1](#)).

MON (compound **1**) and its derivatives (**3**, **4**, **5**, **6**, **7**, and **8**) exhibited strong activity against the hepatic stage of *P. berghei* infection *in vitro*, which exceeded that shown by primaquine – a commonly used drug in treating malaria. Among these derivatives, compounds **8** (IC₅₀ = 0.0007 μM) and **4** (IC₅₀ = 0.0029 μM) displayed the highest potency, followed by MON (IC₅₀ = 0.0055 μM) and derivative **3** (IC₅₀ = 0.0064 μM), both showing comparable activity ([Table 1](#)). Derivative **5** (IC₅₀ = 1.489 μM) had the lowest activity from among the compounds tested, whilst derivatives **6** (IC₅₀ = 0.6233 μM) and **7** (IC₅₀ = 0.0816 μM) displayed intermediate potency hepatic parasites ([Table 1](#)). In addition to nanomolar IC₅₀ values against *P. berghei*, the CC₅₀ values of most compounds against the Huh7 cell line remain in the micromolar range. Compounds **3** and **4** exhibit high selectivity (SI > 1300), although not as high as that observed for the unmodified MON (SI = 3181.8). It is important to highlight that compound **8**, despite having the lowest CC₅₀ value among the tested compounds, remains the most selective compound in the entire series of analogs studied, due to its extraordinarily high antimalarial activity (SI = 4428.6).

Concerning the SAR (structure–activity relationship) analysis, it should be emphasized that the derivatives showing higher activity than

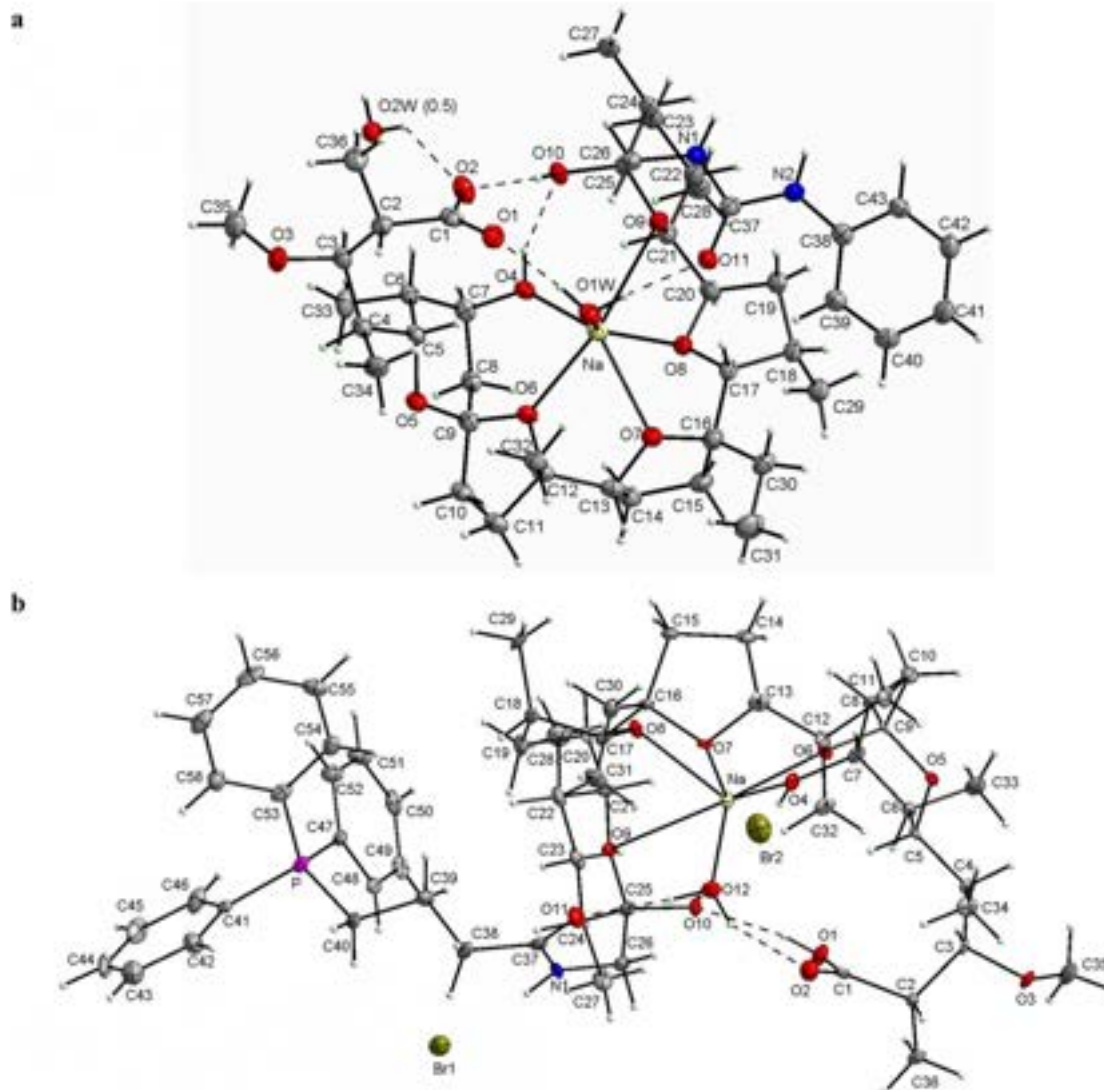


Fig. 2. The crystal structure of: (a) sodium salt of C-26 phenyl urea monensin **4**, (b) sodium salt of C-26 monensin conjugate with TPP⁺ **5**. Ellipsoids are at the 50 % probability level.

unmodified **MON** belong to the group of its C26-analogues (compounds **4** and **8**). Amide **3** also exhibited high antimalarial activity, which was similar to that of **MON**. We can see that chemical modification of position C26 of **MON** gives better results in the context of antimalarial activity, than modification of its C1 position. An exception is compound **5**, which is a C26-conjugate of **MON** with TPP⁺. This compound's decreased activity is probably due to its high steric hindrance, which impedes effective penetration into the parasite cells. Ester compounds **6** and **7** also show lower biological activity than **MON**. This may suggest that potent antimalarial derivatives of **MON** must contain an unhindered carboxyl group, which will enable effective ion transport via an electrogenic or electroneutral mechanism. It is worth noting that the derivatives obtained through chemical modification of the C26 position of **MON** (except for compound **5**) exhibit high selectivity indices (SI > 1300) which means they preferentially target parasite cells over reference Huh7 cell line. Of particular distinction is compound **8**, which is

not only the most active compound in the entire series (with an IC₅₀ value approximately 5 times lower than that of **MON**) but also the most selective one (SI > 4000).

3. Conclusions

In summary, in this work, we describe the possibility of using the C26-aminomonensin (compound **2**) as a convenient starting substrate for the synthesis of various analogues, such as amides or ureas (compounds **3–5**). This significantly contributes to the search for new, highly bioactive analogues of this ionophore. For comparative purposes, C1 ester derivatives of **MON** (compounds **6**, and **7**) and its C26-O-phenyl urethane (compound **8**) were also obtained. Compounds **4** and **5** were obtained in crystalline form, which enabled sXRD analysis to be performed. Both molecules form complexes with the sodium cation and occur in a pseudo-cyclic structure, which is stabilized by numerous

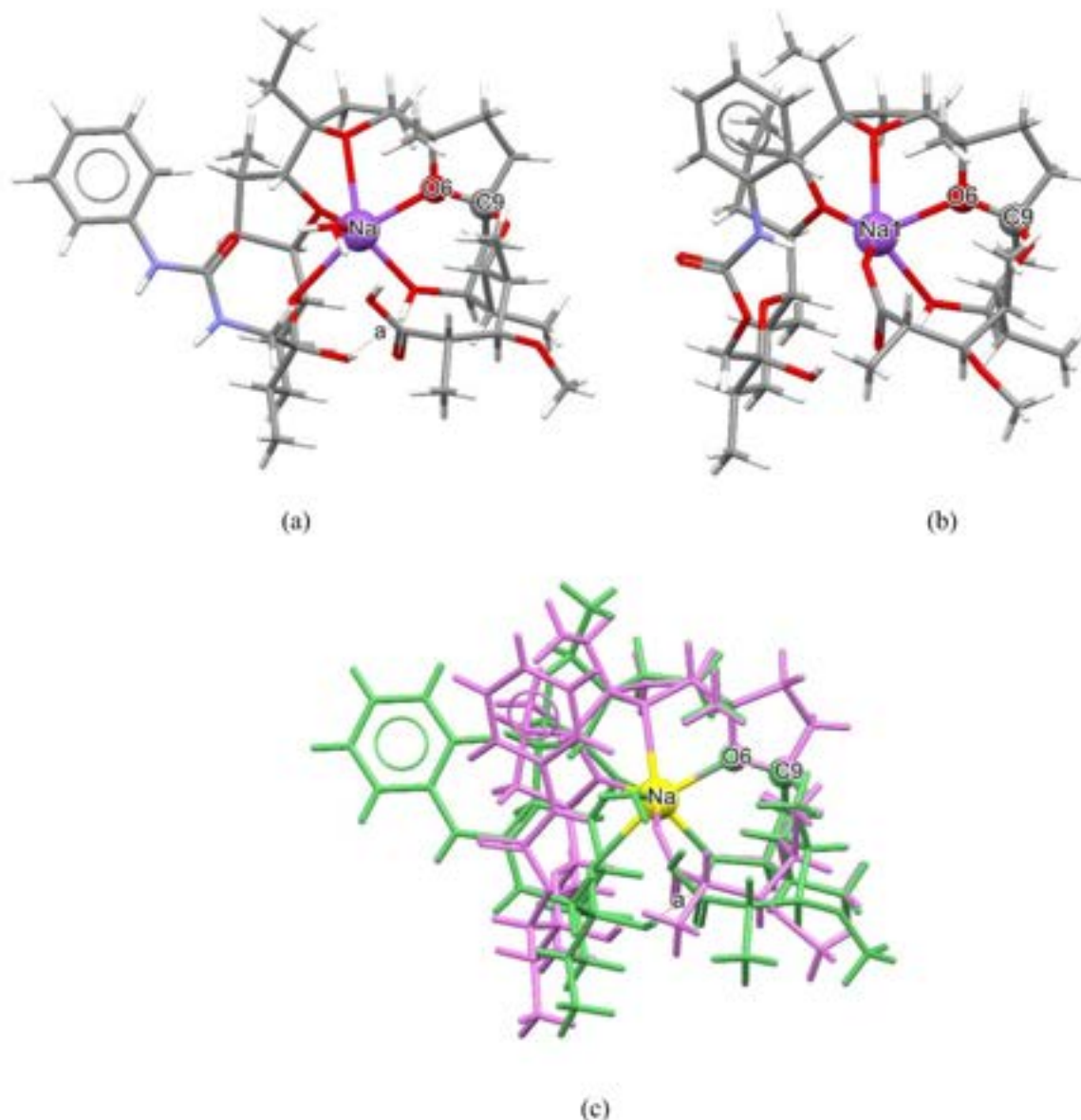


Fig. 3. Comparison of the conformations of monensin sodium salts in crystals: (a) urea **4**, (b) urethane **8** and (c) overlap **4** (green) and **8** (pink), atoms Na, O6 and O9 overlap. Monensin A phenylurethane sodium salt **8** was reproduced using data downloaded from the Cambridge Crystallographic Data Centre CCDC: 778665. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

hydrogen bonds. The substituent at C26 is not involved in the coordination process. In addition, **MON** and its aromatic derivatives (**3–8**) were screened *in vitro* for their antiparasitic activity against the hepatic stage of *P. berghei* infection. It is worth noting that these compounds showed higher biological activity than primaquine used as a reference. Most derivatives showed activity at the nanomolar level, which, in the case of compounds **4** and **8**, even exceeded that of unmodified **MON**. Furthermore, many of the obtained compounds demonstrated high selectivity, meaning they preferentially target parasite cells over the reference Huh7 cell line. The selectivity index for compound **8** exceeded a value of 4000, making it not only more active but also more selective than the unmodified ionophore. These results

underscore the strong antiparasitic potential of **MON** and its derivatives, supporting their development as promising candidates for discovery of new antimalarial drugs.

4. Experimental section

4.1. General procedures

All reagents were purchased from two sources – Merck or Trimex Chemicals S.A., and used without further purification. Deuterated solvents for NMR analysis (CDCl_3 , CD_2Cl_2 , CD_3OH , and CD_3CN) were stored over 3 Å molecular sieves for several days. Reaction mixtures

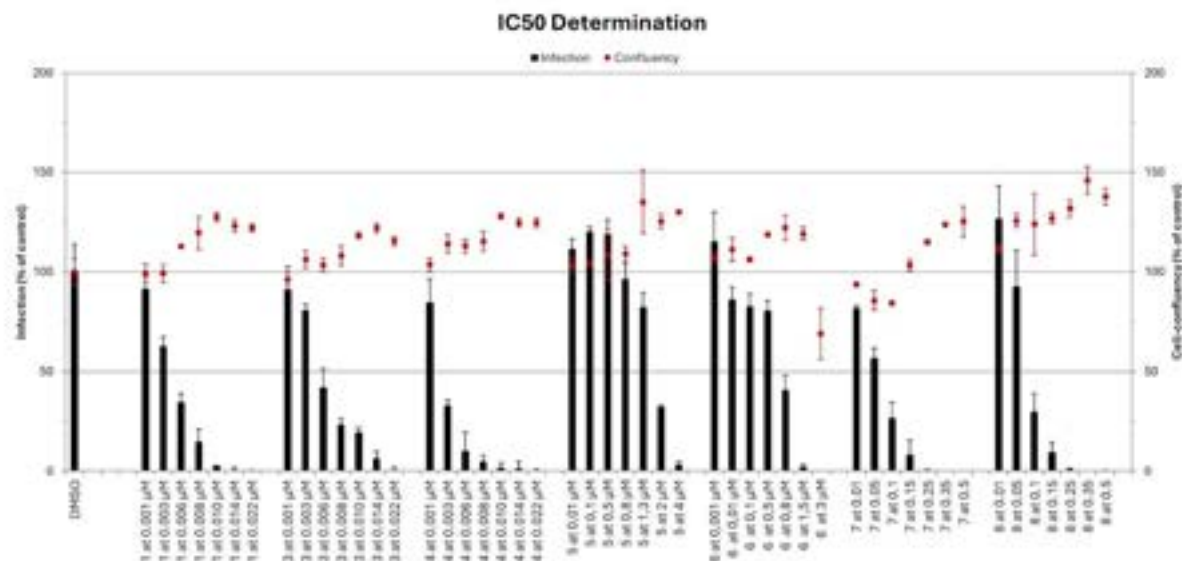


Fig. 4. Dose-dependent response of **MON** (compound **1**), and its derivatives **3**, **4**, **5**, **6**, **7**, and **8**, against the hepatic stage of *P. berghei* infection. Total parasite load (infection scale, bars) and cell viability (cell confluency scale, data) are shown. Calculated IC_{50} values are presented in Table 1.

