

The preparation of artemisinin-cholesterol conjugates as potential new drugs for treatment of intractable forms of tuberculosis and malaria

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Preface

This thesis is submitted in an article format in accordance with the General Academic Rules (A.13.7.3) of the North-West University. An article in the form of a manuscript is included in this dissertation:

Chapter 3: Article for submission

Preliminary evaluation of artemisinin-cholesterol conjugates as potential drugs for treatment of intractable forms of malaria and tuberculosis

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ABSTRACT

Malaria and tuberculosis (TB) are two lethal infectious diseases that continue to plague mankind and claim many lives. These diseases are more prevalent in developing countries especially in Africa and Asia. Malaria alone is estimated to have infected 214 million and killed 438,000 people in 2014. The majority of the cases were in Africa (88%) which also accounted for most deaths (90%). However, it appears that the mortality due to malaria is decreasing given that in 2013 584,000 deaths are estimated to have occurred.

Chemotherapy remains the most effective malaria control strategy. A number of drugs used for treatment of malaria have become ineffective because of resistance, mainly from the most virulent malaria parasite, *Plasmodium falciparum* (*Pf*). Artemisinin and its derivatives (collectively called artemisinins) are currently the most active drugs, but because of their short half-lives are used in combination with longer acting partner drugs in artemisinin combination therapies (ACTs). One aim associated with introduction of ACTs was to inhibit development of resistance. Nevertheless, reports of increased parasite clearance times associated with ACTs are now widely reported and it is clear that incipient development of resistance to artemisinins is taking place. This is a daunting development since there are currently no alternative drugs to artemisinins.

More devastating are the 1.4 million deaths and 10.4 million new TB cases reported to have occurred in 2015. The majority of these cases were also in Africa (26%) and Asia (61%). Socio-economic factors hamper TB eradication in endemic regions. Moreover, the development of *Mycobacterium tuberculosis* (*Mtb*) strains, the causative agent of TB, resistant to current drugs vastly complicates TB control. Multidrug-resistant TB (MDR-TB), extensively drug-resistant TB (XDR-TB) and sporadic totally drug-resistant TB (TDR-TB) have emerged. These *Mtb* strains are resistant to first-line and second-line anti-TB drugs. Although bedaquiline and delamanid have recently been approved conditionally for use in treatment of MDR-TB, these drugs are still undergoing advanced clinical trials and their complete safety profiles still need to be established.

Despite obvious differences in the life cycles of the pathogens of malaria and TB, cholesterol is vital during their development. The malaria parasite constantly diverts and salvages cholesterol during its liver stages. Cholesterol appears to be significant in the membrane architecture and forging nutrient passages into the parasite. In *Mtb* cholesterol is a carbon source and is metabolised by the bacterium. A putative cholesterol transporter, Mce4, actively shuttle this molecule into the bacterium.

Artemisinins are oxidant drugs that interrupt electron transfer in the redox systems of the

malaria parasite. Interference of these drugs with glutathione reductase and related reductases in the malaria parasite leads to greatly impaired redox homeostasis. In *Mtb*, mycothiol reductase and ergothionine are involved in redox homeostasis, and it is likely that artemisinins will act against these systems as well. Therefore, a single drug that targets these pathogens is in principle attainable.

We herein report the synthesis of artemisinin-cholesterol conjugates with varied linkers. The compounds were screened *in vitro* against *Pf*, *Mtb* and the normal mammalian HEK293 embryonic kidney cell line. Antimalarial activities (IC_{50}) against *Pf* chloroquine (CQ) CQ-sensitive NF54, and CQ-resistant K1 and W2 strains ranged from 0.03 – 2.6, 0.03 – 1.9 and 0.02 – 1.7 μ M, respectively. Most of the compounds were relatively insoluble that may have contributed to the low activities relative to comparator artemisinins. The most active were compounds **14** and **15** against all strains. All the compounds showed no cross resistance and were not cytotoxic, with selectivity indices between the mammalian cells and the parasites ranging from 28.9 – 3903.

Activities against *Mtb* H37Rv cultures were assessed by counting the colony forming units (CFU/ml) and then noting percentage inhibition. Cultures were treated with compounds at 10 and 80 μ M concentrations resulting in growth inhibition ranging from 3 – 38% and 18 – 52%, respectively. Compounds **15** and **23** were the most active in displaying 38 and 31% inhibition at 10 μ M and 52 and 47% inhibition at 80 μ M, respectively.

Although the antimalarial activities of the artemisinin-cholesterol conjugates herein are less than the artemisinin comparator drugs, the appreciable antimalarial and especially antimycobacterial activities noted here will help in the development of conjugates exploiting putative transporters in each of *Pf*, and other malaria parasites such as *P. vivax*, and *Mtb*. The immediate aims are therefore to improve aqueous solubilities of the compounds and to perform *in vivo* antimalarial and antimycobacterial assays. Activities of compounds **15** and **23** will be assessed in infected macrophage models. Subsequent studies will be carried out to assess the influx of these compounds into granulomas, and their activities against dormant forms of *Mtb*.

Keywords: Malaria, tuberculosis, artemisinins, cholesterol, conjugates

OPSOMMING

Malaria en tuberkulose (TB) is twee dodelike infeksies wat die mensdom treiter en vele lewens eis. Hierdie siektes is meer volop in ontwikkelende lande, veral in Afrika en Asië. Daar word beraam dat, in 2014, malaria 214-miljoen mense geïnfekteer het en 438,000 sterftes veroorsaak het. Die meeste gevalle is in Afrika (88%) aangeteken wat ook vir die meeste (90%) sterftes verantwoordelik was. Dit blyk egter dat die mortaliteit weens malaria verminder omrede daar vir 2013 584,000 sterftes beraam is.

Chemoterapie is steeds die mees effektiewe strategie vir die beheer van malaria. Sommige geneesmiddels wat vir die behandeling van malaria aangewend word, is egter oneffektief as gevolg van weerstandbiedendheid, hoofsaaklik deur die mees virulente malariaparasiete, *Plasmodium falciparum* (Pf). Artemisinien en derivate daarvan (getiteld artemisiniene) is tans die aktiefste geneesmiddels, maar weens hul kort halfleeftyd word hulle in kombinasie met langerwerkende geneesmiddels in artemisinien-kombinasieterapie (AKT) gebruik. Een doel met die bekendstelling van AKT is om die ontwikkeling van weerstandbiedendheid te voorkom. Ongeag hiervan word verlengde opruimingstye van parasiete aangeteken en is dit duidelik dat weerstandbiedendheid teen artemisiniene ontwikkel. Hierdie is 'n gedugte verwikkeling omdat daar tans geen alternatiewe geneesmiddels vir die artemisiniene bestaan nie.

Nog meer vernietigend is die 1.4-miljoen sterftes en 10.4-miljoen nuwe TB gevalle wat in 2015 aangeteken is. Die meeste van dié gevalle was ook in Afrika (26%) en Asië (61%). Sosio-ekonomiese faktore verhinder die uitdelging van TB in endemiese areas. Die ontwikkeling van *Mycobacterium tuberculosis* (Mtb)-lyne, die veroorsakende organisme in TB, wat weerstandbiedend is teen huidig-gebruikte geneesmiddels, kompliseer die beheer van TB. Meervoudige-geneesmiddel-weerstandige TB (MGW-TB), uitgebreide-geneesmiddel-weerstandige TB (UGW-TB) en sporadiese totaal-geneesmiddel-weerstandige TB (TGW-TB) het verskyn. Hierdie Mtb-lyne is weerstandbiedend teen eerste-linie en tweede-linie TB-geneesmiddels. Alhoewel bedakilien en delamanied onlangs voorwaardelik vir die behandeling van MGW-TB goedgekeur is, ondergaan hierdie geneesmiddels steeds gevorderde kliniese toetse en hul veiligheidsprofile moet steeds bevestig word.

Ten spyte van die duidelike verskille in die lewensiklusse van die malaria- en TB-patogene, is cholesterol noodsaaklik vir hul ontwikkeling. Die malariaparasiet omlei en herwin cholesterol deurentyd gedurende sy lewerstadië. Cholesterol is belangrik vir membraanargitektuur en ingang van voedingstowwe in die parasiet in. In Mtb is cholesterol 'n koolstofbron en word deur die bakterium gemetaboliseer. Die voorgestelde cholesteroltransporter, Mce4, pomp hierdie molekule aktief in die bakterium in.

Artemisiniene is oksiderende-geneesmiddels wat elektronoordrag in redokksisteme van die

malariaparasiet belemmer. Die invloed van hierdie geneesmiddels op glutatioonreduktase en verwante reduktases in die malariaparasiet versteur redokshomeostase in 'n groot mate. In *Mtb* is mikotiolreduktase en ergotionien betrokke by redokshomeostase, en dit is dus waarskynlik dat artemisiniene ook teen hierdie sisteme aktief sal wees. 'n Enkele geneesmiddel wat beide patogene teiken is dus in beginsel haalbaar.

Hiermee lewer ons verslag van die sintese van artemisinien-cholesterolkonjugate met varieerbare skakels. Die geneesmiddels is geëvalueer *in vitro* teen *Pf*, *Mtb* en die normale soogdier embrioniese-niersellyn, HEK293. Antimalaria-aktiwiteite (IC_{50}) teen *Pf* chlorokien (CK) CK-sensitiewe NF54, en CK-weerstandbiedende K1- en W2-lyne strek van 0.03 – 2.6, 0.03 – 1.9 en 0.02 – 1.7 μ M, onderskeidelik. Die meeste verbindings was redelik onoplosbaar wat moontlik kon bydra tot die lae aktiwiteite verkry met die verwysings artemisiniene. Die mees aktiewe verbindings teen alle lyne was **14** en **15**. Geen van die verbindings het kruisweerstandbiedendheid getoon nie en was ook nie sitotoksies nie, met selektiwiteitsindekse tussen die soogdierselle en die parasiete van 28.9 – 3903.

Aktiwiteite teen *Mtb* H37Rv-kulture is geëvalueer deur die kolonievormende eenhede te tel (CFU/ml) en gevolglik die persentasie inhibisie te noteer. Die kulture is met 10 en 80 μ M konsentrasies van die verbindings behandel wat tot groei-inhibisie van 3 – 38% en 18 – 52% gelei het. Verbindings **15** en **23** was die aktiefste en toon 38 en 31% inhibisie by 10 μ M, en 52 en 47% inhibisie by 80 μ M, onderskeidelik.

Alhoewel die antimalaria-aktiwiteite van die artemisinien-cholesterolkonjugate laer is as die artemisinien verwysingsgeneesmiddels, sal die betekenisvolle antimalaria- en veral antimikobakteriële-aktiwiteite wat hier aangeteken is, behulpsaam wees met die ontwikkeling van konjugate wat voorgestelde transporters in *Pf*, ander malariaparasiete soos *P. vivax* en *Mtb* uitbuit. Die primêre doelstelling is dus om wateroplosbaarheid van die verbindings te verbeter en om die antimalaria- en antimikobakteriële-aktiwiteite *in vivo* te bepaal. Aktiwiteite van verbindings **15** en **23** moet bepaal word in geïnfekteerde makrofaagmodelle. Daaropvolgende studies moet uitgevoer word om die influks van hierdie verbindings in granulomas te meet, asook die aktiwiteite teen dormante vorms van *Mtb*.