Table 1

IC_{50} , CC_{50} and SI values of **MON**, its derivatives and primaquine against the hepatic stage of *P. berghei* infection and Huh7 cells.

No.	Antiplasmodial activity		Cytotoxicity	
	IC_{50} (nM) ^a		CC_{50} (nM) ^b	SI ^b
unoxensin 1	5.5 ± 1.4		17500 ± 5700	3181.8
3	6.4 ± 1.0		8400 ± 1900	1312.5
4	2.9 ± 1.0		4200 ± 400	1448.3
5	1489 ± 162.6		75300 ± 10100	50.6
6	623.3 ± 183.0		5800 ± 300	9.3
7	81.6 ± 18.4		33300 ± 7400	383.6
8	0.7 ± 0.004		3100 ± 200	4428.6
Primaquine	8428 ± 3384		138600 ± 25400	16.4

^a Data shown are mean values ± SD of two or three independent experiments.

^b SI (selectivity index) was calculated using the formula: SI = CC_{50} (nM)/ IC_{50} (nM).

were stirred using Teflon-coated magnetic stir bars and were monitored by thin layer chromatography (TLC) using aluminum-backed plates (Merck 60 F₂₅₄). TLC plates were visualized by UV-light (254 nm), followed by treatment with phosphomolybdic acid (PMA) (5 % in absolute ethanol) and gentle heating. Products of the reactions were purified using CombiFlash® Rf + Lumen Flash Chromatography System (Teledyne Isco) with integrated ELS and UV detectors. All solvents used in flash chromatography were of HPLC grade (Merck) and were used as received. Solvents were removed using a rotary evaporator.

NMR spectra were recorded on a Varian 400 (¹H NMR at 400 MHz, ¹³C NMR at 101 MHz, and ³¹P 162 MHz) or Bruker AvanceNEO 600 (¹H NMR at 600 MHz and ¹³C NMR at 151 MHz) magnetic resonance spectrometer. ¹H NMR spectra are reported in chemical shifts downfield from TMS using the respective residual solvent peak as internal standard (CDCl₃ δ 7.26 ppm; CD₂Cl₂ δ 5.32 ppm; CD₃OD δ 3.31 ppm; CD₃CN δ 1.94 ppm). ¹H NMR spectra are reported as follows: chemical shift (δ, ppm), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, dd = doublet of doublets, dt = doublet of triplets, td = triplet of doublets, tt = triplet of triplets, dq = doublet of quartets, ddd = doublet of doublets of doublets, m = multiplet), coupling constant(s) in Hz, and integration. Significant peaks are reported within the overlapping −2.00–0.50 ppm region of the ¹H NMR spectra. ¹³C NMR spectra are reported in chemical

shifts downfield from TMS using the respective residual solvent peak as internal standard (CDCl₃ δ 77.16 ppm; CD₂Cl₂ δ 53.84 ppm; CD₃OD δ 49.00 ppm; CD₃CN δ 1.32 ppm). ³¹P NMR spectra are reported in chemical shifts. Line broadening parameters were 0.5 or 1.0 Hz, while the error of chemical shift value was 0.1 ppm.

Electrospray ionization high resolution mass spectra (ESI-HRMS) were recorded on a QTOF (Impact HD, Bruker Daltonics or Bruker microTOF-q) mass spectrometer in the positive ion detection mode. Samples were prepared in dry acetonitrile. The mass range for ESI experiments was from m/z = 400 to m/z = 1350 or to m/z = 1000.

4.2. Synthesis

4.2.1. Isolation of **MON**

The sodium salt of **MON** was isolated from commercially available veterinary premix – Coxidin®, as previously described.¹⁶ The obtained sodium salt of **MON** was then extracted with a solution of sulfuric acid (pH = 1), giving **MON** ready for further synthesis.

4.2.2. Synthesis of **MON** amine (compound **2**)

To a solution of **MON** (11.0 g, 16.39 mmol, 1.0 equiv.) in toluene (120 mL) upon stirring, a solution of 0.1 M Na₂CO₃ was added (150 mL). After that, tosyl chloride (9.37 g, 49.18 mmol, 3.0 equiv.) was added to the resulting mixture in one portion. The resulting solution was stirred at room temperature for the next 72 h. The reaction mixture was then transferred to a separatory funnel and extracted with 0.1 M Na₂CO₃ solution and brine. After that, the organic layer was concentrated under reduced pressure, and directed subsequently to the next stage of the process. The excess of sodium azide (3.19 g, 49.09 mmol, 3.0 equiv.) was dissolved in 30 mL of DMSO (complete conversion of **MON** to tosylate was assumed). The solution of crude tosylate dissolved in 20 mL of DMSO was then added to the mixture. The resulting solution was stirred at room temperature for the next 72 h. The mixture was then diluted with large amount of water (over 300 mL) and extracted several times with methylene chloride. This step can be dangerous due to the possibility of an explosion as a result of the reaction between sodium azide and the chlorinated solvent, so the sequence of operations must be strictly followed. Alternatively, azide can be extracted from the aqueous phase into the ethyl acetate phase. Purification on silica gel using the

CombiFlash system (0 → 40 % EtOAc/n-hexane) gave the pure product as a clear oil. The oil was diluted in n-pentane and evaporated to dryness three times to form an amorphous solid, with an isolated yield of 62 % (7.10 g). Under a nitrogen atmosphere, to a solution of obtained azide (2.0 g, 2.87 mmol, 1.0 equiv.) in methanol upon stirring, the catalytic amount of palladium on carbon (Pd/C) was added. Then, a hydrogen-filled balloon was connected to the system. The reaction was continued until the azide was completely consumed (TLC and ESI-MS control), replacing the hydrogen balloon if necessary (typically 72 h). After this time, the reaction mixture was filtered through celite to remove Pd/C, and concentrated under reduced pressure. Purification on silica gel using the CombiFlash system (0 → 30 % MeOH/CHCl₃) gave the pure product as a clear oil. The oil was diluted in n-pentane and evaporated to dryness three times to form an amorphous solid, with an isolated yield of 63 % (1.22 g), a single spot by TLC; stains green with PMA. The spectroscopic data were in agreement with previously published data.²²

4.2.3. Synthesis of C26-benzoyl amide of MON (compound 3)

MON amine (compound 2, 135 mg, 0.20 mmol, 1.0 equiv.) was dissolved in CH₂Cl₂ (10 mL) and the solution was cooled on an ice bath. Triethylamine (61 mg, 0.60 mmol, 3.0 equiv.) was added dropwise to the reaction mixture, and after that, benzoyl chloride (34 mg, 0.24 mmol, 1.2 equiv.) was added dropwise. After 24 h, the reaction mixture was concentrated on a rotary evaporator in vacuo. Purification on silica gel using the CombiFlash system (0 % → 100 % EtOAc/hexane) gave the pure amide 3 as a clear oil. After twice evaporation to dryness with n-pentane, the oily product was completely converted into white amorphous solid (65 mg, 42 % yield). Isolated as a white amorphous solid, a single spot by TLC. UV-active and stains green with PMA; ¹H NMR (400 MHz, CDCl₃) δ 8.33 – 8.28 (m, 1H), 7.99 – 7.95 (m, 2H), 7.41 – 7.31 (m, 3H), 4.43 (dd, J = 11.6, 1.7 Hz, 1H), 4.31 (ddd, J = 10.8, 5.7, 2.6 Hz, 1H), 4.03 (dd, J = 14.1, 7.3 Hz, 1H), 3.91 – 3.85 (m, 2H), 3.75 (dd, J = 14.1, 5.5 Hz, 1H), 3.71 (d, J = 4.3 Hz, 1H), 3.64 (s, 1H), 3.43 (dd, J = 10.6, 5.0 Hz, 1H), 3.40 – 3.35 (m, 1H), 3.33 (s, 3H), 2.59 (dq, J = 10.0, 6.8 Hz, 1H), 2.35 – 1.16 (m, 23H), 1.53 (s, 3H), 1.13 (d, J = 6.8 Hz, 3H), 1.02 (d, J = 6.9 Hz, 3H), 0.94 (d, J = 6.6 Hz, 3H), 0.88 (d, J = 7.2 Hz, 3H), 0.85 (d, J = 7.1 Hz, 3H), 0.80 (d, J = 6.2 Hz, 3H), 0.65 (t, J = 7.5 Hz, 3H) ppm; ¹³C{¹H} NMR (101 MHz, CDCl₃) δ 184.2, 166.9, 134.6, 130.8, 128.2, 127.4, 107.7, 96.9, 86.3, 85.1, 85.0, 82.9, 82.1, 77.6, 74.5, 70.8, 65.9, 57.8, 47.5, 44.2, 39.2, 38.7, 36.8, 35.8, 35.3, 34.2, 33.9, 33.3, 32.6, 30.4, 29.8, 28.0, 26.6, 17.4, 16.6, 16.3, 15.6, 10.2, 9.9, 8.0 ppm; HRMS (ESI) m/z: [M]⁺ Calcd for C₄₇H₆₇NNaO₁₁ 796.4606; Found 796.4595.

4.2.4. Synthesis of C26-phenyl urea of MON (compound 4)

MON amine (compound 2, 152 mg, 0.23 mmol, 1.0 equiv.) was dissolved in anhydrous toluene (10 mL). Phenyl isocyanate (27 mg, 0.23 mmol, 1.0 equiv.) was added dropwise to the reaction mixture. After 72 h, the reaction mixture was concentrated on a rotary evaporator in vacuo. Purification on silica gel using the CombiFlash system (0 % → 100 % EtOAc/hexane) gave the pure urea 4 as a clear oil. After twice evaporation to dryness with n-pentane, the oily product was completely converted into white amorphous solid (107 mg, 60 % yield). Isolated as a white amorphous solid, a single spot by TLC. UV-active and stains green with PMA; ¹H NMR (401 MHz, CDCl₃) δ 8.31 – 8.25 (m, 1H), 8.19 (s, 1H), 7.48 (dd, J = 8.7, 1.1 Hz, 2H), 7.18 – 7.12 (m, 2H), 6.86 – 6.80 (m, 1H), 4.34 – 4.25 (m, 2H), 3.89 (s, 1H), 3.85 – 3.81 (m, 2H), 3.76 (dd, J = 13.9, 9.7 Hz, 1H), 3.57 (s, 1H), 3.37 (t, J = 5.3 Hz, 1H), 3.35 (s, 3H), 3.29 (dd, J = 10.2, 2.6 Hz, 1H), 3.22 (dd, J = 13.9, 3.1 Hz, 1H), 2.67 – 2.59 (m, 1H), 2.36 (nd, J = 11.5, 6.5 Hz, 1H), 2.28 – 2.18 (m, 2H), 2.15 – 1.22 (m, 20H), 1.55 (s, 3H), 1.21 (d, J = 6.8 Hz, 3H), 1.13 (d, J = 6.9 Hz, 3H), 0.95 – 0.89 (m, 6H), 0.85 – 0.80 (m, 6H), 0.50 (t, J = 7.4 Hz, 3H) ppm; ¹³C{¹H} NMR (101 MHz, CDCl₃) δ 183.5, 157.1, 140.5, 128.3, 120.9, 117.8, 107.4, 97.0, 86.5, 85.5, 85.1, 83.2, 82.3, 77.4, 75.1, 70.6, 66.5, 57.8, 47.4, 44.5, 38.7, 38.3, 36.3, 36.2, 35.3, 34.2, 33.8, 33.2,

32.4, 32.3, 30.0, 29.8, 27.9, 26.9, 17.3, 16.6, 16.5, 15.6, 10.2, 10.1, 7.8 ppm; HRMS (ESI) m/z: [M]⁺ Calcd for C₄₇H₆₈N₂NaO₁₁ 811.4715; Found 811.4703.

4.2.5. Synthesis of C26-amide-conjugate of MON with TPP⁺ (compound 5)

(3-carboxypropyl)triphenylphosphonium bromide (213 mg, 0.50 mmol, 1.0 equiv.) was dissolved in anhydrous DMF (15 mL) and the solution was cooled to – 5 °C. Ethyl chloroformate (48 mg, 0.45 mmol, 0.9 equiv.) and triethylamine (75 mg, 0.74 mmol, 1.5 equiv.) were added dropwise. After 30 min the solution of MON amine (compound 2, 400 mg, 0.60 mmol, 1.2 equiv.) in anhydrous DMF was added dropwise. The mixture was allowed to warm to ambient temperature. After 24 h, the reaction mixture was concentrated on a rotary evaporator. The oily residue was dissolved in a small amount of CH₂Cl₂ and extracted with brine. Organic phases were concentrated under reduced pressure. Purification on silica gel using the CombiFlash system (0 → 30 % MeOH/chloroform) gave the pure product as a clear oil. The oil was diluted in n-pentane and evaporated to dryness three times to form an amorphous solid, with an isolated yield of 43 % (230 mg), a single spot by TLC. UV-active and stains green with PMA; ¹H NMR (401 MHz, CD₃OD) δ 7.93 – 7.87 (m, 3H), 7.83 (tt, J = 5.4, 1.3 Hz, 4H), 7.81 – 7.73 (m, 8H), 4.16 (dt, J = 8.7, 6.7 Hz, 1H), 4.10 (dd, J = 8.7, 2.3 Hz, 1H), 3.91 (d, J = 4.6 Hz, 1H), 3.73 – 3.65 (m, 2H), 3.62 – 3.59 (m, 1H), 3.54 – 3.47 (m, 2H), 3.46 – 3.39 (m, 2H), 3.35 (s, 3H), 3.30 (p, J = 3.3, 1.6 Hz, 2H), 3.22 (d, J = 13.5 Hz, 1H), 2.60 – 2.53 (m, 3H), 2.28 – 1.35 (m, 25H), 1.33 (s, 3H), 1.13 (d, J = 7.0 Hz, 3H), 1.03 – 0.89 (m, 10H) ppm; ¹³C{¹H} NMR (101 MHz, CD₃OD) δ 179.1, 174.1, 136.5, 136.4, 134.9, 134.8, 131.7, 131.6, 120.2, 119.3, 108.9, 98.5, 88.9, 87.9, 86.9, 85.0, 83.5, 79.8, 78.1, 72.8, 69.1, 59.0, 42.1, 40.3, 38.6, 38.2, 37.8, 36.8, 36.4, 36.3, 36.2, 36.1, 35.4, 32.9, 32.5, 30.6, 29.2, 26.2, 20.02, 19.99, 18.3, 16.7, 16.5, 12.9, 12.4, 11.5, 8.5 ppm; ³¹P NMR (162 MHz, CD₃OD) δ 23.5 ppm; HRMS (ESI) m/z: [M]⁺ Calcd for C₅₈H₈₃NO₁₁P⁺ 1000.5698; Found 1000.5732.