Sleutelwoorde: Malaria, tuberkulose, artemisiniene, cholesterol, konjugate

TABLE OF CONTENTS

Preface.....	ii
ACKNOWLEDGEMENTS.....	iii
OPSOMMING.....	vi
TABLE OF CONTENTS.....	viii
LIST OF ABBREVIATIONS.....	xiv
CHAPTER 1.....	1
INTRODUCTION AND PROBLEM STATEMENT.....	1
1.1. Introduction.....	1
1.2. Aims and objectives of the study.....	7
References.....	9
CHAPTER 2.....	14
LITERATURE REVIEW.....	14
2.1. Introduction.....	14
2.2. Malaria.....	14
2.2.1. Epidemiology of malaria.....	14
2.2.2. Life cycle and pathogenesis of malaria.....	15
2.2.2.1. Exoerythrocytic stages.....	16
2.2.2.2. Erythrocyte stages.....	18
2.2.2.3. Mosquito stage.....	19
2.2.3. Malaria symptoms.....	19
2.2.3.1. Cerebral malaria (CM).....	20
2.2.3.2. Anaemia.....	20
2.2.3.3. Malaria-induced acute respiratory distress syndrome (MI-ARDS).....	21
2.2.3.4. Hypoglycemia.....	21
2.2.3.5. Acute renal failure (ARF).....	21
2.2.4. Diagnosis of malaria.....	22
2.2.4.1. Microscopy.....	22
2.2.4.2. Rapid diagnostic test (RDT).....	23
2.2.4.3. Real-time polymerase chain reaction (RT-PCR).....	23

2.2.5. Key Interventions against Malaria.....	23
2.2.5.1. Indoor residual spraying and insecticide treated nets.....	23
2.2.5.2. Larval control	24
2.2.5.3. Intermittent preventive treatment in pregnancy (IPTp).....	25
2.2.6. Vaccination.....	25
2.2.7. Malaria chemotherapy	26
2.2.7.1. Activity against different parasite stages	26
2.2.8. Antimalarial drugs classes	27
2.2.8.1. Arylaminoalcohols.....	27
2.2.8.2. 4-Aminoquinolines	31
2.2.8.3. 8-Aminoquinolines	34
2.2.8.4. Antifolates	36
2.2.8.5. Hydroxynaphthoquinones	39
2.2.8.6. Antibiotics	41
2.2.8.7. Artemisinins	43
2.3. Tuberculosis	51
2.3.1. Epidemiology of tuberculosis (TB)	51
2.3.2. Tuberculosis in South Africa	52
2.3.3. Transmission and pathology of tuberculosis	52
2.3.3.1. Transmission.....	52
2.3.3.2. The life cycle of Tuberculosis	53
2.3.4. Clinical manifestation of tuberculosis	54
2.3.4.1. Primary pulmonary TB	54
2.3.4.2. Miliary TB.....	55
2.3.4.3. Extrapulmonary TB (EPTB).....	55
2.3.5. Diagnosis of TB	56
2.3.5.1. Sputum smear microscopy (SSM).....	56
2.3.5.2. Chest X-ray.....	56
2.3.5.3. TB Culture.....	56
2.3.5.4. Molecular testing.....	57

2.3.5.5. Latent TB infection testing.....	57
2.3.6. TB vaccine.....	58
2.3.7. Chemotherapy of tuberculosis	58
2.3.8. First-line treatment drugs.....	59
2.3.9. Second-line anti-TB drugs	63
2.3.9.1. Group 2 drugs.....	63
2.3.9.2. Group 3 drugs.....	64
2.3.9.3. Group 4 drugs.....	65
2.3.9.4. Group 5 drugs.....	70
2.4. Rational design	75
References	77
CHAPTER 3.....	113
ARTICLE FOR SUBMISSION.....	113
Abstract	115
Introduction.....	116
Chemistry	119
Biological activity	122
Conclusion.....	126
Experimental Section.....	127
Materials and methods	127
Syntheses	128
Preparation of artemisinin-piperazine cholesterol conjugates	131
Preparation of Carboxylates	133
Antimalarial assays	137
Cytotoxicity assay.....	138
Antituberculosis assay.....	138
References	140
CHAPTER 4.....	144
SUMMARY AND CONCLUSION.....	144
References	148

APPENDIX A: SPECTRA.....	151
APPENDIX B: GUIDE FOR AUTHORS.....	181

LIST OF TABLES, FIGURES AND SCHEMES

Figure 1.1: Artemisinin and its clinically used derivatives.....	2
Figure 1.2: Anti-TB drugs	5
Figure 1.3: Mycobactin-artemisinin conjugate.....	7
Figure 2.1: The life cycle of the malaria parasite.....	16
Figure 2.2: Transfer of sporozoites from mosquito to the human host.	18
Figure 2.3: Quinine 1 and quinidine 2	28
Figure 2.4: The enantiomers of mefloquine 3 comprising the racemic mixture used for treatment of malaria.	29
Figure 2.5: Halofantrine 4 and lumefantrine 5	30
Figure 2.6: Chloroquine 6	31
Figure 2.7: Piperaquine 7	33
Figure 2.8: Amodiaquine 8	34
Figure 2.9: Primaquine 9	35
Figure 2.10: Tafenoquine 10 , bulaquine 11 and NPC 1161 12	36
Figure 2.11: Biosynthesis of tetrahydrofolate and the targets of Class I and Class II antifolates.	37
Figure 2.12: Dapsone 13 and sulfadoxine 14	38
Figure 2.13: Pyrimethamine 15 , proguanil 16 , cycloguanil 17 , chlorproguanil 18 and chlorcycloguanil 19	39
Figure 2.14: Atovaquone 20	40
Figure 2.15: Pyronaridine 21	41
Figure 2.16: Doxycycline 22	42
Figure 2.17: Clindamycin 23	42
Figure 2.18: Artemisinin 24	43
Figure 2.19: Dihydroartemisinin 25 , artemether 26 , arteether 27 and artesunate 28	44
Figure 2.20: Proposed mechanism of action of artemisinins.....	46
Figure 2.21: Thapsigargin 34	48
Figure 2.22: The cofactor model of the artemisinin mechanism of action.....	49
Figure 2.23: Global incidences of Tuberculosis (TB) in 2014.....	52
Figure 2.24: The life cycle of the tuberculosis bacteria.....	54
Figure 2.25: Rifampicin 35	59
Figure 2.26: Isoniazid 36	61
Figure 2.27: Pyrazinamide 37	62
Figure 2.28: Ethambutol 38	62
Figure 2.29: Streptomycin 39 , amikacin 40 , kanamycin 41 and capreomycin 42	63
Figure 2.30: Ofloxacin 43 , levofloxacin 44 , gatifloxacin 45 and moxifloxacin 46	65

Figure 2.31: Ethionamide 47 and prothionamide 48 .	66
Figure 2.32: Cycloserine 49 , D-alanine 50 and terizidone 51 .	67
Figure 2.33: <i>p</i> -Aminosalicylic Acid 52 .	67
Figure 2.34: Biosynthesis of tetrahydrofolate and inhibition by <i>p</i> -aminosalicylic acid.	69
Figure 2.35: Linezolid 53 .	70
Figure 2.36: Clofazimine 54 .	71
Figure 2.37: Bedaquiline 55 .	72
Figure 2.38: Delamanid 56 .	73
Figure 2.39: SQ109 57 .	74
Figure 2.40: PA-824 58 .	74
Figure 2.41: The mycobactin-artemisinin conjugate 59 .	75
Figure 1: Artemisinin 1 and its current clinical derivatives, the hemiacetal dihydroartemisinin (DHA) 2 , the lactol ether artemether 3 and hemiester artesunate 4 .	115
Figure 2: Artemisinins and analogues that are active against Mtb in vitro: the mycobactin-dihydroartemisinin conjugate 5 and the steroidal tetraoxane 6 .	116
Table 1. IC ₅₀ values of compounds against asexual blood stages of NF54, K1 and W2 strains of Plasmodium falciparum and their cytotoxicity against WI-38 HFLF cells.	124
Table 2: The antimycobacterial activity of compounds against Mtb H37Rv determined by CFU enumeration.	126
Scheme 1: Preparation dihydroartemisinin-cholesterol conjugates.	119
Scheme 2: Preparation of artemisinin-piperazine cholesterol conjugates.	121
Scheme 3: Preparation of carboxylates.	122

LIST OF ABBREVIATIONS

ACP	NADH-dependent enoyl acyl carrier protein
ACT	Artemisinin Combination Therapy
ACTs	Artemisinin Combination Therapies
AFs	Antifolates
AgDENV	An. gambiae densonucleosis virus
AlrA	D-alanine racemase
AQ	Amodiaquine
ARF	Acute Renal Failure
BCG	Bacille Calmette-Gièrin
BF ₃ ·Et ₂ O	Boron trifluoride diethyl etherate
CDA	Chlorproguanil/dapsone with artesunate
CDCl ₃	Chloroform- <i>d</i>
CFU	Colony forming units
CFU/mL	Colony forming units per milliliter
CHMP	Committee for Medicinal Products for Human Use
CM	Cerebral Malaria
CNS	Central nervous system
CSP	Circumsporozoite protein
CQ	Chloroquine
CQR	CQ-resistant
CQS	CQ-sensitive
CYP	Cytochrome P

D-Ala	D-alanine
DCC	N,N'-Dicyclohexylcarbodiimide
DCM	Dichloromethane
Ddl	D-alanine:D-alanine ligase
DDT	Dichlorodiphenyltrichloroethane
DEE	Diethyl ether
DFHR	Dihydrofolate reductase
DHA	Dihydroartemisinin
DHF	Dihydrofolate
DHOD	Dihydroorotate dehydrogenase
DHPS	Dihydropteroate synthase
DMAP	N,N-Dimethylpyridin-4-amine
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DMSO- <i>d</i> ₆	dimethyl sulfoxide- <i>d</i> ₆
DST	Drug sensitivity testing
DV	Digestive vacuole
EDTA	Ethylenediaminetetraacetic acid
EGT	Ergothionine
EPF	Entamopathogenic fungi
EPTB	Extrapulmonary TB
ER	Endoplasmic reticulum
ETC	Electron transport chain

EtOAc	Ethyl acetate
EtOH	Ethanol
FAS1	Fatty acid synthase 1
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FPPIX	Ferroprotoporphyrin IX
G6PD	Glucose-6-phosphate dehydrogenase
GSH	Glutathione
GSK	GlaxoSmithKline
GSSG	Glutathione disulfide
HBsAg	Hepatitis B surface antigen
HIV	Human immunodeficiency virus
HIV/TB	Human immunodeficiency virus and TB coinfections
HMS	Hexose monophosphate shunt
HRMS	High resolution mass spectrometry
Hz	Hertz
IC ₅₀	50% Inhibitory concentration
inhA	NADH-dependent enoyl acyl carrier protein (ACP) reductase
IPTp	Intermittent Preventive Treatment in pregnancy
IPTp-SP	IPTp with sulfadoxine-pyrimethamine
IR	Infrared
IRS	Indoor residual spraying
ITNs	Insecticide-treated nets

LLINs	Longer lasting insecticide nets
LTBI	Latent Tuberculosis infection
<i>M. vaccae</i>	<i>Mycobacterium vaccae</i>
MDG	Millennium Development Goals
MDR-TB	Multidrug-resistant TB
MeOH	Methanol
MgSO ₄	Magnesium sulphate
MSH	Mycothiol
MI-ARDS	Malaria-Induced Acute Respiratory Distress Syndrome
MIC	Minimum inhibitory concentration
MoA	Mechanism of action
mp	Melting point
MR	Mycothiol reductase
MRC	South African Medical Research Council
mRNA	Messenger Ribonucleic acid
<i>Mtb</i>	<i>Mycobacterium tuberculosis</i>
MTBC	Mycobacterium Tuberculosis Complex
MTT	3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide
NAD ⁺	Nicotinamide
NADPH	Nicotinamide adenine dinucleotide phosphate (Reduced)
NaHCO ₃	Sodium hydrogen carbonate
NDH-2	NADH dehydrogenase
NMR	Nuclear Magnetic Resonance

NO	Nitric oxide
NRF	South African National Research Foundation
PAS	para-Aminosalicylic acid
PABA	para-Aminobenzoic acid
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
<i>Pf</i>	<i>Plasmodium falciparum</i>
<i>Pfcr1</i>	<i>P. falciparum</i> chloroquine resistance transporter gene
<i>PfKelch13</i>	<i>P. falciparum</i> Kelch13
<i>Pfmdr1</i>	<i>P. falciparum</i> multidrug-resistant gene 1
<i>Pfmdt</i>	<i>P. falciparum</i> metabolite drug transporter gene
<i>pfTetQ</i>	<i>P. falciparum</i> GTPase TetQ gene
Pgh1	P-glycoprotein homologue 1
pHRP-2	<i>Plasmodium</i> histidine-rich protein 2
pLDH	<i>Plasmodium</i> lactate dehydrogenase
ppm	Parts per million
pRBCs	Parasitised Red Blood Cells
PV	Parasitophorous vacuole
QRDR	Quinolone resistance-determining region
RDT	Rapid Diagnostic Test
RI	Resistance index
RPMI	Roswell Park Memorial Institute
RNA	Ribonucleic acid

ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
RT-PCR	Real-Time Polymerase Chain Reaction
SA	South Africa
SERCA	Serca-endoplasmic reticulum Ca ²⁺ ATPase
SI	Selectivity index
SSM	Sputum smear microscopy
ssRNA	Single stranded RNA
TB	Tuberculosis
TDR-TB	Totally-drug resistant TB
TEA	Triethylamine
THF	Tetrahydrofuran
TMS	Tetramethylsilane
TrxR	Thioredoxin reductase
UPR	Unfolded Protein Response
USA	United States of America
WHO	World Health Organization
XDR-TB	Extensively-drug resistant TB
ZNS	Zeihl-Neelsen stain

CHAPTER 1

INTRODUCTION AND PROBLEM STATEMENT

1.1. Introduction

Infectious diseases continue to beleaguer mankind despite progress made in recent years towards their eradication. Human immunodeficiency virus (HIV), tuberculosis (TB) and malaria are amongst the most lethal infectious diseases that continue to claim many lives annually (Murray et al., 2014, Vitoria et al., 2009). Their prevalence is more rampant in the developing world with its insufficiency of resources to control and eradicate them (Ruxin et al., 2005). Adding to the complexity is the persistent resistance of pathogens to drugs currently used in treatment strategies (Spellberg et al., 2008).

In 2000, the World Health Organisation (WHO) set goals to start reversing frequency of infection by diseases such as malaria and TB so as to bring incidence to near zero by 2015 (McArthur, 2014). Positive outcomes have been reported regarding reversal but incidence was still alarmingly high in 2015. The 2015 WHO Malaria report indicates that there were an estimated 214 million cases of malaria and 438 000 deaths due to malaria in 2014. Most of the cases were in the African region (88%) which also accounted for 90% of deaths. During the same year, malaria also killed about 306 000 children under the age of five of which 292 000 were in the African region (WHO, 2015a).

Malaria is a vector-borne infection transmitted by female *Anopheles* mosquitoes. This disease is caused by a protozoan *Plasmodium*; of which five species are responsible for human malaria. These are *P. malariae*, *P. ovale*, *P. knowlesi*, *P. vivax* and *P. falciparum*. *P. vivax* and *P. falciparum* account for most cases of malaria and the latter accounts for the majority of malaria associated deaths (Cox, 2010). *P. vivax* has the ability to remain dormant in the liver and may cause active disease days, months or years after the initial infection; this parasite is largely associated with relapse cases of malaria (Mueller et al., 2009). *P. falciparum* also has an intrinsic ability to develop resistance against most drugs used in malaria chemotherapy.

Eradication of malaria represents an enormous challenge and prospects for its realisation appear improbable in the near future. There is currently no vaccine against malaria but there has been significant progress made in the development of the RTS,S/AS01 vaccine; recent Phase III clinical trials show positive results. Success of this vaccine will add to the current control and treatment methods (Rts, 2015, Agnandji, 2011). The use of insecticide treated nets and residual spraying have contributed to the reduction in malaria morbidity and mortality globally.

The chemotherapy of malaria involving pure substances dates back to the 19th century when quinine was first isolated from the bark of the fever tree and shown to possess antimalarial properties. Synthetic quinolines eventually emerged in the 1930s, and were used alongside quinine. The most effective of these, chloroquine (CQ) was widely used until the 1960s, when the malaria parasite was first noted to develop resistance in South-East Asia (Achan et al., 2011). The rapid spread of the resistance to other parts of the world led to concerted efforts to find alternatives and many drugs emerged. Most of these also tended to become ineffective in the face of resistance. However, in the 1970s effective, unique and fast acting antimalarial artemisinin was isolated in China from *Artemisia annua* L., a herb long used in traditional Chinese medicine for treatment of fevers and chills. Unfortunately, artemisinin had limitations such as poor solubility and short half-life, and its derivative dihydroartemisinin (DHA) was developed and converted into the oil-soluble ethers artemether and arteether, and the water-soluble hemisuccinyl ester artesunate (Figure 1.1) (Van Agtmael et al., 1999). These derivatives (collectively called artemisinins), although they possessed better antimalarial activities than artemisinin, also had limitations such as short half-lives, neurotoxicity and conversion back to DHA *in vivo*.

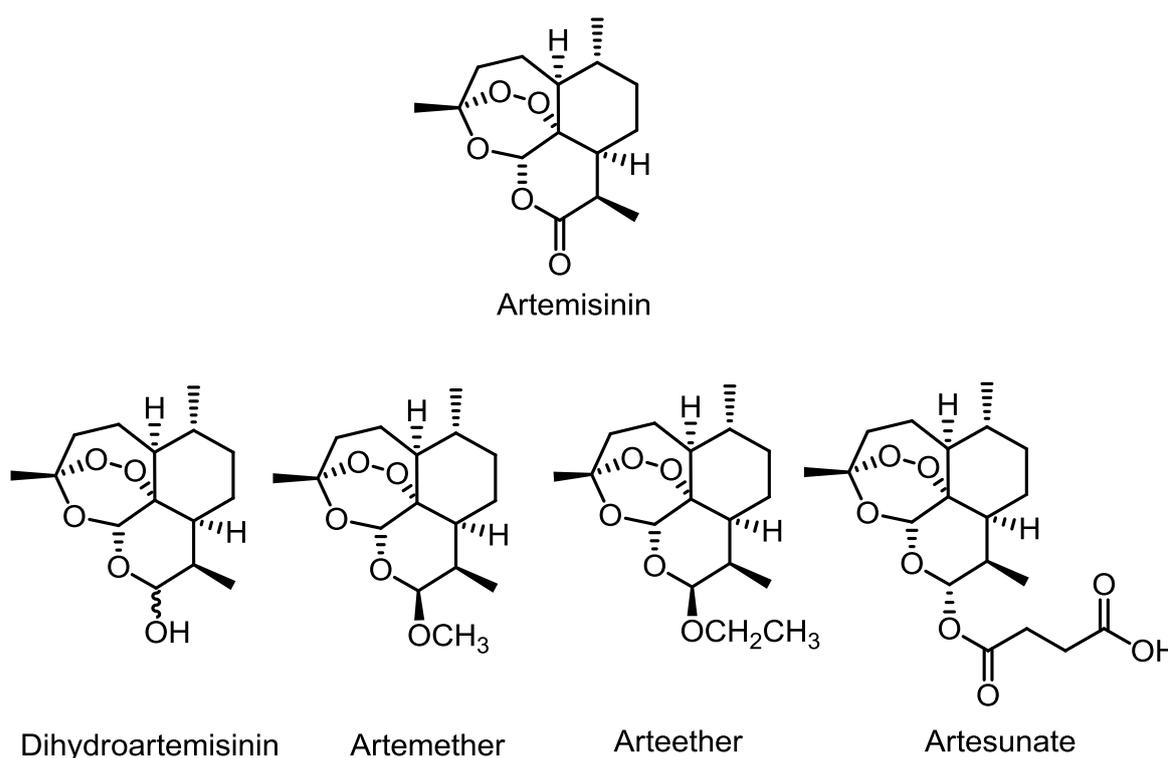


Figure 1.1: Artemisinin and its clinically used derivatives.

All of the artemisinin derivatives are currently used in treatment of malaria and are still effective in most endemic areas. However, cases of increased parasite clearance times were reported in the Greater Mekong region which is indicative of *P. falciparum* resistance to artemisinins. With

mechanisms of resistance recently uncovered, it is clear now that resistance to artemisinins has emerged. Artemisinins appear to induce parasite dormancy at the early intraerythrocytic ring stage; once drug pressure has subsided, reactivation occurs and the parasite resumes in normal growth programme (Paloque et al., 2016). The emergence of resistance has occurred despite artemisinins being used in artemisinin combination therapies (ACTs). The ACTs concept was developed in order to delay emergence of resistance to artemisinin derivatives through combination with a long acting partner drug. Spread of these artemisinin-resistant strains to other parts of the world, especially Africa, will greatly complicate malaria control programmes (Nosten and White, 2007, Bosman and Mendis, 2007). Therefore, there is an urgent need for new antimalarial drugs that should be active against all current drug resistant strains.

Equally catastrophic are morbidity and mortality due to tuberculosis. The 2015 Global Tuberculosis Report indicates that an estimated 1.5 million deaths due to TB and 9.6 million new TB cases occurred worldwide in 2014. These figures, as in the case of malaria, mostly come from the African (28%) and South-East Asian (58%) regions. In the African region, the cases of TB are more frequent than anywhere else in the world with 281 TB cases per 100 000 people compared to an estimated global average of 133 TB cases per 100 000 people. The African region is also plagued by high HIV/TB coinfection cases which accounted for 0.4 million deaths and 12% new TB cases in 2014. However, it is worth noting that TB deaths have decreased by 47% since 1990 and cases have been decreasing by 1.5% per year since 2000 (WHO, 2015b).

TB infection is caused by species of the Mycobacterium Tuberculosis Complex (MTBC). The species that infects human lungs is caused by *Mycobacterium tuberculosis* (*Mtb*) strains of the MTBC (Fogel, 2015). Infectious bacteria are transmitted from one person to another through coughing, sneezing, singing and in rare cases contact with body fluids. Upon inhaling airborne *Mtb*, bacilli travel to the lungs where they are engulfed by alveolar macrophages. This process triggers an innate immune response with concomitant recruitment of monocytes. Some of the macrophages differentiate into specialized cells such as multinucleate giant cells, epithelioid cells and lipid-rich foamy macrophages. This happens simultaneously with extensive vascularization. The response also triggers the recruitment of the lymphocytes, e.g. T cells. The amalgamation of these cells forms a granuloma, the hallmark of TB infection, which halts replication of bacilli and renders them dormant. Lymphocytes are found on the periphery of granuloma due to the formation of a fibrous cuff around the central cells (Ramakrishnan, 2012, Russell et al., 2010). Blood capillaries are also retracted from the centre of the granuloma limiting both oxygen supply and other molecules (e.g. drugs) from reaching the centre. Hypoxia may induce bacilli dormancy within the necrotic granuloma. This has an adverse impact on TB treatment since dormant bacilli are not sensitive to drugs and retraction of blood capillaries

inhibits the delivery of drugs to the centre of the granuloma (Dartois, 2014). Dormant bacilli may remain in the lungs for years without causing progression to disease. This is called latent TB infection (LTBI) and occurs in 90% of primary infections of individuals who are immunocompetent. LTBI leads to active TB in 5-15% of infected people especially with the loss of immunity as is the case in HIV-positive individuals (WHO, 2015b).

LTBI poses a great challenge in TB eradication programmes. There is currently no effective TB vaccine for adolescents and adults (Brandt et al., 2002, Sterne et al., 1998). The only available vaccine is the Bacille Calmette-Gièrin (BCG) vaccine which only offers partial protection in children but loses efficacy over time. Over 16 candidate vaccines are in clinical trials and it is hoped that these will help in control of TB before adulthood (Ginsberg et al., 2016). On the other hand, chemotherapy of TB faces many challenges ranging from low output of new drugs, long treatment regimens to the development of strains of *Mtb* resistant to current anti-TB drugs (Koul et al., 2011).

Strains of *Mtb* such as multidrug-resistant TB (MDR-TB), extensively-drug resistant TB (XDR-TB) and totally-drug resistant TB (TDR-TB) have emerged. MDR-TB is defined as being caused by strains of *Mtb* which are resistant to the first-line drugs isoniazid and rifampicin; an estimated 480 000 cases of MDR-TB were reported in 2014. In addition to MDR-TB, XDR-TB incorporates MDR-TB and additionally resistance to second-line fluoroquinolones and one of the injectable aminoglycosides i.e. amikacin, capreomycin or kanamycin (Sotgiu et al., 2009). TDR-TB has been reported in India and Italy and this is defined as MDR-TB resistant to all second-line drugs (Velayati et al., 2009, Maeurer et al., 2014, Migliori et al., 2007). The spread of these strains will have dire consequences for TB chemotherapy as new drugs are not yet available. Only bedaquiline and delamanid have recently received conditional approval for use in the treatment of MDR-TB; these are the first new TB drugs to be introduced in over forty years. However, these drugs are still undergoing phase III clinical trials; full data for their use in HIV positive patients is not yet available, the pediatric studies are still ongoing and data on combination regimens with other drugs is also not yet available (Brigden et al., 2015). A recent phase II study has also shown that there were more deaths in patients treated with bedaquiline than in the placebo groups while delamanid was found to induce hepatotoxicity and QT prolongation (Diacon et al., 2014, Gler et al., 2012). Taken together, the data already available and toxicity findings would seem to limit effective use of these drugs in treatment regimens. Thus, the problems with these newer drugs mean that efforts must be redoubled to discover and develop new and effective TB drugs.