4.2.6. Synthesis of C1-ester-conjugate of MON with TPP⁺ (compound 6)

MON (1.0 g, 1.49 mmol, 1.0 equiv.) was dissolved in toluene (30 mL) and the solution was heated to 90 °C on a heating mantle. After 15 min DBU (386 mg, 2.53 mmol, 1.7 equiv.) was added dropwise to the reaction mixture, and after another 20 min 1,4-dibromobutane (965 mg, 4.47 mmol, 3.0 equiv.) was added dropwise. The solution changed its color to brownish after a few minutes. After 24 h, the reaction mixture was concentrated on a rotary evaporator. Purification on silica gel using the CombiFlash system (0 % → 50 % EtOAc/hexane) gave the pure ester as a clear oil. After twice evaporation to dryness with n-pentane, the product remained in an oily form (700 mg, 58 % yield). This compound (400 mg, 0.50 mmol, 1.0 equiv.) was then dissolved in CH₃CN (30 mL) and the triphenylphosphine (1.50 mmol, 3.0 equiv., 393 mg) was added. The solution was heated under reflux on a heating mantle for 72 h. After this time, the reaction mixture was concentrated on a rotary evaporator. Purification on silica gel using the CombiFlash system (0 % → 40 % acetone/CHCl₃) gave compound 6 as clear oil. After twice evaporation to dryness with n-pentane, the oily products were completely converted into white amorphous solid (74 mg, 14 % yield). Isolated as a white amorphous solid, a single spot by TLC. UV-active and stains green with PMA; ¹H NMR (400 MHz, CD₃CN) δ 7.91 – 7.84 (m, 3H), 7.83 – 7.76 (m, 6H), 7.76 – 7.69 (m, 6H), 5.78 (d, J = 1.3 Hz, 1H), 5.53 – 5.50 (m, 1H), 4.37 (ddd, J = 9.5, 7.2, 4.1 Hz, 1H), 4.19 – 4.07 (m, 4H), 4.06 (d, J = 3.5 Hz, 2H), 4.02 – 3.94 (m, 2H), 3.82 (dt, J = 5.4, 2.6 Hz, 1H), 3.69 (dd, J = 5.3, 2.9 Hz, 1H), 3.67 – 3.55 (m, 4H), 3.51 – 3.41 (m, 2H), 3.38 (ddd, J = 10.1, 4.0, 1.7 Hz, 2H), 3.19 (s, 3H), 2.83 (ddd, J = 13.8, 6.8, 2.8 Hz, 1H), 2.60 (s, 1H), 2.34 (s, 1H), 2.31 – 2.23 (m, 3H), 2.18 – 1.26 (m, 13H), 1.47 (s, 3H), 1.19 (s, 2H), 1.10 (d, J = 6.9 Hz, 3H), 0.98 (d, J = 7.0 Hz, 3H), 0.93 – 0.84 (m, 9H), 0.75 (d, J = 6.8 Hz, 3H), 0.72 (d, J = 7.1 Hz, 3H) ppm; ¹³C{¹H} NMR (101 MHz, CD₃CN) δ 176.0, 135.8, 135.8, 134.6, 134.5, 131.1, 131.0, 119.6, 118.7, 108.4, 99.0, 86.6, 86.4, 84.6, 81.8, 81.6, 76.3, 74.7, 69.6, 69.1, 67.0, 63.8, 58.4, 55.2, 41.3, 39.9, 36.8, 36.5, 36.3, 35.5, 35.1, 34.6, 33.4, 33.2, 32.2, 30.4, 30.1, 30.0,

29.7, 29.6, 28.3, 26.8, 22.6, 22.1, 20.0, 20.0, 16.4, 16.1, 14.5, 13.2, 11.5, 10.8, 8.1 ppm; ^{31}P NMR (162 MHz, CD_3CN) δ 25.2 ppm; HRMS (ESI) m/z [M] $^+$ Calcd for $\text{C}_{50}\text{H}_{84}\text{O}_{11}\text{P}^+$ 987.5746; Found 987.5745.

4.2.7. Synthesis of *C1*-benzyl ester of MON (compound 7)

MON (0.5 g, 0.75 mmol, 1.0 equiv.) was dissolved in toluene (20 mL) and the solution was heated to 90 °C on a heating mantle. After 15 min DBU (193 mg, 1.27 mmol, 1.7 equiv.) was added dropwise to the reaction mixture, and after another 20 min benzyl bromide (382 mg, 2.24 mmol, 3.0 equiv.) was added dropwise. The solution changed its color to brownish after a few minutes. After 24 h, the reaction mixture was concentrated on a rotary evaporator in vacuo. Purification on silica gel using the CombiFlash system (0 %–50 % EtOAc/hexane) gave the pure ester 7 as a clear oil. After twice evaporation to dryness with *n*-pentane, the oily product was completely converted into white amorphous solid (438 mg, 77 % yield). Isolated as a white amorphous solid, a single spot by TLC. UV-active and stains green with PMA; ^1H NMR (401 MHz, CDCl_3) δ 7.39–7.27 (m, 5H), 5.19 (d, J = 12.4 Hz, 1H), 5.12 (d, J = 12.4 Hz, 1H), 4.37 (d, J = 9.3 Hz, 1H), 4.27 (td, J = 8.0, 3.3 Hz, 1H), 4.04 (dd, J = 9.1, 2.3 Hz, 1H), 3.07 (s, 1H), 3.84 (d, J = 4.7 Hz, 1H), 3.81–3.72 (m, 2H), 3.61–3.56 (m, 2H), 3.47–3.43 (m, 2H), 3.23 (s, 3H), 2.70 (ddd, J = 13.5, 6.9, 3.9 Hz, 2H), 2.31–2.23 (m, 1H), 2.18–1.30 (m, 20H), 1.35 (s, 3H), 1.20 (d, J = 7.0 Hz, 3H), 0.98 (d, J = 6.9 Hz, 3H), 0.95 (d, J = 7.0 Hz, 3H), 0.89 (t, J = 7.4 Hz, 3H), 0.87–0.81 (m, 9H) ppm; ^{13}C (^1H) NMR (101 MHz, CDCl_3) δ 175.2, 136.1, 128.4, 120.2, 128.0, 107.5, 96.8, 87.2, 86.2, 85.6, 83.5, 81.4, 76.4, 76.3, 71.2, 68.0, 67.4, 66.2, 58.1, 40.9, 39.0, 37.0, 36.7, 36.0, 35.1, 34.7, 34.4, 33.5, 32.7, 32.2, 31.0, 29.5, 27.8, 25.6, 17.3, 16.0, 15.6, 12.2, 11.6, 11.0, 6.0 ppm; HRMS (ESI) m/z [M + Na] $^+$ Calcd for $\text{C}_{51}\text{H}_{86}\text{NaO}_{11}$ 783.4654; Found 783.4661.

4.2.8. Synthesis of *C26*-phenyl *O*-urethane of MON (compound 8)

MON (compound 1, 200 mg, 0.30 mmol, 1.0 equiv.) was dissolved in anhydrous toluene (10 mL). Phenyl isocyanate (34 mg, 0.28 mmol, 0.95 equiv.) was added dropwise to the reaction mixture. After 2 weeks, the reaction mixture was concentrated on a rotary evaporator in vacuo. Purification on silica gel using the CombiFlash system (0 %–50 % EtOAc/hexane) gave the pure urethane 8 as a clear oil. After twice evaporation to dryness with *n*-pentane, the oily product was completely converted into white amorphous solid (127 mg, 54 % yield). Isolated as a white amorphous solid, a single spot by TLC. UV-active and stains green with PMA; ^1H NMR (401 MHz, CD_3CN) δ 7.94 (s, 1H), 7.48–7.41 (m, 2H), 7.31–7.25 (m, 2H), 7.06–6.99 (m, 1H), 4.24–4.17 (m, 2H), 4.09 (dd, J = 10.1, 1.5 Hz, 1H), 4.02 (d, J = 11.4 Hz, 1H), 3.93 (d, J = 4.2 Hz, 1H), 3.76 (dd, J = 9.7, 2.9 Hz, 1H), 3.67 (s, 1H), 3.57 (dd, J = 10.0, 5.2 Hz, 1H), 3.41–3.37 (m, 1H), 3.33 (s, 3H), 2.58 (p, J = 6.8 Hz, 1H), 2.19–1.27 (m, 24H), 1.39 (s, 3H), 1.15 (d, J = 6.8 Hz, 3H), 1.02 (d, J = 6.9 Hz, 3H), 0.90 (d, J = 3.8 Hz, 3H), 0.89 (d, J = 4.2 Hz, 3H), 0.87–0.81 (m, 9H) ppm; ^{13}C (^1H) NMR (101 MHz, CD_3CN) δ 177.4, 154.7, 139.9, 129.9, 123.9, 120.1, 119.6, 108.5, 97.7, 87.5, 87.4, 86.2, 84.3, 82.6, 78.0, 77.0, 72.1, 68.3, 68.2, 58.6, 41.5, 39.7, 37.8, 37.4, 36.7, 35.8, 35.4, 35.3, 34.1, 33.8, 33.4, 31.9, 31.0, 28.7, 27.1, 18.0, 16.7, 16.3, 14.0, 12.0, 11.3, 8.7 ppm; HRMS (ESI) m/z [M + Na] $^+$ Calcd for $\text{C}_{51}\text{H}_{87}\text{NNaO}_{12}$ 812.4555; found 812.4573.

4.3. X-ray measurements

The X-ray intensity data for compounds 4, and 5 were collected using graphite monochromatic Mo K_α radiation on a four-circle κ geometry Xcalibur diffractometer with Sapphire2 area CCD detector. Data collections were made using the CrysAlisPro 1.171.42.93a. Integration, scaling of the reflections, corrections for Lorentz and polarization effects and absorption corrections were performed using the CrysAlisPro 1.171.42.93a program.²⁸ Using Olex2,²⁹ the structure was solved by the direct methods using SHELXT-2014/5³⁰ and refined using SHELXL-2018/3 program.³¹ The hydrogen atoms joined to carbon atoms were

introduced in their geometrical positions and treated as rigid. The H atoms involved in the hydrogen bonds were refined if they gave reasonable hydrogen bonds, otherwise they were constrained. It was not possible to locate all solvent molecules in crystal 4 due to their high disorder. Since it was not possible to correctly model the disordered molecules, we performed a “squeeze” treatment to remove the contribution of these molecules to the scattering. The final difference Fourier maps showed no peaks of chemical significance. Details of the data collection parameters, crystallographic data and final agreement parameters are collected in Table S1. The hydrogen bonds are summarized in Tables S2–S3. Visualizations of the structures were made with the Diamond 3.0.³²

4.4. In vitro assessment of hepatic stage antiparasitic activity

The compounds' antiparasitic activity was assessed *in vitro* in Huh7 cells, a human hepatoma cell line provided by Cenix Bioscience GmbH and maintained in complete culture medium, consisting of Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10 % (v/v) fetal bovine serum (FBS), 1 % (v/v) penicillin/streptomycin, 1 % (v/v) glutamine, 1 % (v/v) non-essential amino acids, and 10 mM 2-(4-(2-hydroxyethyl)piperazine-1-yl) ethanesulfonic acid (HEPES) pH 7 (all from Gibco).

Stock solutions of the test compounds (10 mM) were prepared in dimethyl sulfoxide (DMSO) and serially diluted in infection medium – complete RPMI 1640 supplemented with 0.8 $\mu\text{g}/\text{mL}$ amphotericin B and 50 $\mu\text{g}/\text{mL}$ gentamicin (both from Thermo Fisher Scientific) – to achieve the desired test concentrations. Huh7 cells were seeded at a density of 1×10^4 cells per well in 96-well plates and incubated at 37 °C with 5 % CO_2 and humidified atmosphere for 24 h. After incubation, the culture medium was removed, and the test compounds, diluted in infection medium, were added to the cells in triplicate. The cells were incubated with the compounds for 1 h at 37 °C with 5 % CO_2 and humidified atmosphere. DMSO and Primaquine were used as the negative and positive controls, respectively. Cells were then infected with 1×10^4 luciferase-expressing *P. berghei* sporozoites, freshly extracted from the salivary glands of infected female *Anopheles stephensi* mosquitoes reared in the GIMM insectarium. The plates were centrifuged at $1800 \times g$ for 5 min at room temperature and then incubated at 37 °C with 5 % CO_2 and humidified atmosphere. Cell viability was assessed 46 h later using the AlamarBlue assay, according to the manufacturer's instructions (Invitrogen). Parasite burden was determined 48 h after sporozoite infection by a bioluminescence assay (Biotium). Fluorescence and bioluminescence were measured with a microplate reader (Tecan Spark). IC_{50} values were calculated using GraphPad Prism software through nonlinear regression analysis of normalized dose-response curves.

4.5. In vitro assessment of compounds cytotoxicity

The cytotoxicity of the test compounds to Huh7 cells was assessed under similar conditions to those described above. Briefly, cells were seeded in 96-well plates at 1×10^4 cells per well and incubated in complete RPMI at 37 °C with 5 % CO_2 and humidified atmosphere for 24 h. The culture medium was then replaced with compound-containing complete medium, supplemented with 0.8 $\mu\text{g}/\text{mL}$ amphotericin B and 50 $\mu\text{g}/\text{mL}$ gentamicin, at the desired test concentrations. DMSO in amounts equivalent to those present in the different compound dilutions was used as a negative control. After a 46 h incubation, cell viability was assessed using the AlamarBlue assay, and fluorescence was measured using a microplate reader. CC_{50} values were determined by nonlinear regression analysis of normalized dose-response curves using GraphPad Prism software.

CRediT authorship contribution statement

Michał Sulik: Writing – review & editing, Writing – original draft,

Methodology, Investigation, Data curation, Conceptualization. Eyob A. Workneh: Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. Sofia Santana: Investigation. Bárbara Teixeira: Methodology, Investigation. Miguel Prudêncio: Writing – original draft, Validation, Methodology, Conceptualization. Jan Janczak: Writing – original draft, Visualization, Validation, Software, Investigation, Data curation. Adam Huczyński: Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bmc.2025.118177>.

Data availability

Data will be made available on request.