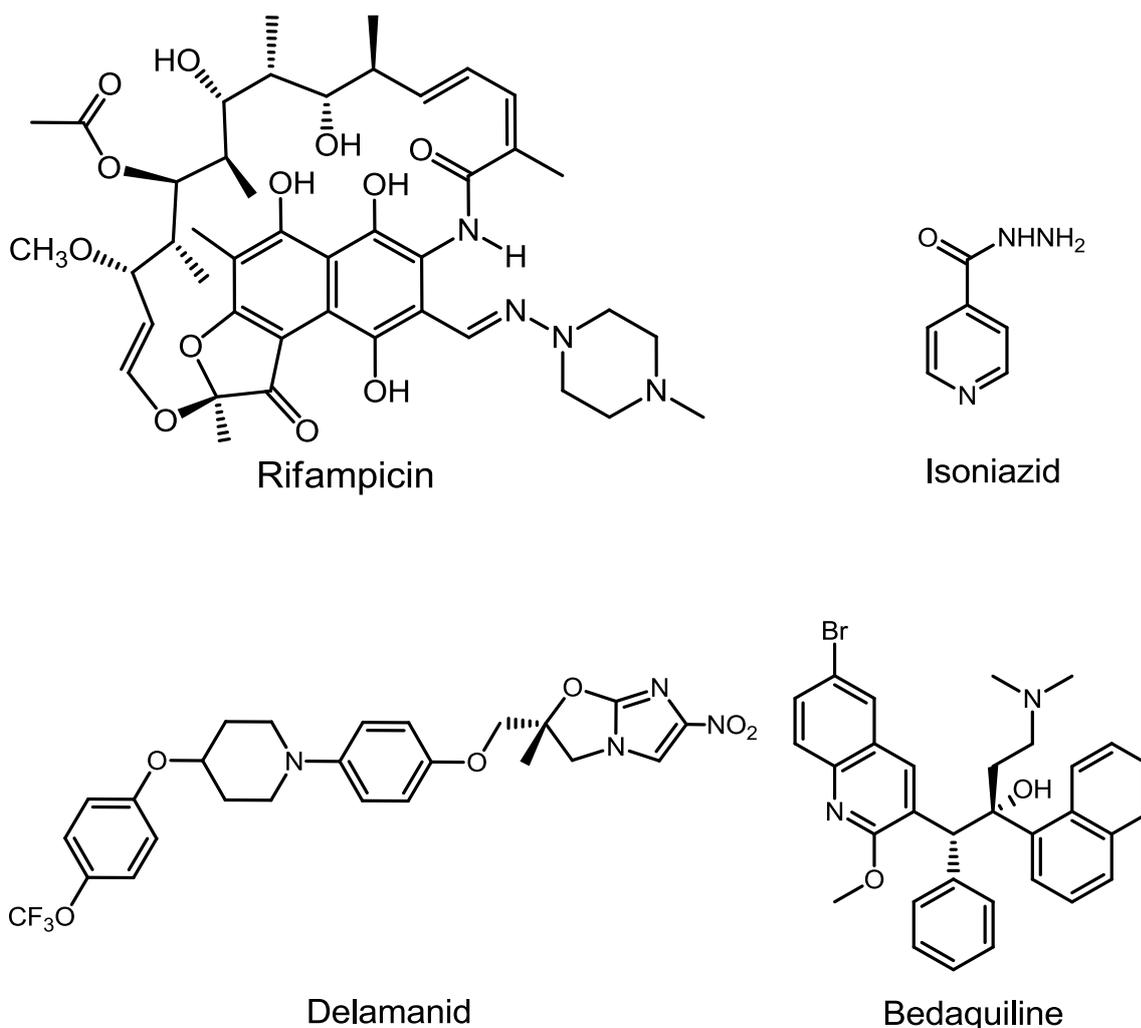


Figure 1.2: Chemical structures of Anti-TB drugs.

Although TB is clearly different from malaria, the life cycles of the respective pathogens converge at the need for cholesterol during development stages. In *Mtb*, cholesterol is a carbon source vital for bacilli survival. Studies with mice have shown that *Mtb* has a transporter called Mce4 which transports cholesterol into the bacterium. During initial infection *Mtb* associates with lipid-rich sites of plasma membranes of macrophages and is predominantly associated with these regions to access cholesterol. In addition, *Mtb* tend to be found around cholesterol-rich foamy macrophages in the granulomas and these are additional sources of cholesterol for *Mtb*. *Mtb* may also obtain its cholesterol as an insoluble crystal form from extracellular spaces (Pandey and Sasseti, 2008). Acquired cholesterol is metabolized to provide carbon that is used for bio-synthesis of molecules required for growth. Cholesterol also appears to be essential for driving virulence of *Mtb* (Ouellet et al., 2011, Brzostek et al., 2009). On the other hand, the malaria parasite diverts cholesterol from the high-density lipoproteins and also associates with the parasite endoplasmic reticulum to salvage newly synthesised lipids during the liver stage of parasite development. Upon invasion of the erythrocyte, the malaria parasite takes up and

incorporates cholesterol from erythrocyte membranes into its parasitophorous vacuole membrane. This is vital for nutrient trafficking into the parasite particularly in the early stages of erythrocyte invasion (Frankland et al., 2006, Tokumasu et al., 2014, Grellier et al., 1991, Labaied et al., 2011).

An interesting conjugate was synthesised from dihydroartemisinin and mycobactin which was potent against both malaria and TB (Miller et al., 2011) (Figure 1.3). It was proposed that mycobactin encapsulates and carries iron into the parasite and this activates artemisinin which then induces its antimalarial activity. This 'bioactivation' is presumed to be associated with the formation of reactive oxygen-based free radicals which cause intracellular damage in the parasite (Miller et al., 2011). These findings are also in line with a number of other studies which have attributed the activity of artemisinins to the generation carbon-centred radicals which are held to cause intracellular damage by alkylating vital biomolecules (O'Neill et al., 2010). However, a thorough review by Haynes *et al.* highlights some inconsistencies with this model; it is apparent that the C-centred radicals based on literature precedent cannot alkylate protein targets (Haynes et al., 2013). Alternatively, it has been proposed that artemisinins act as oxidant drugs that intercept electrons in the redox homeostasis pathway in the malaria parasite. Artemisinins oxidise the reduced flavin cofactor FADH₂ required to reduce enzymes such as glutathione reductase and thioredoxin reductase. These enzymes reduce glutathione disulfide (GSSG) to glutathione (GSH) and GSH in turn reduces reactive oxygen species (ROS) so as to maintain redox homeostasis. Interference by artemisinins with this process leads to build up of cytotoxic ROS that leads to parasite death (Haynes et al., 2012). In *Mtb* a homologue of glutathione reductase called mycothiol reductase is involved in redox homeostasis (Saini et al., 2016, Kumar et al., 2011). It is equally likely then that artemisinins will also act against this redox homeostasis system in a similar fashion to the GSH redox system in the malaria parasite.

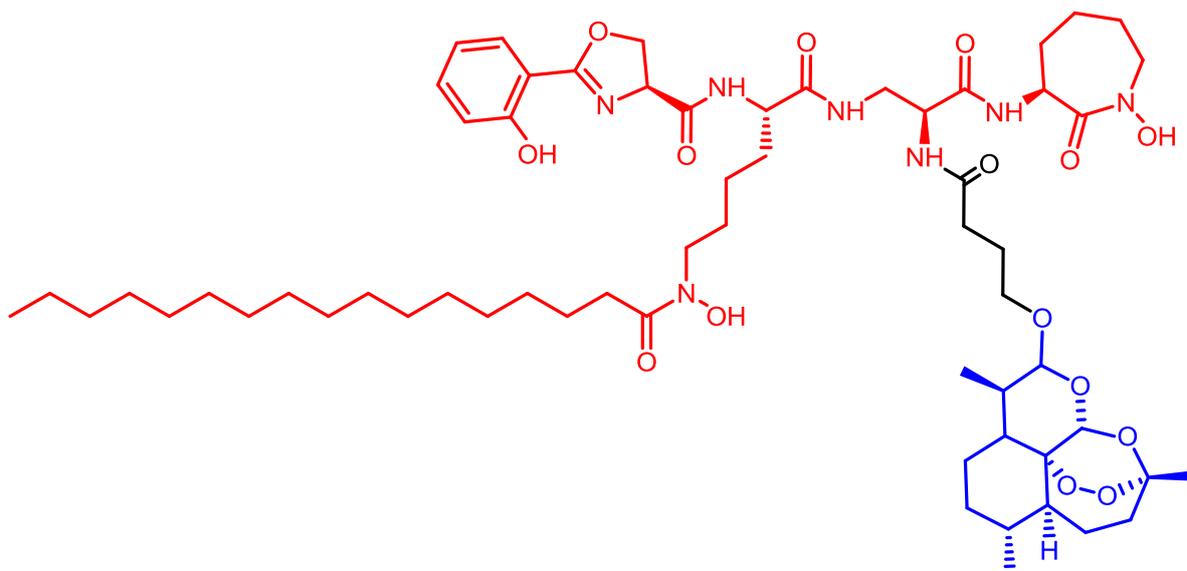


Figure 1.3: Mycobactin-arteisinin conjugate. Mycobactin part (red) and artemisinin portion (blue).

With the above considerations in mind, a single drug that can be used for treatment of malaria and TB is in principle attainable. Since both cholesterol and maintenance of redox homeostasis are vital for survival of these pathogens, an artemisinin derivative can be conjugated to the cholesterol to induce intracellular ROS. In the malaria parasite, it is anticipated that this conjugate will be drawn via the cholesterol transporter into the parasite during either the liver or erythrocyte stage of development. This will allow for the artemisinin component to act during early infection in the liver stage and halt further development of the parasite. Interference with the erythrocyte parasites will prevent transmission of malaria. In TB, the conjugate is anticipated to involve the *mce4* transporter to bring the conjugate into the bacterium thereby enabling the artemisinin component to perturb redox homeostasis, thereby leading to ROS build-up and death of the bacterium.

1.2. Aims and objectives of the study

The aims of this study are to synthesise and assess *in vitro* activities of a series of artemisinin-cholesterol conjugates against CQ-sensitive (CQS) and CQ-resistant (CQR) strains of *P. falciparum* and against culture mutant *Mtb* H37RvMA::*gfp* strains. Should the artemisinin-cholesterol conjugates be active against *Mtb*, then an attempt will be made to assess the ability of selected conjugates to penetrate infected macrophages and kill *Mtb* within the macrophage. A selected group of these compounds also will be used to assess antimalarial activity against the liver stage of parasite development.

The following objectives are set in order to achieve these aims:

- To synthesise and characterise artemisinin-cholesterol conjugates.
- To investigate antimalarial activity against *in vitro* CQS and CQR strains of *Plasmodium falciparum* (*Pf*).
- To determine 90% minimum inhibitory concentration and intracellular efficacy against *Mtb* H37Rv strain.
- If compounds are active according to the foregoing screens, to assess efficacy against macrophages infected with *Mtb*.
- To select artemisinin-cholesterol conjugates that are active against *Pf* and assess their activity against liver-stages of the malaria parasite.
- To assess cytotoxicity towards human and animal cells.

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CHAPTER 2

LITERATURE REVIEW

2.1. Introduction

The ongoing war waged by humanity against infectious diseases suffers from the current inadequacy of most measures currently available for their eradication. Morbidity and mortality due to infectious diseases are still at alarmingly high proportions. Infectious diseases such as malaria and tuberculosis continue to be the leading causes of death annually. These diseases afflict most poverty-stricken parts of the world where there is a tragic lack of fundamental resources required at the very least for elementary disease control. Socio-economic and governmental factors in general incapacitate health care systems required for disease management. Coupled with these logistical factors is the intrinsic ability of pathogens to develop resistance against drugs used in routine treatment. In this chapter the current situation in the control of malaria and tuberculosis is reviewed. Current epidemiology, control measures, chemotherapy, successes and challenges are discussed.

2.2. Malaria

2.2.1. Epidemiology of malaria

The World Health Organisation (WHO) 2015 Malaria report marks a significant point in the progress towards eradication of malaria coinciding with the deadlines set for the Millennium Development Goals (MDG) (WHO, 2015e). MDG target 6c “to have halted and begun to reverse the incidence of malaria by 2015” has been met and this is a positive step towards eventual eradication of malaria. In addition to this, satisfactory progress has been made in meeting the goals set by more ambitious parallel programme like the Roll Back Malaria and the World Health Assembly to bring deaths to near zero and to reduce malaria burden by 75% in 2015 respectively (WHO, 2015e).

The 2015 WHO Malaria report indicates there were approximately 214 million cases and 438,000 deaths due to malaria in 2014. The majority of cases were from Africa (88%); others were from South-East Asia (10%) and the Eastern Mediterranean region (2%). Africa accounted for 90% of the deaths reported in 2014 and deaths have decreased by 66% between 2000 and 2013. Malaria took the life of a child every 2 minutes, and 306,000 children died of malaria in 2014, 292,000 of whom were from Africa. The favourable change in these statistics has led to the estimate that 1.2 billion cases and 6.2 million deaths have been averted since 2000. Elimination of malaria also appears to be following the right trajectory: 33 countries are now

reported to have fewer than 1000 malaria cases when compared to only 15 countries in 2000. About 57 out of 106 countries have reduced disease incidence by at least 75% compared to 2000 (WHO, 2015e). These achievements need to be maintained so that the elimination phase can continue. Since the elimination phase cannot be indefinite, it is important that eradication of malaria be made a global priority (Anstey et al., 2009, Klepac et al., 2015).

2.2.2. Life cycle and pathogenesis of malaria

Malaria is a vector-borne infection caused by a protozoan parasite of the genus *Plasmodium* transmitted by female *Anopheles* mosquitoes. Five species of *Plasmodium* responsible for human malaria infection are *P. knowlesi*, *P. ovale*, *P. malariae*, *P. vivax* and *P. falciparum*. The latter two are responsible for most global cases of malaria and *P. falciparum* is the most fatal, accounting for the majority of deaths. *P. vivax* and *P. ovale* are difficult to control and detect due to innate ability to remain dormant in the liver as hypnozoites for days, months or years after infection (Anstey et al., 2009, Richter et al., 2010). *P. vivax* is the second leading cause of human malaria infection because of its ability to re-infect and cause relapse (Mueller et al., 2009). *P. knowlesi*, recently found to infect humans, is the simian form of malaria prevalent in South-East Asia (Sabbatani et al., 2010).

The development of the malaria parasite alternates between a vertebrate and a mosquito host through intricate biological phases. Between and within these hosts the parasite undergoes asexual (in a vertebrate) and sexual (in a mosquito) development, respectively. Asexual stage includes exoerythrocytic and erythrocytic stages. The erythrocytic form of an asexual stage is associated with malaria symptoms while the sexual stage only takes place in the mosquito (Figure 2.1). These different stages are briefly described below.

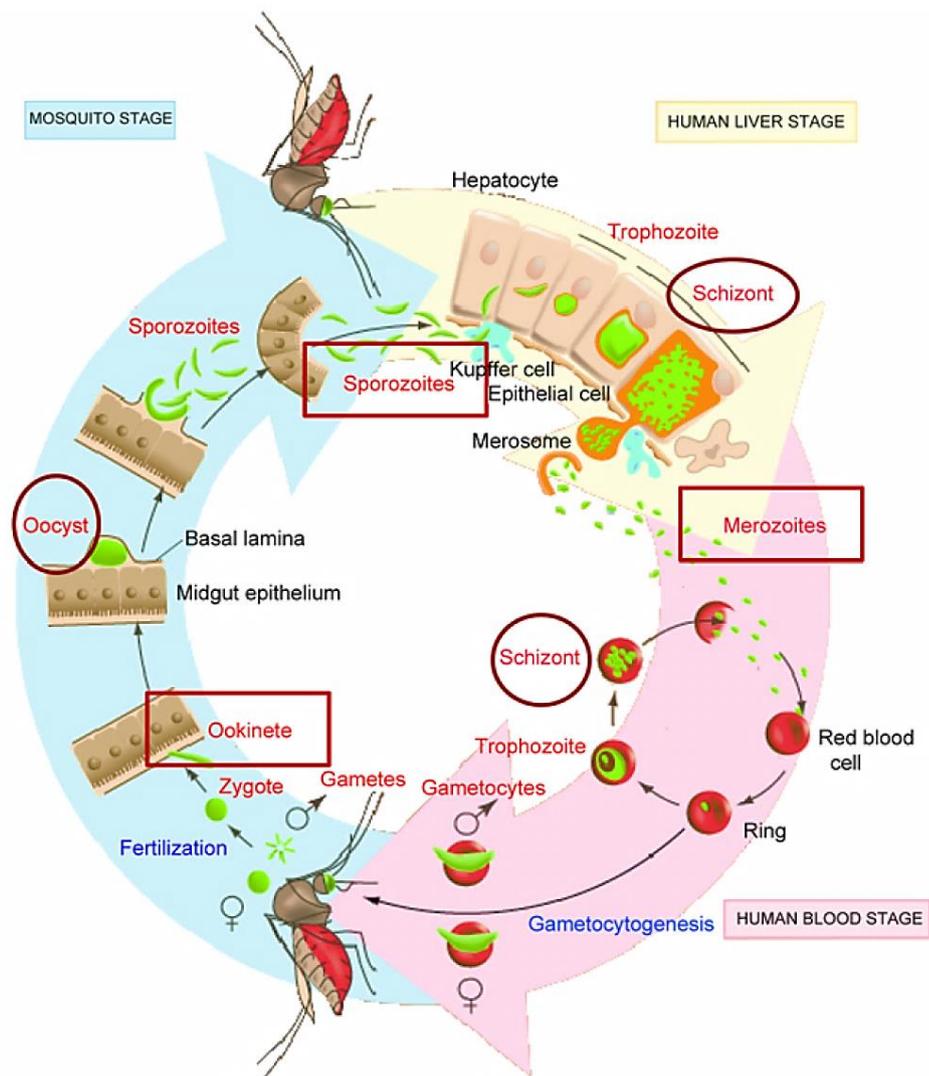


Figure 2.1: The life cycle of the malaria parasite (Coppens, 2011).

2.2.2.1. Exoerythrocytic stages

A. Sporogenesis and transmission

Sporogenesis takes place in the mosquito following fusion of the gametes. When the mosquito takes a blood meal of an infected individual it takes up erythrocytes that are infected with gametocytes. The drop in temperature and changes in environmental conditions like pH in the mosquito leads to maturation of the gametocytes into male and female gametes (Billker et al., 1997). These gametes then fuse to form a diploid zygote that then differentiates to form motile ookinets in the mosquito midgut. The ookinets traverse the midgut and arrest on the basal lamina of the midgut. Here the sessile ookinets form oocysts which undergo mitotic divisions leading to formation of sporozoites. Sporozoites rupture the oocyst and enter the hemocoel in which the hemolymph circulates them around the *Anopheles* body. When the hemolymph reaches the salivary glands, the sporozoites attach and here they await transfer into human host during feeding by the mosquito (Lindner et al., 2013, Aly et al., 2009, Matuschewski, 2006).

B. Exoerythrocyte

During the blood meal, the mosquito releases sporozoites either into the skin or blood capillaries of the human host (Figure 2.2a). In the former instance, sporozoites have to traverse cells to enter the bloodstream before transport to the liver. For the latter, however, sporozoites are quickly taken to the liver within a few minutes (15-30 min) of injection. In the liver sporozoites invade hepatocytes. Hepatocyte invasion occurs in three possible ways: (i) sporozoites pass through fenestrations between hepatoendothelia following arrest by protruding heparan sulfate proteoglycan (Kappe et al., 2003, Sultan, 2010, Coppi et al., 2007); (ii) sporozoites may enter Kupffer cells and become enclosed by a parasitophorous vacuole (PV) and drain into hepatocytes (Pradel and Frevert, 2001); (iii) intra-hepatocyte PV-enclosed sporozoites invade neighboring hepatocytes by penetrating the plasma membrane of adjoining hepatocytes (Kaplan et al., 2003) (Figure 2.2b). Once in the hepatocytes the PV-enclosed sporozoites grow and rapidly differentiate into thousands of merozoites. For *P. vivax* and *P. ovale* infections, some of the sporozoites do not differentiate and remain in dormant forms called hypnozoites. Hypnozoites account for relapse seen with *P. vivax* days, months or even years after initial infection (Markus, 2015).

The rapid growth of the parasite in the hepatocytes requires nutrient supply. The parasite achieves this by associating with the endoplasmic reticulum (ER) in the hepatocyte where it scavenges newly synthesised lipids (Bano et al., 2007). In addition the parasite salvages the intrahepatic cholesterol which it uses to strengthen its membranes and to modulate porosity through formation of structures like lipid rafts. Membrane porosity allows for additional nutrients to enter the parasite (Labaied et al., 2011). This is important for parasite survival. The hepatocyte eventually ruptures to release merozoites into the bloodstream which then infect erythrocytes.

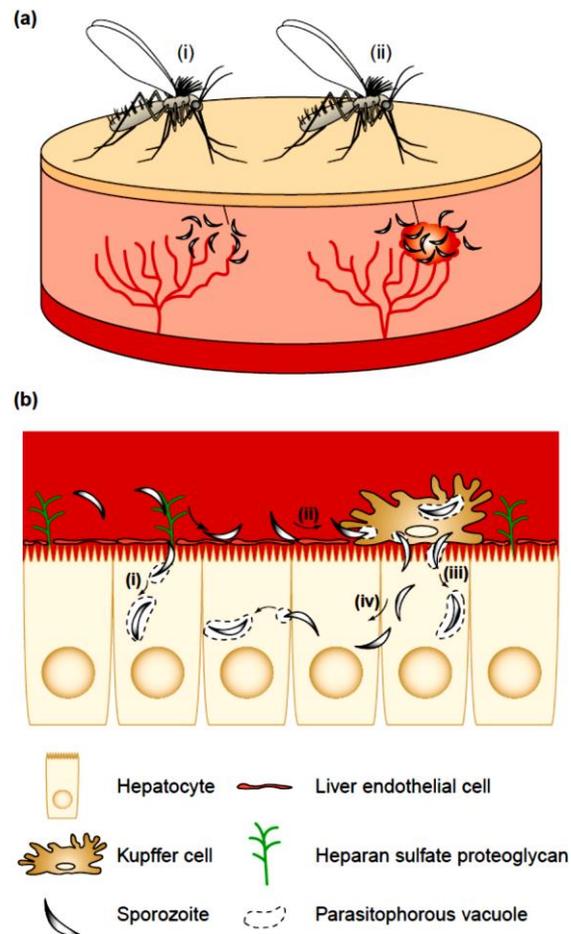


Figure 2.2: Transfer of sporozoites from mosquito to the human host. a: Mosquito injects sporozoites into the (i) skin or (ii) the capillaries. b: Different ways in which sporozoites invade hepatocytes (Kappe et al., 2003).

2.2.2.2. Erythrocyte stages

Merozoites enter erythrocytes through proteolytic processing of the merozoite surface proteins and shedding of the surface coat (Smith et al., 2000, Cowman and Crabb, 2006, O'Donnell and Blackman, 2005). Merozoites initially develop into ring forms and then trophozoites. Mature trophozoites differentiate into schizonts which rupture, releasing 16-32 merozoites into the bloodstream and these infect more erythrocytes. At this point in the life cycle, a patient will present with chills, fevers and prostration due to erythrocyte rupture. The cycle repeats differently depending on the *Plasmodium* species which has infected an individual. For *P. falciparum*, *P. vivax* and *P. ovale* the periodicity is 48 h while for *P. malariae* it is 72 h (Wiser, 2011).

This stage is also marked by a need for nutrients to support parasite growth. The parasite has developed a number of strategies for acquisition of nutrients. The intraerythrocyte parasite digests the cytoplasm and haemoglobin of the infected erythrocytes to release amino acids

which are used in protein synthesis. The parasite also develops a network of tubules that are in close proximity with the extracellular environment and these may divert more nutrients into the parasite (Tilley et al., 2011). In addition the parasite diverts host cholesterol from the erythrocyte membrane, high density lipoproteins, cholesterol-rich detergent-resistant membrane and exosomes to support its nutrient supply system. Cholesterol becomes incorporated into the PV membrane to maintain membrane porosity and to allow nutrients to traffic into the parasite (Tokumasu et al., 2014, Labaied et al., 2011, Grellier et al., 1991). These nutrient acquisition strategies result in the growth of the parasite and the release of the merozoites for further infection.

During this trophic phase some merozoites also differentiate into sexually-committed schizonts resulting in male (microgametocyte) and female (macrogametocyte) gametocytes. These gametocytes remain in the peripheral erythrocytes where they mature and await uptake by the female mosquito when she takes another blood meal (Smith et al., 2000).

2.2.2.3. Mosquito stage

The drop in temperature by about 5 °C, the increase in pH from 7.4 in human to more alkaline pH 8 in the mosquito's midgut and the presence of xanthuric acid lead to microgametogenesis from microgametocytes (Bhattacharyya and Kumar, 2001, Ramasamy et al., 1997). The process occurs within 20 min and results in the release of up to 8 motile microgametes from male gametocytes in the process known as exflagellation (Ramasamy et al., 1997, Raabe et al., 2009). Motile microgametes fertilise macrogametes through the fusion of surface proteins HAP2 and P48/45. This fertilisation process results in the formation of the zygote. The zygote undergoes mitotic divisions resulting in motile ookinete 16-20 h after ingestion of the blood meal. The ookinetes traverse the peritrophic matrix through chitinase hydrolysis, and then implant on the basal lamina of the midgut epithelium (Vinetz, 2005). Here they differentiate into oocyst in a nutrient-dependent process that takes 12-36 h. The oocysts undergo mitotic nuclear divisions concomitant with the formation of small clefts known as sporoblasts. This process results in production of thousands of sporozoites which eventually bud from the sporoblasts entering the hemolymph. Circulation of the hemolymph carries the sporozoites around the body and finally to the salivary glands where they arrest and await transfer to the vertebrate host (Wang et al., 2005). This marks the completion of the life cycle of parasite development.