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Chemical modification of monensin as a source of potent antiplasmodial agents

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Supplementary materials

Figure S1. The ¹³ C{ ¹ H} NMR (151 MHz) spectrum of 2 in dichloromethane-d ₂ .	S2
Figure S2. The ¹ H NMR (600 MHz) spectrum of 2 in dichloromethane-d ₂ .	S2
Figure S3. The ¹³ C{ ¹ H} NMR (101 MHz) spectrum of 3 in chloroform-d.	S3
Figure S4. The ¹ H NMR (400 MHz) spectrum of 3 in chloroform-d.	S3
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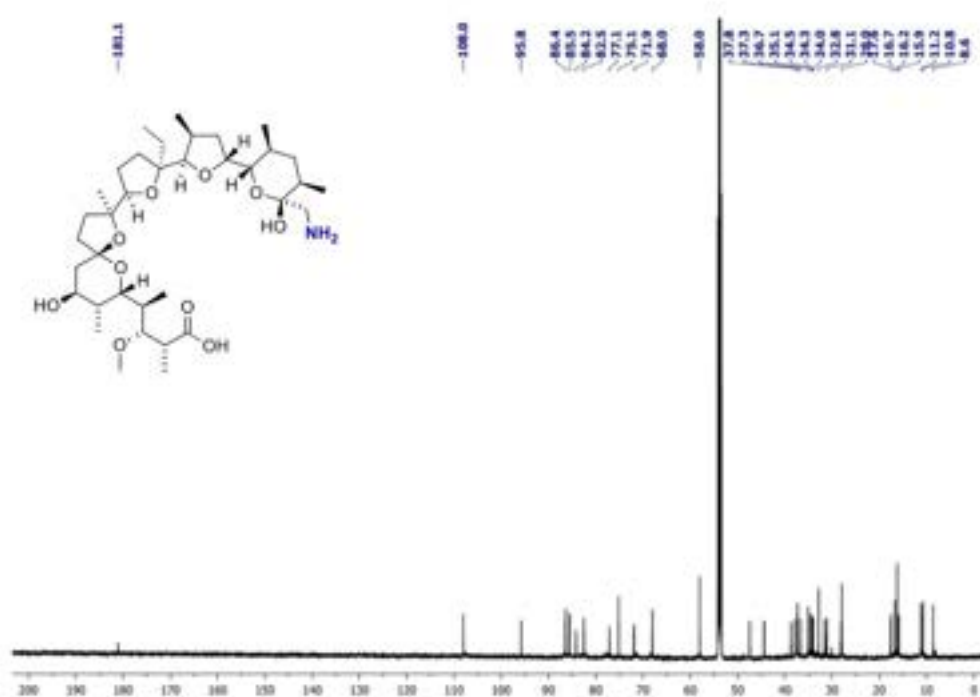


Figure S1. The ^{13}C NMR (151 MHz) spectrum of **2** in dichloromethane- d_2 .

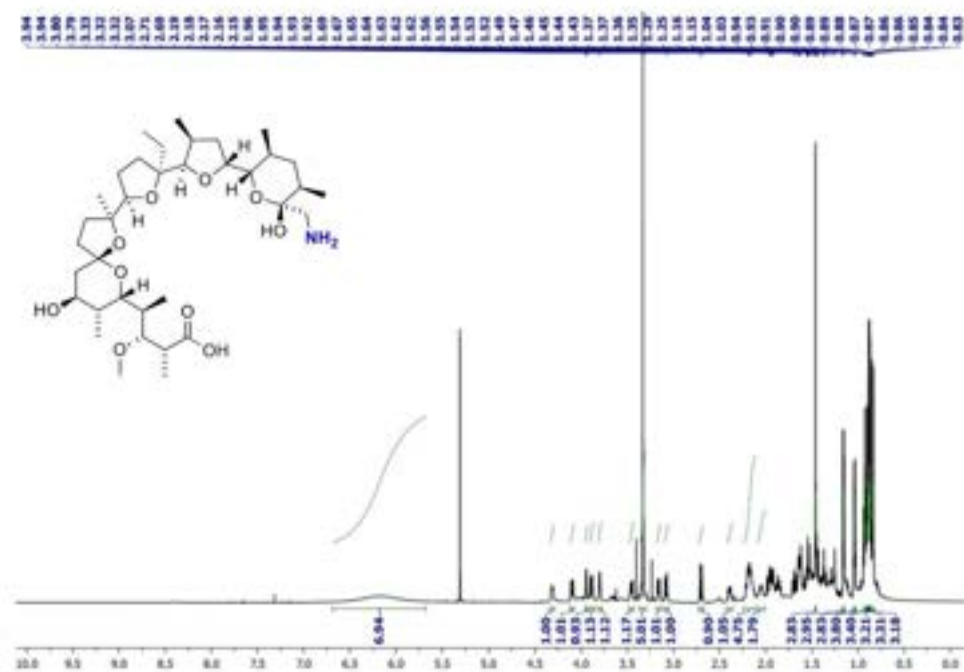


Figure S2. The ^1H NMR (600 MHz) spectrum of **2** in dichloromethane- d_2 .

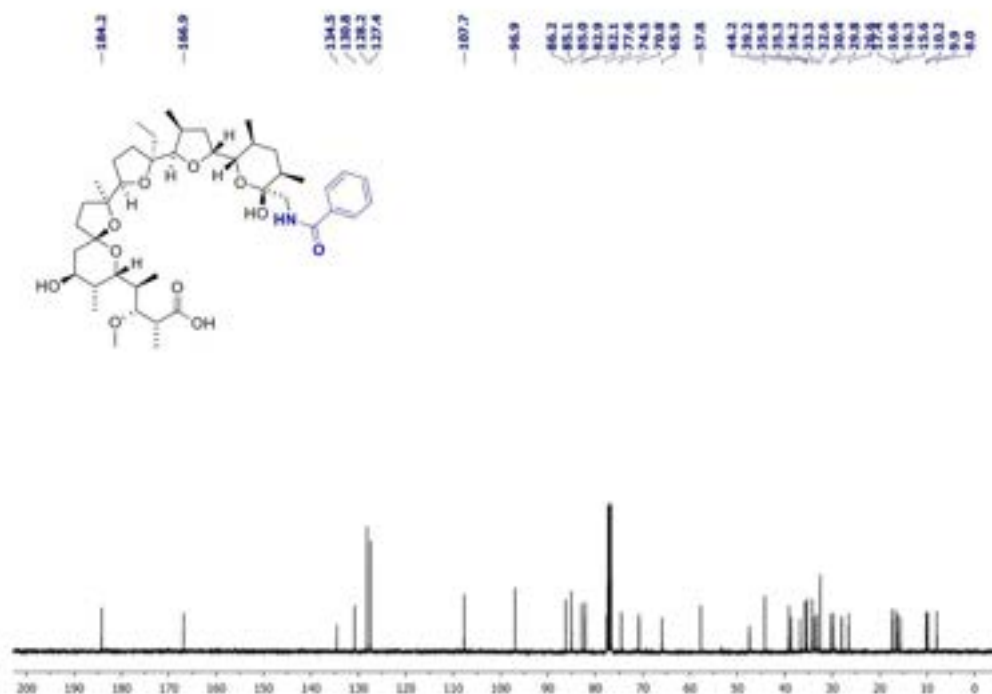


Figure S3. The ^{13}C (^1H) NMR (101 MHz) spectrum of **3** in chloroform- d .

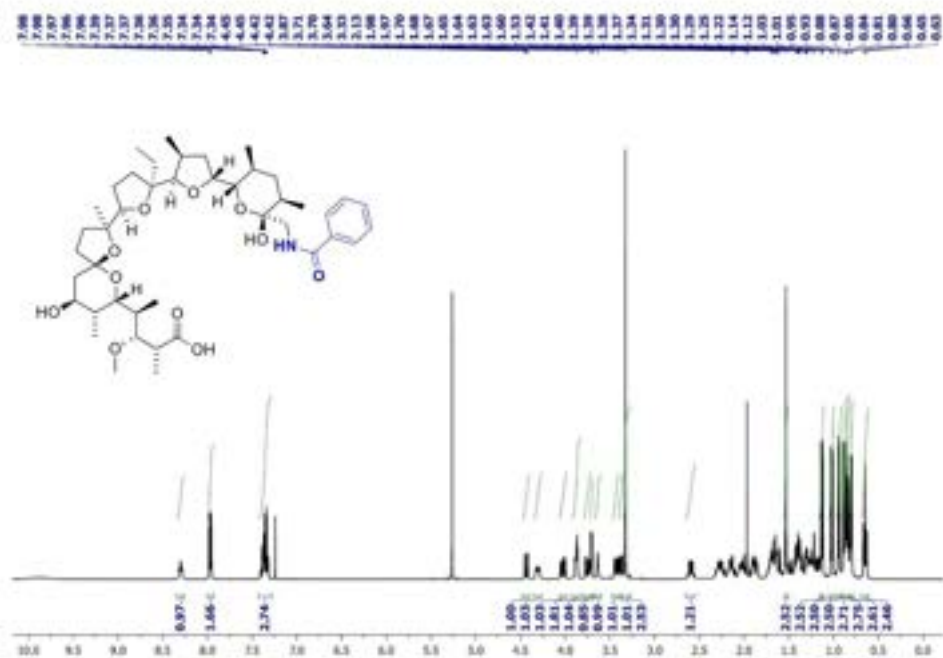
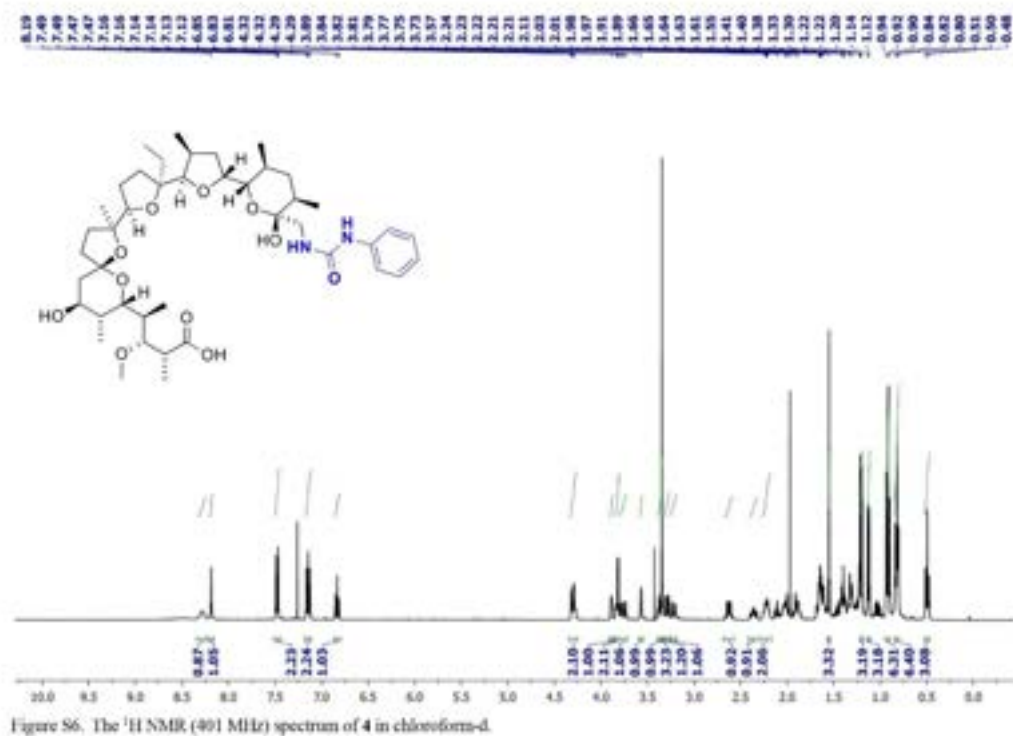


Figure S4. The ^1H NMR (400 MHz) spectrum of **3** in chloroform- d .



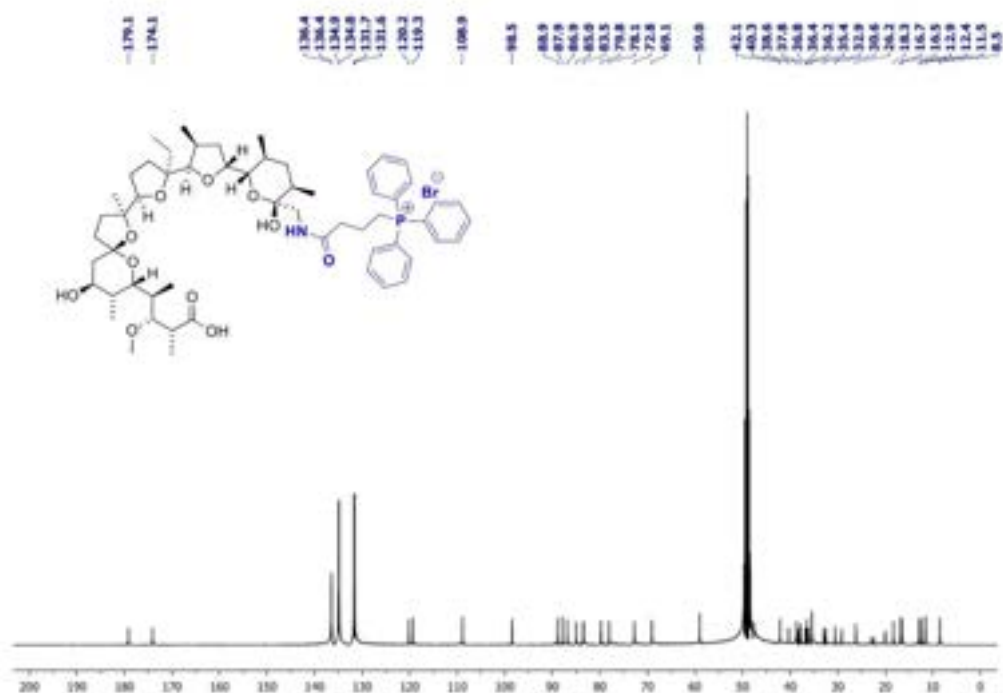


Figure S7. The ^{13}C (^1H) NMR (101 MHz) spectrum of **5** in methanol- d_4 .

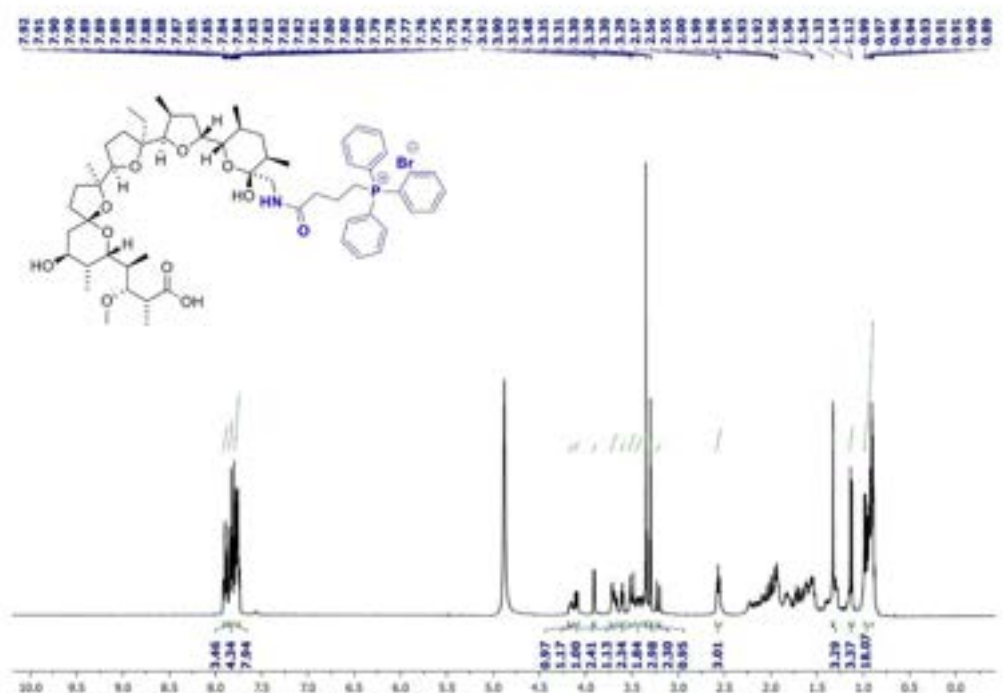
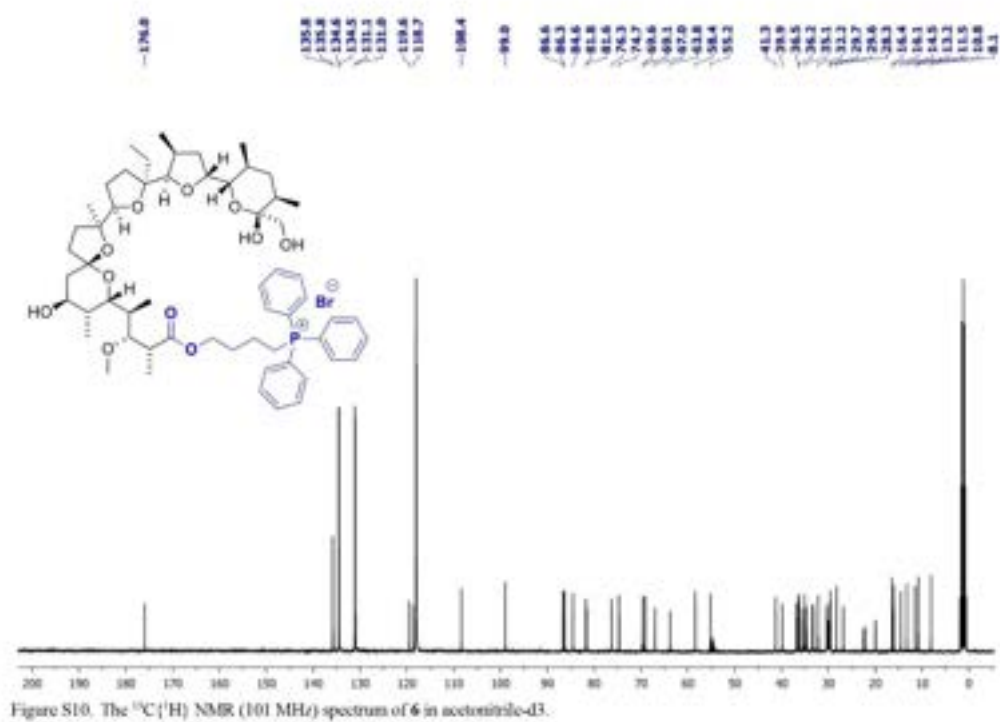
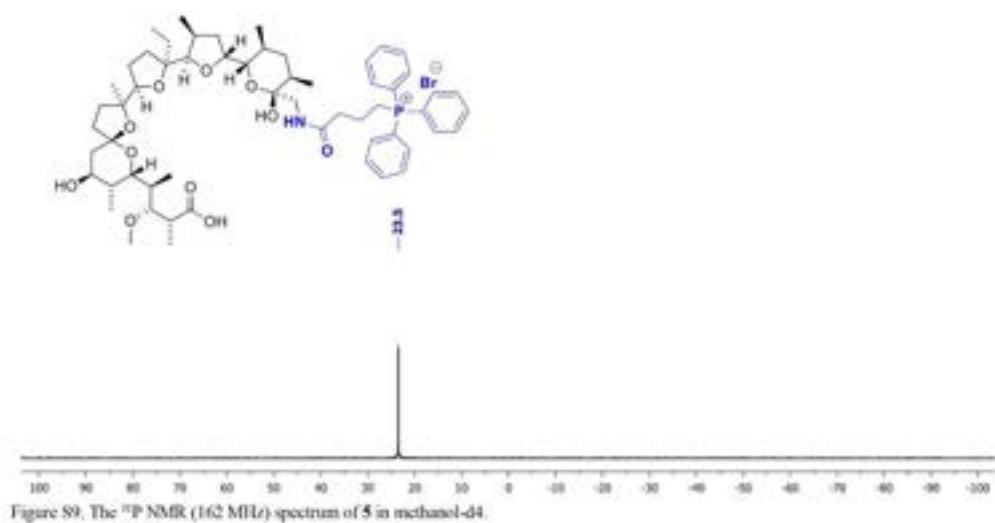
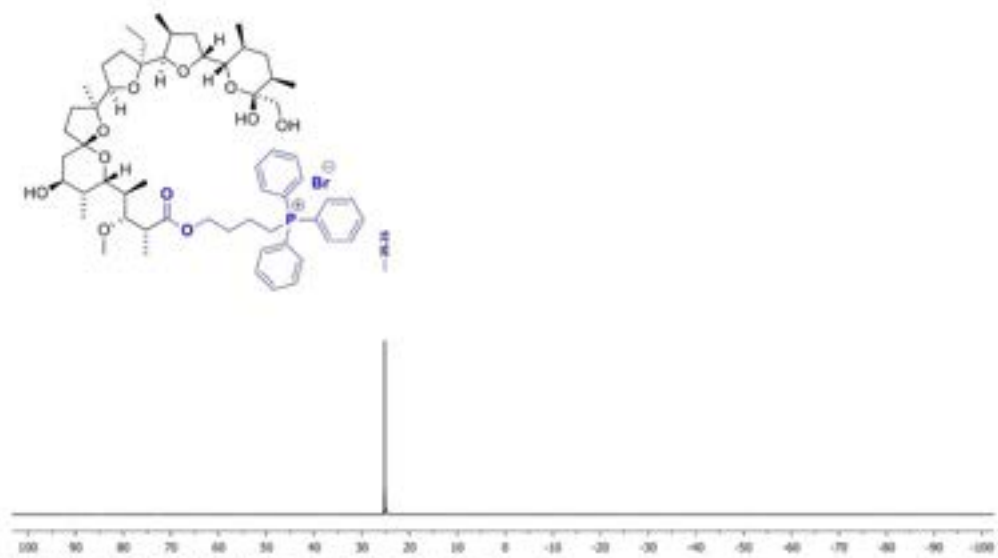
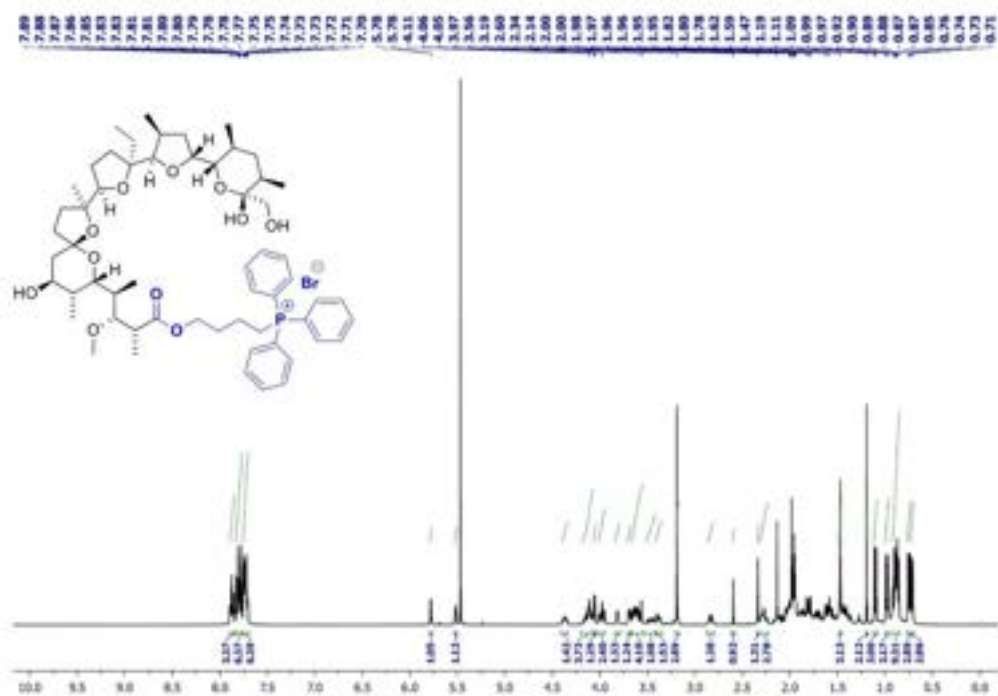
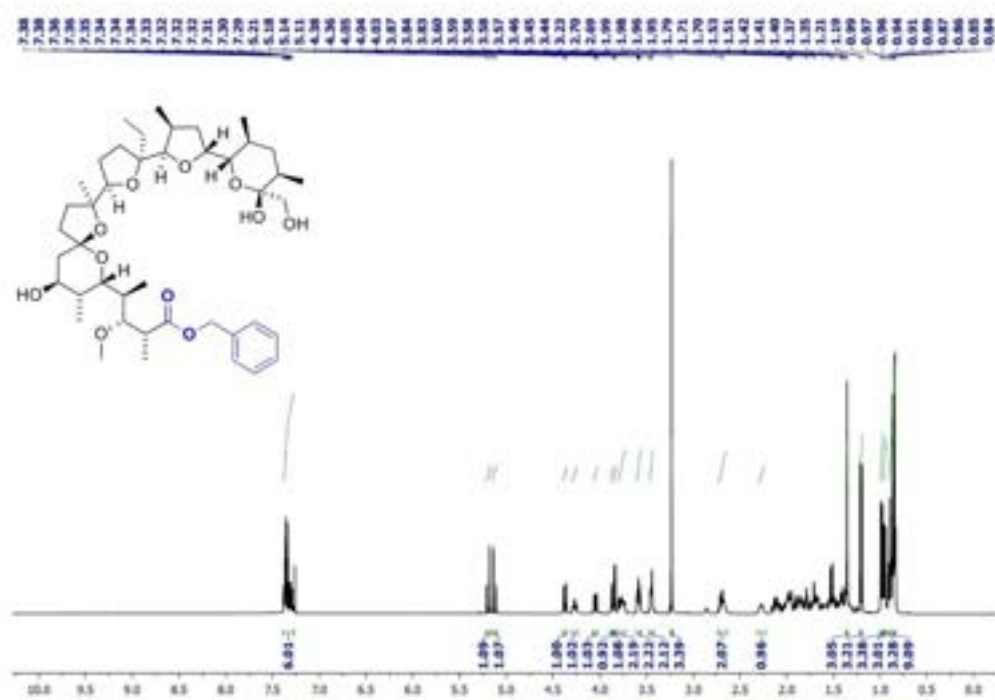
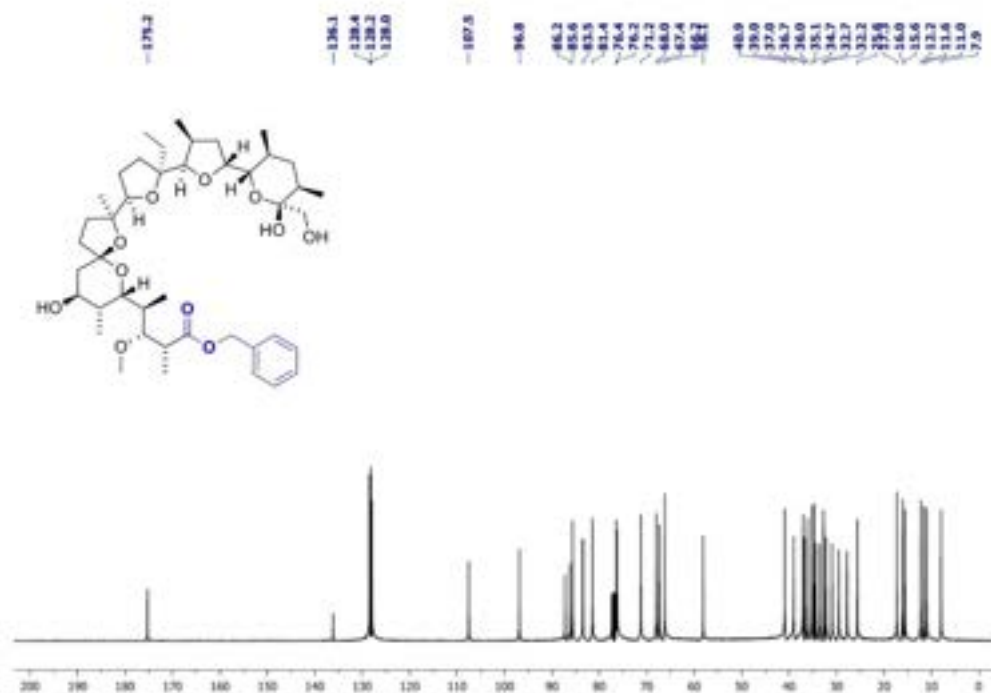


Figure S8. The ^1H NMR (401 MHz) spectrum of **5** in methanol- d_4 .







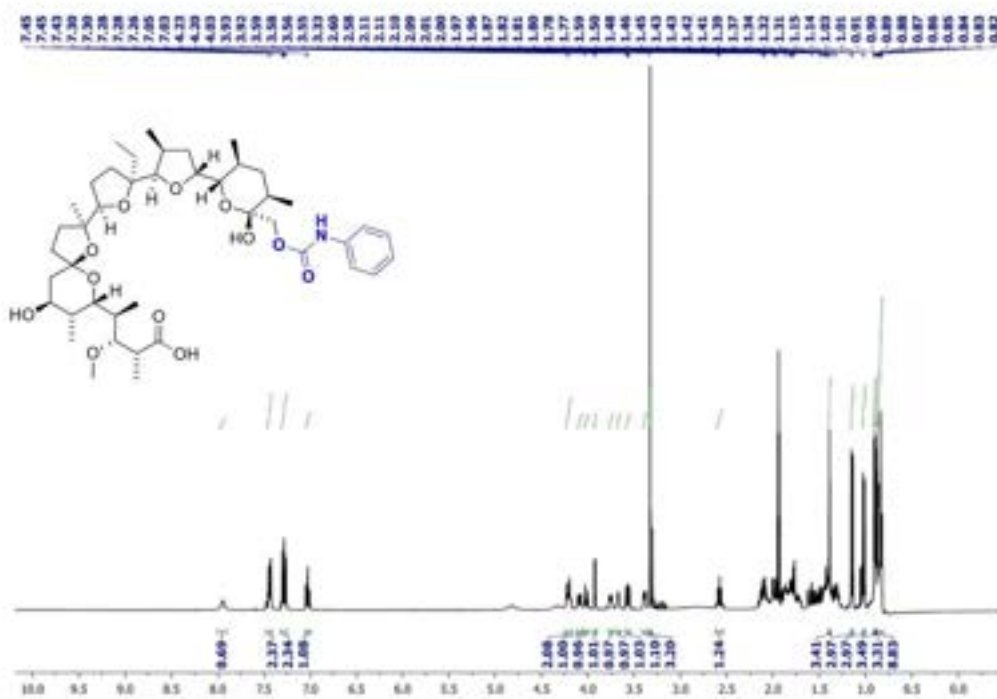
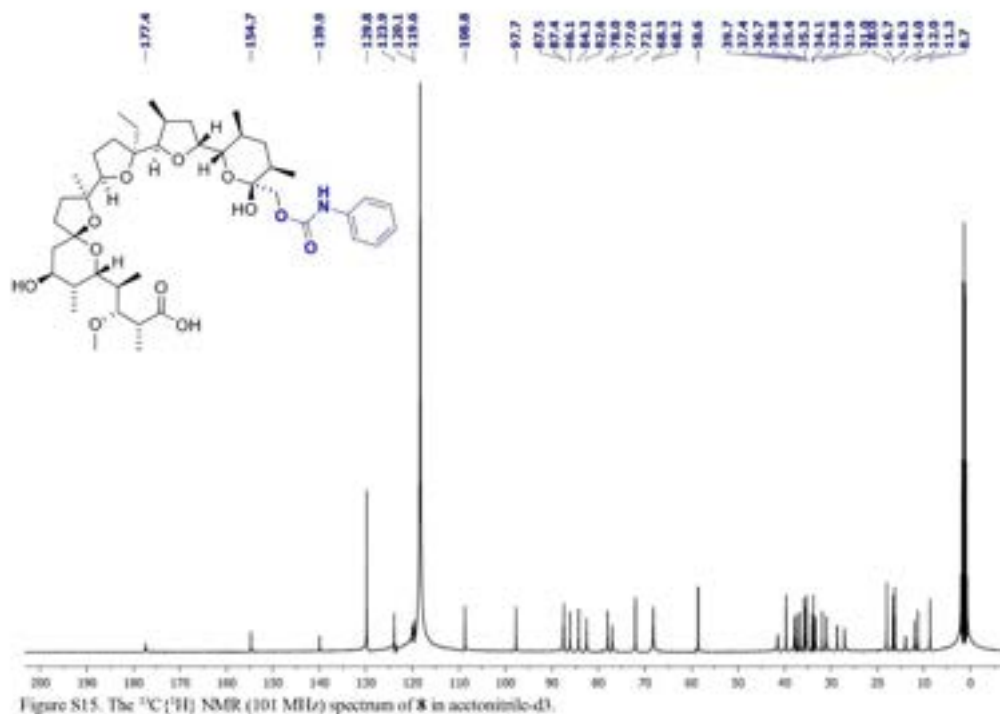


Table S1. Crystal data, collection data and structure refinement parameters for compounds **4**, and **5**.