2.2.3. Malaria symptoms

Symptoms of malaria manifest at different time points depending on the parasite species that has infected the person. For malaria caused by *P. falciparum* infections, symptoms appear after 9-12 days, 12-17 days in *P. vivax* and 18-40 days in *P. malariae*. The onset of malaria may present nonspecific symptoms and may make it difficult to notice the disease early on.

However, the cardinal symptom of malaria is fever which is common for malaria caused by any of the *Plasmodium* species. Fever leads to manifestation of symptoms like chills, sweats, fatigue, nausea, vomiting and convulsions. Fever episodes in malaria due to *P. falciparum*, *P. vivax* and *P. ovale* occur every 48 h while with *P. malariae* they occur every 72 h (Wiser, 2011). Malaria due to *P. falciparum* causes the most severe form of the disease and leads to a number of other complications. Infections by *P. vivax* and *P. ovale* can also be fatal if not properly managed (Walker and Colledge, 2013). Some of the fatal complications that develop from the infection by *P. falciparum* are now described.

2.2.3.1. Cerebral malaria (CM)

CM is a complication resulting from the infection by *P. falciparum*. This complication is found mostly in African children under the age of 5 (about 1120 cases per 100 000 malaria cases per year) and in adults in endemic areas (Idro et al., 2010). WHO defines CM as the persistent coma 1 h after the correction of hypoglycemia and seizures with asexual forms of *P. falciparum* evident in peripheral blood smears and no other encephalopathy causes (Idro et al., 2005). CM results from sequestration of parasitised red blood cells (pRBCs) in the cerebral microvasculature. Sequestration occurs by platelet-mediated clumping of pRBCs to other pRBCs and by rosetting formed when uninfected erythrocytes bind to pRBCs in cerebral microvasculature. These coalescences decrease perfusion and result in hypoxia and hypercarbia (Adams et al., 2014, Dondorp et al., 2004, van der Heyde et al., 2006). The common features of CM in children are coma, seizures and malarial retinopathy. In adults, CM manifests as part of multi-organ disease with patients presenting with malaise, delirium followed by coma, fever, joints and body aches, pulmonary edema, renal failure, chronic hepatitis B infection and severe acidosis. Some outcomes of CM include epilepsy, speech impairment, cognitive sequelae and mortality (Idro et al., 2005, Idro et al., 2010).

2.2.3.2. Anaemia

Anaemia is frequently reported in children living in holoendemic areas of Sub-Saharan Africa and in pregnant women (Oladeinde et al., 2012, Tay et al., 2013). It can be defined as a hemoglobin concentration less than 5 g/dL in the presence of the blood parasite. During the erythrocyte stages of parasite development hemoglobin is degraded and converted to non-toxic hemozoin. Hemozoin is released into the blood stream during merozoites egress from the pRBCs and is phagocytised by monocytes, macrophages and neutrophils (Perkins et al., 2011). Phagocytosis leads to an altered innate inflammatory response that result in enhanced production of chemokines and cytokines as well as elevated levels of nitric oxide (NO) and reactive oxygen species (ROS). The altered innate inflammatory response leads to suppression of erythropoiesis that leads in turn to anaemia and then death (Chang and Stevenson, 2004).

2.2.3.3. Malaria-induced acute respiratory distress syndrome (MI-ARDS)

MI-ARDS is more common in adults than young children and can be caused by severe infections by *P. falciparum*, *P. vivax* and *P. knowlesi* (Taylor et al., 2012). MI-ARDS is caused by disruption of alveolar-capillary membranes followed by leaking of plasma fluid into interstitia and alveoli. Recently it has been shown that haemozoin increases pulmonary inflammation which leads to MI-ARDS in a mouse model (Deroost et al., 2013). MI-ARDS can persist even after the patients have awoken from coma and are on antimalarial chemotherapy. MI-ARDS is higher in pregnant women and poses high mortality risk during fasting periods (McGready et al., 2014). The symptoms include tachypnea, dyspnea, hypoxemia and loss of weight (Bartoloni and Zammarchi, 2012).

2.2.3.4. Hypoglycemia

Hypoglycemia is more common in severe *P. falciparum* infections and mainly affects children and pregnant women. Hypoglycemia occurs in 8-30% cases of severe malaria (Thien et al., 2006). Hypoglycemia is an independent cause of mortality in children and it is a frequent complication during admission (Madrid et al., 2015). Hypoglycemia-associated mortality in Africa has been estimated to occur in 1 out of 2 children with severe malaria (Osonuga et al., 2011). The main probable cause of hypoglycemia in these countries may be malnourishment associated with abject poverty. The WHO defines hypoglycemia as glycemic concentrations below 2.5 mmol/L in healthy children, 3 mmol/L in malnourished children or 2.2 mmol/L in newborns (Barennes et al., 2016, WHO, 2013b).

Hypoglycemia often recurs even after the correct glycemic concentration is restored. It was believed that this was associated with quinine chemotherapy as quinine triggers hyperinsulinemia which lowers blood glucose levels (Okitolonda et al., 1987). However, it was recently found that there was no association between hypoglycemia and quinine chemotherapy (Ogetii et al., 2010). This implies that other factors cause hypoglycemia in malaria. Hypoglycemia may result from impaired gluconeogenesis as a result of increased concentration of alanine and lactate in severe malaria patients (Thien et al., 2006). In addition, prolonged fasting in pregnant women with non-severe malaria has been found to increase the risk of hypoglycemia (van Thien et al., 2004). The resulting fatalities of hypoglycemia are brain damage, a number of neurological sequelae and renal impairment (English et al., 1998).

2.2.3.5. Acute renal failure (ARF)

ARF is another complication due to infection by *P. falciparum* but it has also been noted in infections with *P. vivax* and *P. malariae* (Badiane et al., 2014, Das, 2008). It is a condition where the creatinine concentration is greater than 3 mg/dL and when urine volume is less \approx 400 mL in a day. This complication is more common in adults than children. Pregnant women with

high parasitaemia are particularly prone to developing ARF complication. Other conditions that may lead to ARF are jaundice, use of NSAIDs and dehydration (Mishra and Das, 2008, Badiane et al., 2014).

The mechanisms involved in the development of ARF are not fully defined. It is hypothesised that pRBCs with knobs adhere to one another and end up obstructing the microcirculation leading to ARF. Other causes are due to cytoadhesion of thrombospondin to vascular endothelial system, acute tubular necrosis due to ischemia and increased vasoconstriction due to catecholamine. Increased vascular permeability as well as problems associated with glomerular and tubular pathology may also contribute to causes of ARF either separately or in combination. The complications that result from this condition include severe anaemia, multiple organ dysfunctions, thrombocytopenia and renal failure due to oliguria and pulmonary edema. The mortality rate due to ARF is as high as 45% but proper implementation of early treatment reduces this risk to 10% (Mishra and Das, 2008).

2.2.4. Diagnosis of malaria

In 2010, the WHO has urged that all suspected cases of malaria receive diagnostic testing to confirm the disease (WHO, 2010a). Proper diagnosis will help reduce the burden of overtreatment especially in cases of misdiagnosis; it will also reduce the risk of resistance to the artemisinin combination therapies (ACTs) and will further reduce unnecessary costs of treatment in endemic areas. The diagnostic methods used include microscopy, rapid diagnostic testing and quantification of the polymerase chain reaction. These are briefly reviewed in this section.

2.2.4.1. Microscopy

Microscopy remains the gold standard diagnostic method for malaria with large scale use worldwide (WHO, 2010b). This method involves the observation of Giemsa-stained thin and thick blood films to assess the presence of the malaria parasite. Microscopy has several advantages including the ability to distinguish the species of the *Plasmodium*, stages of infection and the presence of mixed species. Microscopy can also detect low levels of parasitaemia in asymptomatic individuals or even early during infection with sensitivity in the range of 5-50 parasites/ μ L. The disadvantages of the use of microscopy are that it requires an experienced technician for operation; it is labor-intensive and may delay commencement of therapy. It requires continuous external assessment to maintain quality of the results. In the face of new diagnostic methods, it is becoming relatively less desirable for rapid diagnosis (Chiodini, 2014, Wilson, 2013, Murphy et al., 2013).

2.2.4.2. Rapid diagnostic test (RDT)

The use of RDT has drastically increased in the past few years and its use in many endemic countries has also increased (WHO, 2015e). The technique is based on immunographic detection of soluble malaria antigens. The targeted antigens are *Plasmodium* histidine-rich protein 2 (pHRP-2) (specific for *P. falciparum* or *P. vivax*), lactate dehydrogenase (pLDH) (specific for *P. falciparum* and all *Plasmodium* species) and *Plasmodium* aldolase (also panspecific). The increasing application of RDT is favored for ease of use, cost-effectiveness, high accuracy and short turnaround times (at least 15 min). Disadvantages of RDT include lack of parasite specificity for *P. ovale*, *P. malariae* and *P. knowlesi*; inability to distinguish between different stages of the parasite; inability to determine the level of parasitaemia and propensity to indicate false positives due to persistence of PfHRP2 antigen in the blood even when parasite load is low (Mouatcho and Goldring, 2013, Wilson, 2012, Murray et al., 2008). These drawbacks make RDT unlikely to replace microscopy, which is used to confirm results obtained by RDT (Wilson, 2013).

Infections due to *P. knowlesi* have been difficult to detect with RDT but recent data shows that RDTs OptiMAL and BinaxNOW kits are sensitive to this malaria parasite, with the latter being much more sensitive (van Hellemond et al., 2009).

2.2.4.3. Real-time polymerase chain reaction (RT-PCR)

RT-PCR is a sensitive diagnostic method and can also be used to confirm results of microscopy and RDT (Shokoples et al., 2009). It is based on the amplification of the single stranded RNA (ssRNA) genes of *Plasmodium* species using genus specific primers. RT-PCR is highly sensitive and can detect 20 parasites/mL of blood sample (Andrews et al., 2005). Its specificity helps in distinguishing between different parasite species in a mixed sample as the ssRNA primers are species specific (Dormond et al., 2011). RT-PCR is expensive and requires established laboratories and an experienced technician for operation. This makes its use in the endemic settings challenging.

2.2.5. Key Interventions against Malaria

2.2.5.1. Indoor residual spraying and insecticide treated nets

Vector control is an integral part of the malaria control program. Vector control has contributed immensely towards the reduction of malaria morbidity and mortality among African children (Kleinschmidt et al., 2009a). The use of vector control measures such as insecticide-treated nets (ITNs), indoor residual spraying (IRS), biological control of vectors and vaccination are at the forefront in malaria control programs. ITNs and IRS are now widely used in most endemic countries especially in Sub-Saharan Africa. They are both cost effective and complementary where they are applied (Kleinschmidt et al., 2009b).

Through the distribution of ITNs to the groups at risk, namely children under the age of five and pregnant women, it has been found that there had been a significant decline in malaria morbidity and mortality in some parts of Africa (Kleinschmidt et al., 2009a). ITNs are effective in that they enhance the protection at an individual level. Their ability to inhibit and kill mosquitoes through their insecticides helps evade the bite and blood meal by mosquitoes (Okumu and Moore, 2011). There has also been a concern with their use as it had been found that some strains of *Anopheles* have developed resistance to insecticides that are used for these nets. Moreover, wear and tear of the nets means that they have to be frequently replaced. Longer lasting insecticide nets (LLINs) prolong the lifetime of the insecticide-treated nets. LLINs last for periods of 3-5 years and their use has proven fruitful according to recent reports (WHO, 2007a).

The use of IRS also results in reduction in malaria transmission since it kills most of the mosquitoes in the households (WHO, 2015d). The repellency and toxicity of the IRS to mosquitoes render it effective as vector control (Okumu and Moore, 2011). Conventional IRS contains dichlorodiphenyltrichloroethane (DDT) which has been discouraged for use because of toxicity. IRS containing organophosphates, carbamates and pyrethroids can be used as alternatives but they are more expensive (Walker, 2000). IRS is effective in that it can destroy mosquitoes which have entered the household for feeding with the human blood or those who have fed and are resting on the walls for escape (WHO, 2015c). The major drawback with the IRS is that the efficacy drops rapidly and may require reapplication every 3-4 months (e.g. pyrethroids) or 6-12 months (e.g. DDT). In a recent study use of IRS with ITNs appeared to offer added efficacy (Okumu and Moore, 2011). Therefore the use of these methods in combination will offer much protection and will also reduce the risk of the vector developing resistance to the insecticides. It is advised that these be used together and when used in combination nets and IRS should contain different insecticides (West et al., 2014, Okumu and Moore, 2011).

2.2.5.2. Larval control

There is a growing interest in the control of malaria at the larval stage through means such as manipulation of mosquito habitat, larvicides or even biological control. Forty eight countries reported the use of one of these larval control methods in national malaria control program according to the 2015 WHO Malaria report (WHO, 2015e). The field data for some of the methods is yet to be reported but the currently applied methods are promising as they appear to be environmentally friendly, act in different stages of development of the vector and thus impair selection pressure and they are inexpensive.

A number of larval control strategies are currently explored. The use of larvivorous fish in the ponds or habitats of mosquitoes interfere with mosquitoes population increase by consuming larvae (Kamareddine, 2012). Viral pathogens such as *An. gambiae* densovirus virus

(AgDNV) can be passed down the generations and potentially act as an “evolution-proof biopesticide”. This will interfere with the developmental phases of mosquitoes (Ren and Rasgon, 2010). Another method under increasing consideration is entamopathogenic fungi (EPF) which act as adultacidal agents and kill mosquitoes before parasite transmission, interfere with the blood feeding tendency and impair fecundity of the vectors and with implication to intercept the survival of resistant strains (Scholte et al., 2005, Abdul-Ghani et al., 2012). Larval control is promising for control and prevention of transmission of malaria but its current coverage and impact is not yet known (WHO, 2015e).

2.2.5.3. Intermittent preventive treatment in pregnancy (IPTp)

The WHO has recommended the use of IPTp as one of intervention strategies for pregnant women who are vulnerable to malaria in endemic areas (WHO, 2015e). In 2012 the WHO recommended that IPTp be implemented and applied for every antenatal visit given that treatment is separated by at least 1 month (WHO, 2012a). Of the 75% of women who attended antenatal clinics, it was found that only 26% received at least two doses of IPTp with sulfadoxine-pyrimethamine (IPTp-SP). This number is low given that the number of pregnant women at risk to malaria in endemic areas is high (van Eijk et al., 2013). This number is still below the proportion of all pregnant women who have to receive IPTp. IPTp is also under-implemented as only 44% of 88% of pregnant women visiting antenatal care received IPTp in 2012 (WHO, 2012b). However, the 2015 WHO malaria report indicates that about 52% of eligible pregnant women received IPTp (WHO, 2015e). This is an important improvement given that IPTp-SP shows great efficacy in moderate to high *P. falciparum* transmission settings in Sub-Saharan Africa despite SP resistance in these areas. The gains in these areas are reflected by the reduced maternal clinical malaria and neonatal malaria mortality. The fact that IPTp-SP has been found to be cost effective also makes it an intervention of choice in pregnant women (Sicuri et al., 2010). In order to meet WHO recommendations for proper implementation of IPTp-SP and to limit the growing resistance to SP joint efforts from the governments, health staff and antenatal attendees are required (van Eijk et al., 2013).

2.2.6. Vaccination

There is currently no licensed vaccine against malaria. A number of vaccines are at different preclinical or clinical stages of development. Of these vaccines, RTS,S/AS01 is the most advanced. RTS,S/AS01 was first developed by GlaxoSmithKline (GSK) in collaboration with the Walter Reed Army Institute for Research in the 1980s. In July 2015 RTS,S/AS01 received positive scientific support and approval by the Committee for Medicinal Products for Human Use (CHMP) of the European Medicines Agency (WHO, 2016). The approval by CHMP makes RTS,S/AS01 the first vaccine ever developed for malaria and/or any parasitic disease.

RTS,S/AS01 is a hybrid recombinant protein vaccine made up of carboxy terminus of *P. falciparum* circumsporozoite protein (CSP) “R”, the T-cell epitopes of the CSP “T” and the hepatitis B surface antigen (HBsAg) “S”. RTS is expressed in *Saccharomyces cerevisiae* and fused with the free HBsAg’s S protein to form RTS,S virus-like particles. At the point-of-use a 25 µg formulation of RTS,S with an adjuvant AS01 is administered (WHO, 2016, Moorthy and Ballou, 2009).

Phase III clinical trials involving over 15,000 children and infant participants from 11 sites with moderate to high transmission in Sub-Saharan Africa have recently been completed. The results of this study show that RTS,S/AS01 is both efficacious and safe for use (Agnandji et al., 2011). In January 2016 the WHO recommended implementation of RTS,S/AS01 in Africa following CHMP’s approval. The WHO has urged that use of the vaccine be implemented in 3-5 areas with moderate-to-high transmission in Sub-Saharan Africa. This implementation will also add more data regarding recent efficacy and safety findings. RTS,S/AS01 will be beneficial in African countries. GSK together with the WHO have pledged to make it accessible and affordable to the populations that most need it (WHO, 2016).

2.2.7. Malaria chemotherapy

A variety of antimalarial drugs (antimalarials) are currently used for treatment of malaria and in the advent of malaria vaccine chemotherapy remains the most effective strategy against this disease. Most of the currently used antimalarials are obsolete or becoming ineffective due to ability of the malaria parasite to develop resistance (Schlitzer, 2008). Artemisinins are currently the most active antimalarials in clinical use but resistance by the parasite against them is also increasing (Paloque et al., 2016). Current treatment also use drugs combinations as the strategy to help curb the spread of resistance (Sharma and Awasthi, 2015). Most of the antimalarials currently in use are based on repurposed drugs or those derived from natural sources (Nzila et al., 2011). Overall, there is an urgent need to develop synthetic compounds with different scaffolds possessing distinct modes of action and targeting different biomolecules. In the next section current antimalarials, mechanism of action, resistance, susceptible strains, current use and side effects will be discussed.

2.2.7.1. Activity against different parasite stages

Antimalarials have different mechanism of action (MoA) against different stages of parasite development. Most of the available antimalarials are active against the asexual stage of the parasite. Thus, this specificity of antimalarials for different stages allows them to be classed depending on their MoA. Antimalarials can be classified as:

(i) Tissue schizonticides: these are active against merozoites multiplication in the hepatocytes early during parasite infection. The class consists of antimalarials such as primaquine and

pyrimethamine active in hepatocyte stage albeit to an unknown extent since clinical symptoms are not yet manifest at this stage. Primaquine is also useful in prevention of relapse as in the case of *P. vivax* infections because it is hypnozoitocidal (Nelwan et al., 2015, Vangapandu et al., 2007).

(ii) Blood schizonticides: act against the blood forms of the parasite. Their effects are seen by termination in malaria symptoms prevalent during this stage. Different drugs are active in this stage including the 4-aminoquinolones, 9-aminoacridines and artemisinins (Achan et al., 2011, Delves et al., 2012).

(iii) Gametocytocidal antimalarials: act against gametocytes and in this class drugs such as quinine and artemisinins are active, but evidently with no activity to suppress transmission. Chloroquine and quinine are not active against the *P. falciparum* gametocytes but primaquine and artemisinins possess activity (López-Antuñano, 1999, Achan et al., 2011).

(iv) Sporozoitocides: are effective in prevention of oocyst development within the mosquito and consequently prevent transmission of the parasite to the human host. Two examples of drugs active against this stage are primaquine and proguanil (Vangapandu et al., 2007).

2.2.8. Antimalarial drugs classes

2.2.8.1. Arylaminoalcohols

Quinine

Quinine **1** occurs naturally in the bark of the cinchona tree. Originally known as Jesuits bark, the bark was used for several centuries for the treatment of fevers including malaria from the 1600s. The bark was commonly grounded into powder and mixed with liquid before administration. Quinine, together with its stereoisomer quinidine **2**, was extracted from the bark in 1820. It has since been used as an antimalarial and still remains effective to date. It was replaced by the totally synthetic chloroquine in the 1940s as the main antimalarial but its use became more prominent in the 1980s following the emergence of widespread resistance to chloroquine (Achan et al., 2011). The efficacy of quinine has been declining and the WHO has discouraged the use of quinine monotherapy which is commonly practiced in Sub-Saharan Africa (Achan et al., 2009). It was recommended that quinine be used as a second-line treatment against complicated or severe malaria in combination with doxycycline, tetracycline or clindamycin (WHO, 2010a). This was to be adopted when the first-line treatment with intravenous artesunate, used for severe/cerebral malaria, fails or where such treatment is not available. Seven days treatment with quinine or quinine-clindamycin is safe and recommended for treatment of uncomplicated *P. falciparum* malaria in the first-trimester in pregnant women (WHO, 2015a). Combination with antibiotics has been fruitful as findings from Thailand have

shown high cure rates with 5-7 days treatment course of quinine-doxycycline and quinine-azithromycin (Taylor et al., 1999, Bunnag et al., 1996). The universal application of these combinations is yet to be attained given the cost implications especially in resource limited settings. Thus, poor countries still use quinine as the first-line treatment against severe *P. falciparum* malaria (Obonyo and Juma, 2012). The major side effects from quinine treatment, known as cinchonism, are tinnitus, headache, nausea and in severe cases, vertigo, diarrhea, visual impairment, vomiting and hypoglycemia, especially in pregnant women (Achan et al., 2011).

The mechanism of action of quinine is not clearly defined. It is known that quinine is a blood schizonticide and is gametocytocidal against *P. vivax* and *P. malariae*. The proposed mechanism is similar to that of chloroquine since these compounds are structurally similar. The most common hypothesis is that quinine interferes with haemozoin formation in the digestive vacuole (DV) of the parasite and potentially inhibits haem catalase (Foley and Tilley, 1997, Olliaro, 2001).

Quinine efficacy has been decreasing and treatment failures have been reported from South-East Asia and Africa (Pukrittayakamee et al., 1994, Kofoed et al., 1997). It has been found that point mutation on *pfmdr1* that encodes the transmembrane protein Pgh1 of the parasite DV is responsible for reduced sensitivity to quinine (Sidhu et al., 2005). *In vitro* studies with resistance reversal compounds acting against Pgh1 have shown that the susceptibility of the parasite to quinine is re-established (Trenholme et al., 1975). These findings overall show that *pfmdr1* point mutations are responsible for reduced sensitivity of parasites to quinine (Ciach et al., 2003).

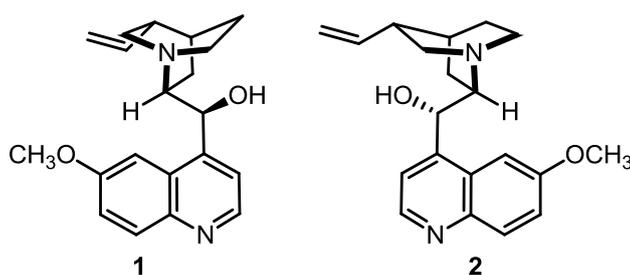


Figure 2.3: Quinine 1 and quinidine 2.

Mefloquine

Mefloquine 3 is an arylaminoalcohol with two asymmetric centers and exists as four stereoisomers, although it is the racemic mixture consisting of the (*R,S*)- and (*S,R*)-enantiomers

that is used. It was developed by the US Army in the late 1970s during the upsurge in chloroquine resistance. Mefloquine was effective against chloroquine resistant (CQR) *P. falciparum* malaria during the time and it was immediately put into malaria therapy (Trenholme et al., 1975). However, within only six years of use in Cambodia and Thailand, treatment failures started to occur (Price et al., 2004). Its use in monotherapy is no longer recommended in this region. In contrast, resistance is infrequent in Africa and mefloquine is still an important antimalarial drug for clinical treatment (Schlitzer, 2008).

Mefloquine has a half-life of about 14-18 days and is well tolerated in prophylaxis. It has been recommended for inclusion in the IPTp but the risk profile is yet to be defined. Adverse effects following mefloquine use include fatigue, insomnia and neuropsychotic disorders such as depression and anxiety (Croft and Herxheimer, 2002). In pregnant women it has been shown that 15 mg/kg of mefloquine causes dizziness. Mefloquine is contra-indicated in patients with the history of epilepsy and psychotic disorders such as depression. Long-term use of the drug appears to contribute to neurological and psychiatric disorders (González et al., 2014).