	4	5
Formula	C ₁₃ H ₆₀ N ₂ NaO ₁₂ ·0.5(H ₂ O)	C ₃₈ H ₆₄ NNaO ₁₂ P·2(Br)
Molecular weight	838.00	1201.04
Temperature (K)	100	100
Crystal system	Trigonal	Orthorhombic
Space group	<i>R</i> 3̄: <i>H</i>	<i>P</i> 2 ₁ 2 ₁ 2 ₁
Unit cell dimensions	<i>a</i> (Å)	26.4195 (8)
	<i>b</i> (Å)	-
	<i>c</i> (Å)	17.5811 (5)
<i>V</i> (Å ³)	10627.4 (7)	5937.8 (4)
<i>Z</i>	9	4
<i>F</i> (000)	4077	2524
<i>D</i> _{calc} (g cm ⁻³)	1.178	1.344
θ range (°)	3.1–25.1°	2.6–24.7°
μ (mm ⁻¹)	0.09	1.46
Crystal size (mm)	0.31 × 0.27 × 0.22	0.27 × 0.22 × 0.16
<i>T</i> _{min} / <i>T</i> _{max}	0.952/1.000	0.879/1.000
Total / unique / obs reffs	33432/10793/7764	91861/14115/11162
<i>R</i> _{int}	0.057	0.096
<i>R</i> [<i>F</i> ² > 2σ(<i>F</i> ²)] ^a	0.054	0.078
<i>wR</i> [<i>F</i> ² all reffs] ^a	0.115	0.189
<i>S</i>	1.01	1.02
Flack parameter	0.0 (2)	0.015 (5)
$\Delta\rho_{\text{max}}$, $\Delta\rho_{\text{min}}$ (e Å ⁻³)	0.22, -0.22	0.73, -1.46

^a $R = \sum [|F_o| - |F_c|] / \sum F_o$, $wR = [\sum [w(F_o^2 - F_c^2)^2] / \sum wF_o^4]^{1/2}$, $w^{-1} = \sigma^2(F_o^2) + (aP)^2 + bP$ where $P = (F_o^2 + 2F_c^2)/3$. The *a* and *b* parameters are 0.0466 and 3.1960 for **4**, and 0.0814 and 25.8110 for **5**.

Table S2. Hydrogen bond geometry for **4** (Å, °).

<i>D</i> — <i>H</i> ··· <i>A</i>	<i>D</i> — <i>H</i>	<i>H</i> ··· <i>A</i>	<i>D</i> ··· <i>A</i>	<i>D</i> — <i>H</i> ··· <i>A</i>
O4—H4O···O10	0.84	1.95	2.715 (3)	151
O10—H10O···O2	0.84	1.84	2.627 (3)	155
N1—H1···O1 ⁱ	0.88	2.12	2.907 (4)	149
N2—H2N···O1 ⁱ	0.88	2.03	2.860 (4)	157
O1W—H1W1···O11	0.87	1.91	2.732 (3)	156
O1W—H2W1···O1	0.87	1.86	2.724 (4)	169
O2W—H1W2···O2	0.87	2.27	2.948 (8)	135
O2W—H1W2···O2W ⁱⁱ	0.87	1.97	2.799 (12)	160

Symmetry codes: (i) $-y+2/3, x-y+1/3, z+1/3$; (ii) $-x+y, -x, z$.

Table S3. Hydrogen bond geometry for **5** (Å, °).

<i>D</i> —H... <i>A</i>	<i>D</i> —H	H... <i>A</i>	<i>D</i> ... <i>A</i>	<i>D</i> —H... <i>A</i>
O1—H1 <i>A</i> ...O10	0.84	1.93	2.728 (7)	159
O4—H4 <i>A</i> ...Br2	0.85 (1)	2.33 (2)	3.151 (5)	161 (6)
O10—H101...Br2	0.84	2.37	3.106 (5)	147
N1—H1 <i>N</i> ...Br1	0.88	2.41	3.285 (6)	174
O12—H12 <i>A</i> ...O11	0.86	1.94	2.792 (8)	176
O12—H12 <i>B</i> ...O2	0.86	2.11	2.898 (8)	152

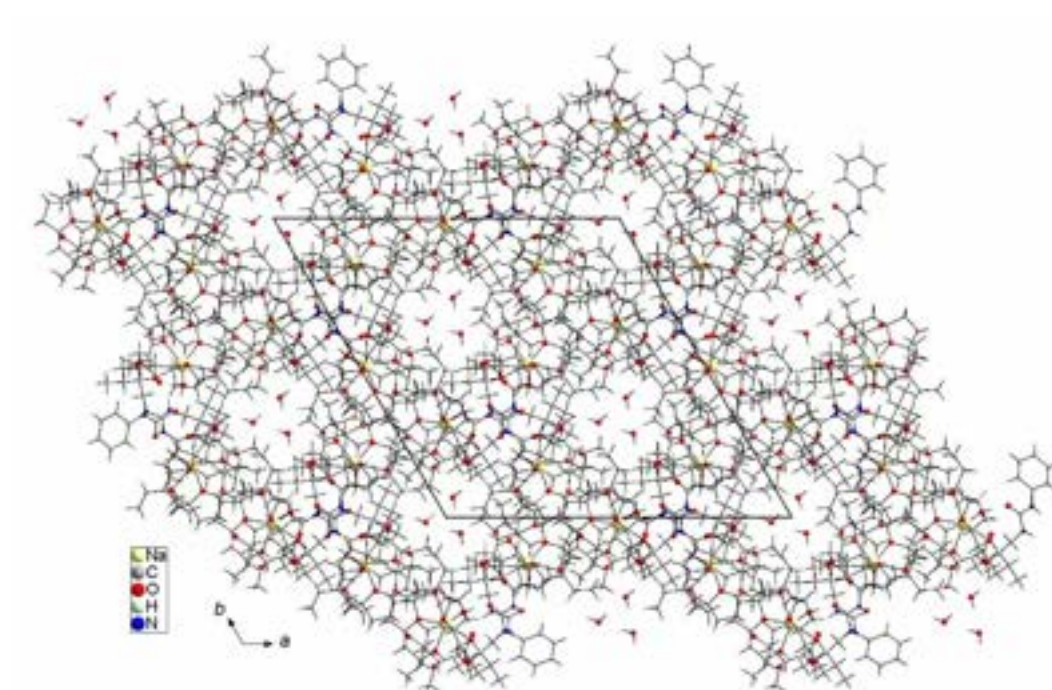


Figure S17. Packing along for 4.

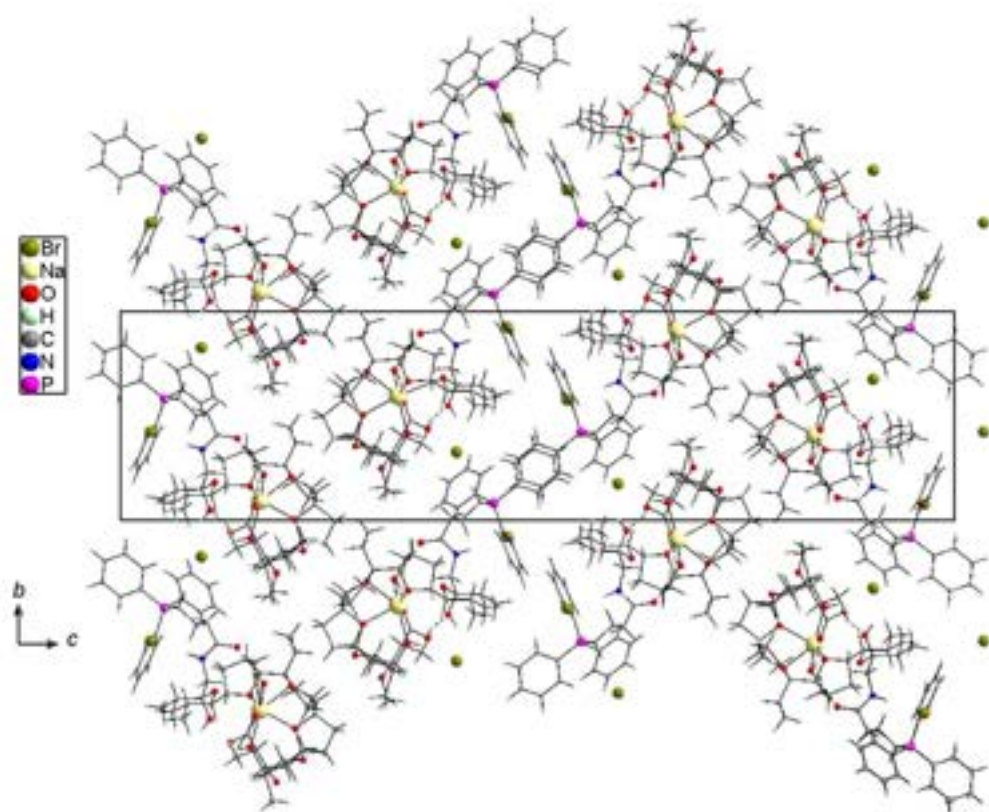


Figure S18. Packing along for 5.

12. Oświadczenia współautorów

Poznań, 12.06.2025

mgr Michał Sulik
Zakład Chemii Medycznej, Wydział Chemii
Uniwersytet im. Adama Mickiewicza w Poznaniu

OŚWIADCZENIE

W związku z ubieganiem się o stopień doktora nauk chemicznych oświadczam, że jestem współautorem publikacji:

- **Michał Sulik**, Michał Antoszczak, Adam Huczyński, Dietmar Steverding „*Antiparasitic activity of ivermectin: Four decades of research into a “wonder drug”*”, European Journal of Medicinal Chemistry 261 (2023) 115838. Opis wkładu doktoranta w powstanie publikacji: Jestem kierownikiem projektu Diamentowy Grant w ramach którego powstał niniejszy artykuł. Jestem odpowiedzialny za wykonanie przeglądu literaturowego dotyczącego właściwości przeciwpasożytniczych ivermektyny na przestrzeni ostatnich 40 lat oraz jej zastosowań w medycynie i weterynarii. Odpowiadałem za napisanie manuskryptu. Mój udział procentowy stanowi **60%**.
- **Michał Sulik**, Diana Fontinha, Dietmar Steverding, Szymon Sobczak, Michał Antoszczak, Miguel Prudêncio, Adam Huczyński „*Unexpected rearrangement of ivermectin in the synthesis of new derivatives with trypanocidal and antiplasmodial activities*”, European Journal of Medicinal Chemistry 263 (2024) 115951. Opis wkładu doktoranta w powstanie publikacji: Jestem kierownikiem projektu Diamentowy Grant w ramach którego powstał niniejszy artykuł. Jestem w pełni odpowiedzialny za syntezę oraz oczyszczanie pojedynczo oraz podwójnie modyfikowanych pochodnych ivermektyny opisanych w pracy. Odpowiadałem za koordynowanie badań w tym kontakt z innymi naukowcami. Przeprowadziłem też analizę otrzymanych wyników, w tym tych z badań biologicznych. Odpowiadałem za napisanie manuskryptu. Mój udział procentowy stanowi **45%**.

Michał Sulik

reprint

- **Michał Sulik**, Dagmara Otto-Ślusarczyk, Michał Antoszczak, Marta Struga, Adam Huczyński „*Ivermectin and its synthetic derivatives – A new class of anticancer agents*”, *European Journal of Medicinal Chemistry Reports* 12 (2024) 100176. Opis wkładu doktoranta w powstanie publikacji: Jestem kierownikiem projektu Diamentowy Grant w ramach którego powstał niniejszy artykuł. Jestem w pełni odpowiedzialny za syntezę oraz oczyszczanie pojedynczo oraz podwójnie modyfikowanych pochodnych ivermektyny opisanych w pracy. Odpowiadałem za koordynowanie badań w tym kontakt z innymi naukowcami. Przeprowadziłem też analizę otrzymanych wyników, w tym tych z badań biologicznych. Odpowiadałem za napisanie manuskryptu. Mój udział procentowy stanowi **60%**.
- **Michał Sulik**, Dagmara Otto-Ślusarczyk, Dietmar Steverding, Marta Struga, Adam Huczyński „*Antiproliferative and trypanocidal activity of ivermectin bioconjugates*”, *ACS Omega* (2025), DOI: 10.1021/acsomega.5c02998, Opis wkładu doktoranta w powstanie publikacji: Jestem kierownikiem projektu Diamentowy Grant w ramach którego powstał niniejszy artykuł. Jestem w pełni odpowiedzialny za syntezę oraz oczyszczanie wszystkich biokoniugatów ivermektyny opisanych w pracy. Odpowiadałem za koordynowanie badań w tym kontakt z innymi naukowcami. Przeprowadziłem też analizę otrzymanych wyników, w tym tych z badań biologicznych. Odpowiadałem za napisanie manuskryptu. Mój udział procentowy stanowi **70%**.
- **Michał Sulik**, Marta Jędrzejczyk, Magdalena Mielczarek-Puta, Gwan Yong Lim, Małgorzata Podsiad, Jakub Hoser, Piotr Bednarczyk, Marta Struga, Adam Huczyński „*Mitochondria-targeting phosphonium salts of monensin A as the source of novel antiproliferative agents*”, *European Journal of Medicinal Chemistry Reports* 14 (2025) 100275. Opis wkładu doktoranta w powstanie publikacji: Jestem w pełni odpowiedzialny za syntezę oraz oczyszczanie wszystkich soli fosfoniowych monenzyny opisanych w pracy. Przeprowadziłem też analizę otrzymanych wyników, w tym tych z badań biologicznych oraz biofizycznych. Odpowiadałem za napisanie manuskryptu. Mój udział procentowy stanowi **40%**.
- **Michał Sulik**, Robert Graniczny, Jan Janczak, Dagmara Kłopotowska, Joanna Wietrzyk, Adam Huczyński „*From Pseudocyclic to Macrocyclic Ionophores: Strategies toward the Synthesis of Cyclic Monensin Derivatives*”, *The Journal of Organic Chemistry* 90, 3, (2025) 1344–1353. Opis wkładu doktoranta w powstanie

Huczyński

Michał Sulik

publikacji: Jestem odpowiedzialny za opracowanie metodologii syntezy oraz oczyszczania azydowej oraz aminowej pochodnej monenzyny. Pełniłem rolę opiekuna pracy laboratoryjnej lic. Roberta Granicznego, który przeprowadził syntezę cyklicznych pochodnych monenzyny. Odpowiadałem za koordynowanie badań w tym kontakt z innymi naukowcami. Przeprowadziłem też analizę otrzymanych wyników, w tym tych z badań biologicznych. Odpowiadałem za napisanie manuskryptu. Mój udział procentowy stanowi **40%**.

- **Michał Sulik**, Eyob A. Workneh, Sofia Santana, Bárbara Teixeira, Miguel Prudêncio, Jan Janczak, Adam Huczyński „*Chemical modification of monensin as a source of potent antiparasmodial agents*”, *Bioorganic & Medicinal Chemistry* 123 (2025) 118177. Opis wkładu doktoranta w powstanie publikacji: Jestem w pełni odpowiedzialny za syntezę oraz oczyszczanie wszystkich pochodnych monenzyny opisanych w pracy. Odpowiadałem za koordynowanie badań w tym kontakt z innymi naukowcami. Przeprowadziłem też analizę otrzymanych wyników, w tym tych z badań biologicznych. Odpowiadałem za napisanie manuskryptu. Mój udział procentowy stanowi **30%**.

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(czytelny podpis)

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OŚWIADCZENIE

Mgr Michał Sulik jest związany z moją grupą badawczą od wielu lat. Pod moją opieką zrealizował swój licencjat i pracę magisterską, co sprawiło, iż podczas realizacji rozprawy doktorskiej moja rola mogła ograniczyć się głównie do konsultacji wyników jego syntez i badań spektroskopowych oraz współredakcji manuskryptów. Pełniłem również rolę autora korespondującego.

Doktorant nie potrzebował nadzoru i pracował niezwykle samodzielnie. Był On również współodpowiedzialny za redakcję manuskryptów, którą umiejętnie prowadził we współpracy z wieloma naukowcami z Polski i z zagranicy.

Swoje prace realizował w dwóch nurtach, pierwszym dotyczącym modyfikacji ivermektyny – tu realizował własny projekt badawczy: Diamentowy Grant pt. „*Synteza oraz badania właściwości przeciwnowotworowych i przeciwpasożytniczych nowych pochodnych ivermektyny*” (0159/DIA/2020/49), finansowy przez Ministerstwo Edukacji i Nauki.

Drugi kierunek badań związany był z chemiczną modyfikacją monenzyny – tu był głównym wykonawcą w moim projekcie: OPUS 21 pt. „*Koniugaty celujące w mitochondria jako nowe środki przeciwnowotworowe*” (2021/41/B/ST4/00088).

W związku z ubieganiem się mgra Michała Sulika o stopień doktora nauk ścisłych i przyrodniczych w dyscyplinie chemia oświadczam, że jestem współautorem 7 publikacji:

- 1) Michał Sulik, Michał Antoszczak, **Adam Huczyński**, Dietmar Steverding „*Antiparasitic activity of ivermectin: Four decades of research into a “wonder drug”*”, European Journal of Medicinal Chemistry 261 (2023) 115838. Mój udział w przygotowaniu tej publikacji przeglądowej polegał na współredakcji manuskryptu. Pełniłem rolę autora korespondującego. Mój udział procentowy stanowi 10%.
- 2) Michał Sulik, Diana Fontinha, Dietmar Steverding, Szymon Sobczak, Michał Antoszczak, Miguel Prudêncio, **Adam Huczyński** „*Unexpected rearrangement of ivermectin in the synthesis of new derivatives with trypanocidal and antiplasmodial activities*”, European Journal of Medicinal Chemistry 263 (2024) 115951. Mój udział w przygotowaniu tej publikacji polegał na współtworzeniu koncepcji badań, nadzorem nad ich realizacją i współredakcją manuskryptu. Pełniłem rolę autora korespondującego. Mój udział procentowy stanowi 10%.



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- 3) Michał Sulik, Dagmara Otto-Ślusarczyk, Michał Antoszczak, Marta Struga, **Adam Huczyński** „*Ivermectin and its synthetic derivatives – A new class of anticancer agents*”, *European Journal of Medicinal Chemistry Reports* 12 (2024) 100176. Mój udział w przygotowaniu tej publikacji polegał na współtworzeniu koncepcji badań, nadzorem nad ich realizacją i współredakcją manuskryptu. Pełniłem rolę autora korespondującego. Mój udział procentowy stanowi 10%.
- 4) Michał Sulik, Dagmara Otto-Ślusarczyk, Dietmar Steverding, Marta Struga, **Adam Huczyński** „*Antiproliferative and trypanocidal activity of ivermectin bioconjugates*”, *ACS Omega* (2025), DOI: 10.1021/acsomega.5c02998. Mój udział w przygotowaniu tej publikacji polegał na współtworzeniu koncepcji badań, nadzorem nad ich realizacją i współredakcją manuskryptu. Pełniłem rolę autora korespondującego. Mój udział procentowy stanowi 10%.
- 5) Michał Sulik, Marta Jędrzejczyk, Magdalena Mielczarek-Put, Gwan Yong Lim, Małgorzata Podsiad, Jakub Hoser, Piotr Bednarczyk, Marta Struga, **Adam Huczyński** „*Mitochondria-targeting phosphonium salts of monensin A as the source of novel antiproliferative agents*”, *European Journal of Medicinal Chemistry Reports* 14 (2025) 100275. Mój udział w przygotowaniu tej publikacji polegał na współtworzeniu koncepcji badań, nadzorem nad ich realizacją i współredakcją manuskryptu. Pełniłem rolę autora korespondującego. Mój udział procentowy stanowi 10%.
- 6) Michał Sulik, Robert Graniczny, Jan Janczak, Dagmara Kłopotowska, Joanna Wietrzyk, **Adam Huczyński** „*From Pseudocyclic to Macrocyclic Ionophores: Strategies toward the Synthesis of Cyclic Monensin Derivatives*”, *The Journal of Organic Chemistry* 90, 3, (2025) 1344–1353. Mój udział w przygotowaniu tej publikacji polegał na współtworzeniu koncepcji badań, nadzorem nad ich realizacją i współredakcją manuskryptu. Pełniłem rolę autora korespondującego. Mój udział procentowy stanowi 10%.
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str. 2

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OŚWIADCZENIE

W związku z ubieganiem się mgra Michała Sulika o stopień doktora nauk chemicznych oświadczam, że jestem współautorem publikacji:

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- Michał Sulik, Diana Fontinha, Dietmar Steverding, Szymon Sobczak, **Michał Antoszczak**, Miguel Prudêncio, Adam Huczyński „Unexpected rearrangement of ivermectin in the synthesis of new derivatives with trypanocidal and antiplasmodial activities”, European Journal of Medicinal Chemistry 263 (2024) 115951. Mój udział w przygotowaniu tej publikacji polegał na współtworzeniu koncepcji badań, nadzorze nad ich realizacją i współredakcji manuskryptu. Mój udział procentowy stanowi 10%.
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Michał Antoszczak

Poznań, 11.06.2025

mgr Marta Jędrzejczyk
Zakład Chemii Medycznej, Wydział Chemii
Uniwersytet im. Adama Mickiewicza w Poznaniu

OŚWIADCZENIE

W związku z ubieganiem się mgra Michała Sulika o stopień doktora nauk chemicznych oświadczam, że jestem współautorką publikacji:

Michał Sulik, **Marta Jędrzejczyk**, Magdalena Mielczarek-Puta, Gwan Yong Lim, Małgorzata Podsiad, Jakub Hoser, Piotr Bednarczyk, Marta Struga, Adam Huczyński „*Mitochondria-targeting phosphonium salts of monensin A as the source of novel antiproliferative agents*”, European Journal of Medicinal Chemistry Reports 14 (2025) 100275.

Mój udział w przygotowaniu tej publikacji polegał na pomocy w izolowaniu monenzyny oraz redagowaniu tekstu artykułu. Mój udział procentowy stanowi 5%.


(czytelny podpis)

Poznań, 12.06.25

dr Szymon Sobczak
Zakład Chemii Materiałów, Wydział Chemii
Uniwersytet im. Adama Mickiewicza w Poznaniu

OŚWIADCZENIE

W związku z ubieganiem się mgra Michała Sulika o stopień doktora nauk chemicznych oświadczam, że jestem współautorem publikacji:

Michał Sulik, Diana Fontinha, Dietmar Steverding, **Szymon Sobczak**, Michał Antoszczak, Miguel Prudêncio, Adam Huczyński „*Unexpected rearrangement of ivermectin in the synthesis of new derivatives with trypanocidal and antiplasmodial activities*”, *European Journal of Medicinal Chemistry* 263 (2024) 115951.

Mój udział w przygotowaniu tej publikacji polegał na wykonaniu analizy dyfraktometrycznej oraz przygotowania opisu struktury otrzymanego związku. Mój udział procentowy stanowi 5%.


(czytelny podpis)

Poznań, 12.06.2025

lic. Robert Graniczny
Zakład Chemii Medycznej, Wydział Chemii
Uniwersytet im. Adama Mickiewicza w Poznaniu

OŚWIADCZENIE

W związku z ubieganiem się mgra Michała Sulika o stopień doktora nauk chemicznych oświadczam, że jestem współautorem publikacji:

Michał Sulik, **Robert Graniczny**, Jan Janczak, Dagmara Kłopotowska, Joanna Wietrzyk, Adam Huczyński „From Pseudocyclic to Macrocyclic Ionophores: Strategies toward the Synthesis of Cyclic Monensin Derivatives”, *The Journal of Organic Chemistry* 90, 3, (2025) 1344–1353.

Mój udział w przygotowaniu tej publikacji polegał na syntezie substratów wyjściowych (azydowa i aminowa pochodna monenzyny) oraz cyklicznych pochodnych monenzyny. Mój udział procentowy stanowi 25%.

(czytelny podpis)

Robert Graniczny

Warszawa, 10.06.2025

prof. dr hab. Marta Struga
Katedra i Zakład Biochemii, Wydział Lekarski
Warszawski Uniwersytet Medyczny

OŚWIADCZENIE

W związku z ubieganiem się mgra Michała Sulika o stopień doktora nauk chemicznych oświadczam, że jestem współautorką publikacji:

- Michał Sulik, Dagmara Otto-Ślusarczyk, Michał Antoszczak, **Marta Struga**, Adam Huczyński „*Ivermectin and its synthetic derivatives – A new class of anticancer agents*”, *European Journal of Medicinal Chemistry Reports* 12 (2024) 100176. Mój udział w przygotowaniu tej publikacji polegał na nadzorze merytorycznym nad badaniami biologicznymi. Mój udział procentowy stanowi 5%.
- Michał Sulik, Dagmara Otto-Ślusarczyk, Dietmar Steverding, **Marta Struga**, Adam Huczyński „*Antiproliferative and trypanocidal activity of ivermectin bioconjugates*”, *ACS Omega* (2025), DOI: 10.1021/acsomega.5c02998. Mój udział w przygotowaniu tej publikacji polegał na nadzorze merytorycznym nad badaniami biologicznymi. Mój udział procentowy stanowi 5%.
- Michał Sulik, Marta Jędrzejczyk, Magdalena Mielczarek-Put, Gwan Yong Lim, Małgorzata Podsiad, Jakub Hoser, Piotr Bednarczyk, **Marta Struga**, Adam Huczyński „*Mitochondria-targeting phosphonium salts of monensin A as the source of novel antiproliferative agents*”, *European Journal of Medicinal Chemistry Reports* 14 (2025) 100275. Mój udział w przygotowaniu tej publikacji polegał na nadzorze merytorycznym nad badaniami biologicznymi. Mój udział procentowy stanowi 5%.

Marta
Katarzyna
Struga

Elektronicznie
podpisany przez Marta
Katarzyna Struga
Data: 2025.06.10
14:23:31 +02'00'

(czytelny podpis)

Warszawa, 11.06.2025

Dr Dagmara Otto-Ślusarczyk
Katedra i Zakład Biochemii, Wydział Lekarski
Warszawski Uniwersytet Medyczny

OŚWIADCZENIE

W związku z ubieganiem się mgra Michała Sulika o stopień doktora nauk chemicznych oświadczam, że jestem współautorką publikacji:

- Michał Sulik, Dagmara Otto-Ślusarczyk, Michał Antoszczak, Marta Struga, Adam Huczyński „*Ivermectin and its synthetic derivatives – A new class of anticancer agents*”, *European Journal of Medicinal Chemistry Reports* 12 (2024) 100176. Mój udział w przygotowaniu tej publikacji polegał na wykonaniu badań biologicznych na poziomie komórkowym, analizie i opisie wyników. Mój udział procentowy stanowi 15%.
- Michał Sulik, Dagmara Otto-Ślusarczyk, Dietmar Steverding, Marta Struga, Adam Huczyński „*Antiproliferative and trypanocidal activity of ivermectin bioconjugates*”, *ACS Omega* (2025), DOI: 10.1021/acsomega.5c02998. Mój udział w przygotowaniu tej publikacji polegał na wykonaniu badań dotyczących oceny cytotoksyczności. Mój udział procentowy stanowi 5%.

(czytelny podpis)



Warszawa, 11.06.2025

dr n. med. Magdalena Mielczarek-Puta
Katedra i Zakład Biochemii, Wydział Lekarski
Warszawski Uniwersytet Medyczny

OŚWIADCZENIE

W związku z ubieganiem się mgra Michała Sulika o stopień doktora nauk chemicznych oświadczam, że jestem współautorką publikacji:

Michał Sulik, Marta Jędrzejczyk, Magdalena Mielczarek-Puta, Gwan Yong Lim, Małgorzata Podsiad, Jakub Hoser, Piotr Bednarczyk, Marta Struga, Adam Huczyński „*Mitochondria-targeting phosphonium salts of monensin A as the source of novel antiproliferative agents*”, *European Journal of Medicinal Chemistry Reports* 14 (2025) 100275. Mój udział w przygotowaniu tej publikacji polegał na wykonaniu badań biologicznych na poziomie komórkowym, analizie i opisie wyników. Mój udział procentowy stanowi 15%.

(czytelny podpis)

Magdalena Mielczarek-Puta

Warszawa, 11.06.2025

mgr inż. chem. Małgorzata Podsiad
Katedra i Zakład Biochemii, Wydział Lekarski
Warszawski Uniwersytet Medyczny

OŚWIADCZENIE

W związku z ubieganiem się mgr Michała Sulika o stopień doktora nauk chemicznych oświadczam, że jestem współautorką publikacji:

Michał Sulik, Marta Jędrzejczyk, Magdalena Mielczarek-Puta, Gwan Yong Lim, **Małgorzata Podsiad**, Jakub Hoser, Piotr Bednarczyk, Marta Struga, Adam Huczyński „*Mitochondria-targeting phosphonium salts of monensin A as the source of novel antiproliferative agents*”, *European Journal of Medicinal Chemistry Reports* 14 (2025) 100275. Mój udział w przygotowaniu tej publikacji polegał na wykonaniu części badań mikrobiologicznych. Mój udział procentowy stanowi 5%.

(czytelny podpis)

M. Podsiad

Warsaw, 11.06.2015

Gwan Yong Lim
Chair and Department of Biochemistry,
Faculty of Medicine,
Medical University of Warsaw

CO-AUTHOR DECLARATION

In connection with the application of Michał Sulik for the degree of doctor of Chemical Sciences, I declare that I am a co-author of the publication:

Michał Sulik, Marta Jędrzejczyk, Magdalena Mielczarek-Puta, Gwan Yong Lim, Małgorzata Podsiad, Jakub Hoser, Piotr Bednarczyk, Marta Struga, Adam Huczyński „Mitochondria-targeting phosphonium salts of monensin A as the source of novel antiproliferative agents”, European Journal of Medicinal Chemistry Reports 14 (2025) 100275. My contribution to the preparation of this publication consisted of cytotoxicity assessment. My percentage contribution is 5%.

Gwan Yong Lim
(signature)


Wrocław, 4.06.2025

prof. dr hab. Jan Janczak
Instytut Niskich Temperatur i Badań Strukturalnych
Polska Akademia Nauk, Wrocław

OŚWIADCZENIE

W związku z ubieganiem się mgr Michała Sulika o stopień doktora nauk chemicznych oświadczam, że jestem współautorem publikacji:

- Michał Sulik, Robert Graniczny, **Jan Janczak**, Dagmara Kłopotowska, Joanna Wietrzyk, Adam Huczyński „*From Pseudocyclic to Macrocyclic Ionophores: Strategies toward the Synthesis of Cyclic Monensin Derivatives*”, *The Journal of Organic Chemistry* 90, 3, (2025) 1344–1353. Mój udział w przygotowaniu tej publikacji polegał na pomiarze kryształów i oznaczeniu struktur krystalicznych. Mój udział procentowy stanowi 10%.
- Michał Sulik, Eyob A. Workneh, Sofia Santana, Bárbara Teixeira, Miguel Prudêncio, **Jan Janczak**, Adam Huczyński „*Chemical modification of monensin as a source of potent antiplasmodial agents*”, *Bioorganic & Medicinal Chemistry* 123 (2025) 118177. Mój udział w przygotowaniu tej publikacji polegał na pomiarze monokryształów i oznaczeniu struktur krystalicznych. Mój udział procentowy stanowi 10%.


(czytelny podpis)

Wrocław, 05.06.2025

prof. dr hab. Joanna Wietrzyk
Instytut Immunologii i Terapii Doświadczalnej
Polskiej Akademii Nauk im. Ludwika Hirszfelda
we Wrocławiu

OŚWIADCZENIE

W związku z ubieganiem się mgra Michała Sulika o stopień doktora nauk chemicznych oświadczam, że jestem współautorką publikacji:

Michał Sulik, Robert Graniczny, Jan Janczak, Dagmara Kłopotowska, **Joanna Wietrzyk**, Adam Huczyński „From Pseudocyclic to Macrocyclic Ionophores: Strategies toward the Synthesis of Cyclic Monensin Derivatives”, *The Journal of Organic Chemistry* 90, 3, (2025) 1344–1353.

Mój udział w przygotowaniu tej publikacji polegał na udziale w planowaniu metodyki badań *in vitro* oraz interpretacji uzyskanych wyników z badań *in vitro*. Mój udział stanowi 5%.



(czytelny podpis)

Wrocław, 09.06.2025

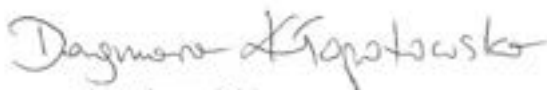
dr inż. Dagmara Kłopotowska
Instytut Immunologii i Terapii Doświadczalnej
Polskiej Akademii Nauk im. Ludwika Hirszfelda
we Wrocławiu

OŚWIADCZENIE

W związku z ubieganiem się mgra Michała Sulika o stopień doktora nauk chemicznych oświadczam, że jestem współautorką publikacji:

Michał Sulik, Robert Graniczny, Jan Janczak, **Dagmara Kłopotowska**, Joanna Wietrzyk, Adam Huczyński „From Pseudocyclic to Macrocyclic Ionophores: Strategies toward the Synthesis of Cyclic Monensin Derivatives”, *The Journal of Organic Chemistry* 90, 3, (2025) 1344–1353.

Mój udział w przygotowaniu tej publikacji polegał na wykonaniu badań aktywności przeciwnowotworowej związków (przygotowaniu komórek do testów, wykonanie testu SRB, wyznaczenie IC50 dla badanych związków na 5 liniach komórkowych). Mój udział procentowy stanowi 10%.


(czytelny podpis)

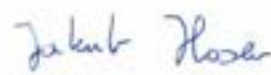
Warszawa, ..12.06.25

mgr Jakub Hoser
Katedra Fizyki i Biofizyki, Instytut Biologii,
Szkoła Główna Gospodarstwa Wiejskiego w Warszawie

OŚWIADCZENIE

W związku z ubieganiem się mgra Michała Sulika o stopień doktora nauk chemicznych oświadczam, że jestem współautorem publikacji:

Michał Sulik, Marta Jędrzejczyk, Magdalena Mielczarek-Puta, Gwan Yong Lim, Małgorzata Podsiad, **Jakub Hoser**, Piotr Bednarczyk, Marta Struga, Adam Huczyński „*Mitochondria-targeting phosphonium salts of monensin A as the source of novel antiproliferative agents*”, European Journal of Medicinal Chemistry Reports 14 (2025) 100275. Mój udział w przygotowaniu tej publikacji polegał na wykonaniu, a także opracowaniu wyników i opisów eksperymentów biofizycznych - pomiaru transportu jonów w poprzek sztucznej dwuwarstwy lipidowej przy pomocy techniki czarnych błon lipidowych oraz pomiaru zużycia tlenu metodą respirometrii o wysokiej rozdzielczości. Mój udział procentowy stanowi 10%.


(czytelny podpis)

Warszawa, 10/6/2025

prof. dr hab. Piotr Bednarczyk
Katedra Fizyki i Biofizyki, Instytut Biologii,
Szkoła Główna Gospodarstwa Wiejskiego w Warszawie

OŚWIADCZENIE

W związku z ubieganiem się mgra Michała Sulika o stopień doktora nauk chemicznych oświadczam, że jestem współautorem publikacji:

Michał Sulik, Marta Jędrzejczyk, Magdalena Mielczarek-Puta, Gwan Yong Lim, Małgorzata Podsiad, Jakub Hoser, **Piotr Bednarczyk**, Marta Struga, Adam Huczyński „*Mitochondria-targeting phosphonium salts of monensin A as the source of novel antiproliferative agents*”, *European Journal of Medicinal Chemistry Reports* 14 (2025) 100275. Mój udział w przygotowaniu tej publikacji polegał na pomocy przy analizie i opisie danych elektrofizjologicznych. Mój udział procentowy stanowi 5%.



(czytelny podpis)

Priv.-Doz. Dr. Dietmar Steverding
Associate Professor
Bob Champion Research & Education Building
Norwich Medical School
University of East Anglia

CO-AUTHOR DECLARATION

In connection with the application of Michał Sulik for the degree of doctor of Chemical Sciences, I declare that I am a co-author of the publication:

- Michał Sulik, Michał Antoszczak, Adam Huczyński, **Dietmar Steverding** „Antiparasitic activity of ivermectin: Four decades of research into a “wonder drug”, European Journal of Medicinal Chemistry 261 (2023) 115838. My contribution to the preparation of this publication consisted of writing the Introduction and the Conclusion, and reviewing and editing the manuscript. My percentage contribution was about 20%.
- Michał Sulik, Diana Fontinha, **Dietmar Steverding**, Szymon Sobczak, Michał Antoszczak, Miguel Prudêncio, Adam Huczyński „Unexpected rearrangement of ivermectin in the synthesis of new derivatives with trypanocidal and antiplasmodial activities”, European Journal of Medicinal Chemistry 263 (2024) 115951. My contribution to the preparation of this publication consisted of determining the trypanocidal and cytotoxic activity of the ivermectin derivatives as well as reviewing and editing the manuscript. My percentage contribution was about 15%.
- Michał Sulik, Dagmara Otto-Ślusarczyk, **Dietmar Steverding**, Marta Struga, Adam Huczyński „Antiproliferative and trypanocidal activity of ivermectin bioconjugates”, ACS Omega (2025), DOI: 10.1021/acsomega.5c02998. My contribution to the preparation of this publication consisted of determining the trypanocidal and cytotoxic activity of the ivermectin bioconjugates. My percentage contribution is about 10%

Norwich, 10/06/2025



Lisbon, 3 June 2025

Prof. Miguel Prudêncio

Instituto de Medicina Molecular João Lobo Antunes, Faculdade de Medicina, Universidade de Lisboa Avenida Prof. Egas Moniz, 1649-028 Lisboa, Portugal

GIMM - Gulbenkian Institute for Molecular Medicine, Avenida Prof. Egas Moniz, 1649-035 Lisboa, Portugal

CO-AUTHOR DECLARATION

In connection with the application of Michał Sulik for the degree of doctor of Chemical Sciences, I declare that I am a co-author of the publication:

- Michał Sulik, Diana Fontinha, Dietmar Steverding, Szymon Sobczak, Michał Antoszczak, **Miguel Prudêncio**, Adam Huczyński „*Unexpected rearrangement of ivermectin in the synthesis of new derivatives with trypanocidal and antiplasmodial activities*”, *European Journal of Medicinal Chemistry* 263 (2024) 115951. My contribution to the preparation of this publication consisted of providing the biological and other materials required for the determination of compound activity against the hepatic stage of *Plasmodium* infection, supervising the researchers in my lab responsible for these assays, co-interpreting the data, and reviewing the manuscript prior to submission. My percentage contribution is 10%.
- Michał Sulik, Eyob A. Workneh, Sofia Santana, Bárbara Teixeira, **Miguel Prudêncio**, Jan Janczak, Adam Huczyński „*Chemical modification of monensin as a source of potent antiplasmodial agents*”, *Bioorganic & Medicinal Chemistry* 123 (2025) 118177. My contribution to the preparation of this publication consisted of providing the biological and other materials required for the determination of compound activity against the hepatic stage of *Plasmodium* infection, supervising the researchers in my lab responsible for these assays, co-interpreting the data, and reviewing the manuscript prior to submission. My percentage contribution is 10%.



Lisbon, June 10, 2025

Eyob A. Workneh
Instituto de Medicina Molecular João Lobo Antunes,
Faculdade de Medicina,
Universidade de Lisboa

GIMM - Gulbenkian Institute for Molecular Medicine,
Avenida Prof. Egas Moniz

CO-AUTHOR DECLARATION

In connection with the application of Michał Sulik for the degree of doctor of Chemical Sciences, I declare that I am a co-author of the publication:

Michał Sulik, **Eyob A. Workneh**, Sofia Santana, Bárbara Teixeira, Miguel Prudêncio, Jan Janczak, Adam Huczyński „*Chemical modification of monensin as a source of potent antiplasmodial agents*”, *Bioorganic & Medicinal Chemistry* 123 (2025) 118177.

My contribution to the preparation of this publication consisted of writing the original draft, Methodology, Investigation, Data curation, and Conceptualization. My percentage contribution is 30%.

(signature)

Eyob Addise Workneh



Lisbon, 11th June of 2025

Dr. Diana Fontinha
Instituto de Medicina Molecular João Lobo Antunes,
Faculdade de Medicina,
Universidade de Lisboa

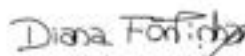
GIMM - Gulbenkian Institute for Molecular Medicine,
Avenida Prof. Egas Moniz

CO-AUTHOR DECLARATION

In connection with the application of Michał Sulik for the degree of doctor of Chemical Sciences, I declare that I am a co-author of the publication:

Michał Sulik, **Diana Fontinha**, Dietmar Steverding, Szymon Sobczak, Michał Antoszczak, Miguel Prudêncio, Adam Huczyński „*Unexpected rearrangement of ivermectin in the synthesis of new derivatives with trypanocidal and antiplasmodial activities*”, European Journal of Medicinal Chemistry 263 (2024) 115951.

My contribution to the preparation of this publication consisted of the assessment of the antiplasmodial activity of the new ivermectin derivatives. My percentage contribution is 5%.


(signature)

Lisbon, 09/06/2025

Sofia Santana
Instituto de Medicina Molecular João Lobo Antunes,
Faculdade de Medicina,
Universidade de Lisboa

GIMM - Gulbenkian Institute for Molecular Medicine,
Avenida Prof. Egas Moniz

CO-AUTHOR DECLARATION

In connection with the application of Michał Sulik for the degree of doctor of Chemical Sciences, I declare that I am a co-author of the publication:

Michał Sulik, Eyob A. Workneh, **Sofia Santana**, Bárbara Teixeira, Miguel Prudêncio, Jan Janczak, Adam Huczyński „*Chemical modification of monensin as a source of potent antiplasmodial agents*”, *Bioorganic & Medicinal Chemistry* 123 (2025) 118177.

My contribution to the preparation of this publication consisted of the determination of IC₅₀ experiments. My percentage contribution is 5%.

(signature)



Lisbon, 4th of June, 2025

Bárbara Teixeira
Instituto de Medicina Molecular João Lobo Antunes,
Faculdade de Medicina,
Universidade de Lisboa

GIMM - Gulbenkian Institute for Molecular Medicine,
Avenida Prof. Egas Moniz

CO-AUTHOR DECLARATION

In connection with the application of Michał Sulik for the degree of doctor of Chemical Sciences, I declare that I am a co-author of the publication:

Michał Sulik, Eyob A. Workneh, Sofia Santana, **Bárbara Teixeira**, Miguel Prudêncio, Jan Janczak, Adam Huczyński „*Chemical modification of monensin as a source of potent antiplasmodial agents*”, *Bioorganic & Medicinal Chemistry* 123 (2025) 118177.

My contribution to the preparation of this publication consisted of determination of CC50 values. My percentage contribution is 5%.


(signature)