Mefloquine is a blood schizonticide and is structurally related to quinine. It also interferes with haem detoxification in the parasite. Resistance is due to increased *pfmdr1* copy number in the parasite (Price et al., 2004). The combination of mefloquine with artesunate led to improved efficacy in the 1990s but this has started to decline as well. It is recommended that mefloquine be used in combination with other drugs especially artemisinins in endemic areas (Wongsrichanalai and Meshnick, 2008, Nosten et al., 2000, Na-Bangchang et al., 2010).

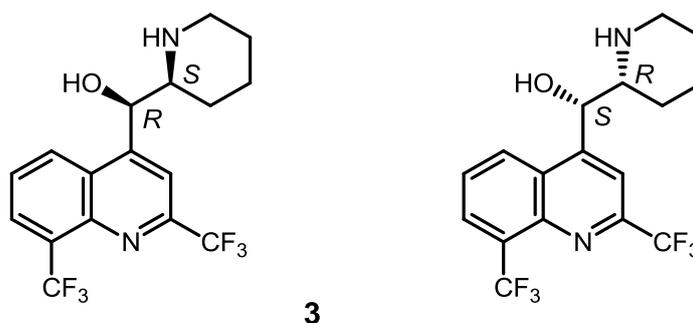


Figure 2.4: The enantiomers of mefloquine **3** comprising the racemic mixture used for treatment of malaria.

Halofantrine and Lumefantrine

Halofantrine **4** and lumefantrine **5** are structurally related arylaminoalcohols used in the treatment of acute uncomplicated *P. falciparum* malaria. Discovered in the 1970s, these antimalarials are active against blood stage multidrug-resistant *P. falciparum* malaria. Halofantrine is a phenanthrenemethanol which metabolises into an active desbutyl-halofantrine

metabolite in the body (Khoo et al., 1998). Halofantrine was used against *P. falciparum* until serious cardiotoxic side effects were reported in 1993; with such toxicity its use was drastically curtailed. Halofantrine causes QT prolongation and is contra-indicated in patients with underlying cardiac disease and metabolic disorders (Bouchaud et al., 2009, Kinoshita et al., 2010).

Lumefantrine **5** however is not cardiotoxic and it is widely used in malaria chemotherapy (Schlitzer, 2008). Lumefantrine is metabolised into an active metabolite desbutyl-lumefantrine in the liver by CYP3A4 and has a peak concentration of over 3 h in oral doses. Its half-life can reach 4 days and this appears to be enhanced by intermittent dosing. It is currently used in combination with artemether under the market name Coartem in artemisinin combination therapy (ACT) for treatment of acute uncomplicated *P. falciparum* malaria. The slow onset of action of lumefantrine allows it to exert its parasiticidal effect when artemether has been cleared the biomass (Wong et al., 2011a, Stover et al., 2012). Like halofantrine, lumefantrine is lipophilic and oral absorption is low which impacts on its effectiveness. This drawback, as in the case of halofantrine administration, is circumvented by taking this drug with fatty food (Djimdé and Lefèvre, 2009, Milton et al., 1989).

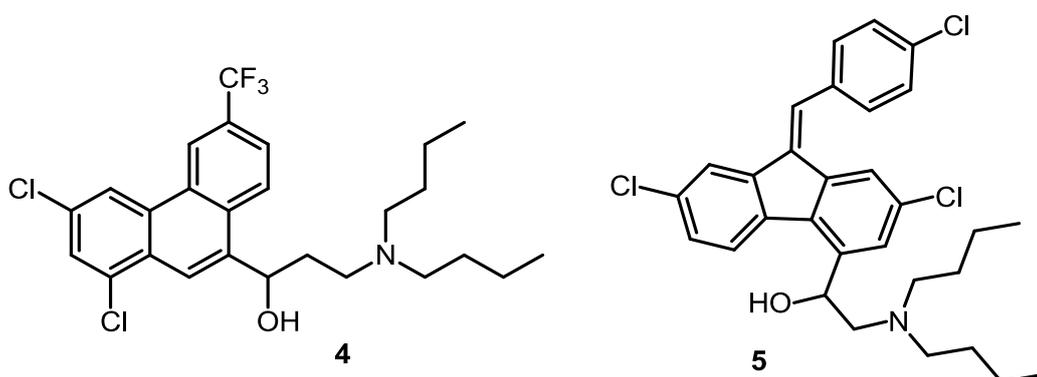


Figure 2.5: Halofantrine **4** and lumefantrine **5**.

Halofantrine and lumefantrine have related MoA and this is proposed to be similar to that of other quinolines. Halofantrine and lumefantrine act in the DV of the parasite and interfere with haemozoin formation. It has been shown that halofantrine inhibits haemozoin formation through interaction with ferroprotoporphyrin IX (FPPIX) (de Villiers et al., 2008). It is proposed that halofantrine form intermolecular hydrogen bonds, and coordinates via the alcohol group with the Fe (III) centre of FPPIX and the phenanthrene has a π -stacking arrangement with the porphyrin ring of FPPIX. This results in accumulation of haem and leads to parasite intoxication. Similar findings are reported with other quinolones and this suggests that lumefantrine likely act through the same mechanism. Lumefantrine is known to inhibit β -hematin formation (de Villiers et al., 2008, Schlitzer, 2008).

Cross-resistance has been reported for halofantrine, lumefantrine and quinine due to *pfmdr1* mutations. The point mutation N86Y on *pfmdr1* is found to result in treatment failures and recrudescence in lumefantrine treatments (Sisowath et al., 2005, Ritchie et al., 1996, Sidhu et al., 2006). Additional mutations such as S1034C, N1042D and D1246Y result in decreased efficacy of arylaminoalcohols in *in vitro* studies (Sisowath et al., 2005). In addition to this, *pfcr1* mutation has also been shown to contribute to the decreased efficacy of arylaminoquinolines in *P. falciparum* resistant strains (Sidhu et al., 2006).

2.2.8.2. 4-Aminoquinolines

Chloroquine

Chloroquine **6** is an important 4-aminoquinoline that was first prepared in the laboratories of Bayer AG in 1934. Its potential for use as an antimalarial drug was first realised after World War II and it was widely used in chemoprophylaxis and chemoprevention for malaria of all *Plasmodium* species. However, the development of resistance was first reported in 1957 on the Thai-Cambodian border. The resistance rapidly spread such that use of chloroquine was essentially proscribed in most malaria endemic areas in Asia (Trape et al., 1998). Many African countries also discontinued the use of chloroquine but recent study shows that chloroquine sensitive strains have returned in countries such as Kenya, Malawi and Tanzania (Lucchi et al., 2015). Current treatment regimen requires a full 3-day standard course and recommended prophylaxis for travellers to endemic settings involves a single dose per week before travel, a single dose every week during the visit and a single dose per week for 4 consecutive weeks upon return (Centers for Disease Control and Prevention, 2016).

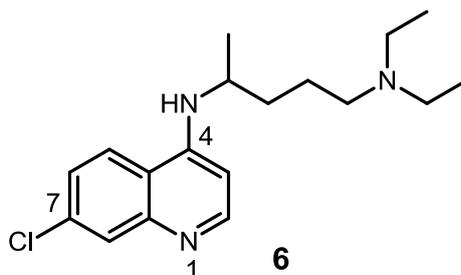


Figure 2.6: Chloroquine **6**.

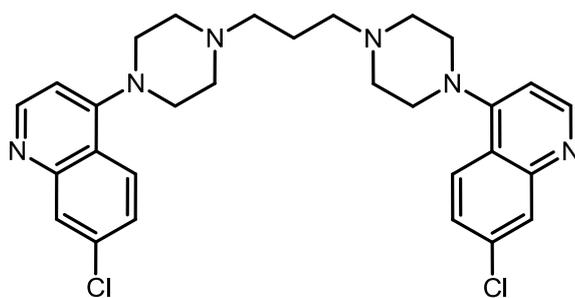
Chloroquine is absorbed from the gut, has a high volume of distribution and almost half of the drug is removed by the kidneys. The rest is biotransformed in the liver into active metabolites *N*-desethyl-chloroquine and *N*-bis-desethyl-chloroquine. Over 20-50% of the *N*-desethyl-chloroquine can be detected in the body upon treatment with chloroquine while <15% of *N*-bis-desethylchloroquine is usually detected. Chloroquine has an elimination half-life of up to 41 days making it to be bioavailable for an extended period of time. It is contraindicated for

individuals with hepatic disease (Projean et al., 2003, Wetsteyn et al., 1995).

Chloroquine is a blood schizonticide that interferes with the haem detoxification process in the DV of the parasite. The parasite breaks down haemoglobin in the erythrocytes leading to the release and accumulation of haem or FPPIX. This is toxic by virtue of redox cycling that generates reactive oxygen species and other effects. Haem dimerises to form haemozoin, the haem dimer that associates into a completely bioinsoluble polymer. It is hypothesised that chloroquine interferes with this process by forming via π - π stacking the FPPIX-chloroquine complex. This complex is toxic to the parasite and leads to its death. At the relatively acidic pH of the DV (4.5-5.5), the basic amino group in the side chain of chloroquine becomes protonated, thus increasing chloroquine retention in the vacuole and enhancing the inhibitory mechanism. Another suggested mechanism is that chloroquine contributes to lethal oxidative stress in the parasite by inhibiting haem catalase activity. This enzyme plays an important role in maintaining the optimal oxidative stress following oxidation of Fe(II) to Fe(III) upon haem oxidation. Taken together, these studies indicate that the MoA of chloroquine is based on interference with the haem detoxification process and that the backbone of chloroquine plays an important role in its activity (Vangapandu et al., 2007).

While *P. falciparum* chloroquine resistant strains had already been reported in Asia, it was only in 1970s that these strains were reported in Sub-Saharan Africa (Trape et al., 1998). The mechanism of resistance is proposed to be due to reduced concentrations of chloroquine in the DV. The K76T point mutation on the *pfCRT* gene, encoding the chloroquine transport protein on the DV membrane likely contributes to increased chloroquine efflux from the DV. In addition, the *pfmdr1* mutations may also increase the chloroquine efflux since such mutations alter Pgh1 expression (Cooper et al., 2007, Atroosh et al., 2012).

Structurally similar to chloroquine is the bisquinoline antimalarial piperazine **7** which was synthesised to circumvent the rising resistance to chloroquine in the 1970s. It was initially used for malaria prophylaxis in China and was effective against *P. falciparum*. Due to its safety and favourable toxicity, piperazine has since been used for treatment of *P. falciparum* malaria in China (Davis et al., 2005). The drug is absorbed from the gastrointestinal gut and is metabolised in the liver. Unlike chloroquine, metabolites of piperazine are not yet defined and the enzymes that facilitate metabolism are also unknown. The half-life is around 9 h and the terminal elimination half-life is 543 h in adults and 324 h in children (Hung et al., 2004). Side effects associated with piperazine include vomiting, QT prolongation at high plasma levels and high blood pressure (Schlitzer, 2008).



7

Figure 2.7: Piperavaquine 7.

Piperaquine is a rapidly acting blood schizonticide which, like chloroquine, acts in the parasite DV. It inhibits haemozoin formation thus leading to accumulation of toxic FPPIX (Davis et al., 2005, Fivelman et al., 2007). Recently, it was found that piperaquine has superior efficacy to chloroquine against blood stage parasites implying cross-resistance with chloroquine is not yet pronounced (Delves et al., 2012). Piperaquine monotherapy has been found to lead to increased gametocytemia and this poses a threat for spread of piperaquine resistance (Pasay et al., 2016). Piperaquine is currently used in ACT with DHA (Artekin) despite some reports that antagonism exists *in vitro* (Fivelman et al., 2007).

Parasite resistance to piperaquine has not yet fully developed. The use of piperaquine in monotherapy in China led to resistance in the 1970s and this was thought to be due to cross-resistance with chloroquine (Hao et al., 2013, Davis et al., 2005). However, it was recently reported that resistance to piperaquine is not associated with *pfcr*, especially the K76T mutation. It is proposed that steric hindrance of piperaquine blocks the transporter-mediated drug efflux (Hao et al., 2013).

Amodiaquine

Amodiaquine **8** was predominantly used for treatment of uncomplicated *P. falciparum* and is still used in some parts of Africa as a replacement for chloroquine. The two bear structural similarities. Amodiaquine has a high volume of distribution and is absorbed from the gastrointestinal tract. It reaches peak plasma concentrations at around 1.75 h following oral administration and has terminal half-life of 5.2 h. It is metabolised by CYP2C8 in the liver into desethyl-amodiaquine and 2-hydroxy-amodiaquine. The former is the most active metabolite and has a half-life over 100 h (Li et al., 2002, Tarning et al., 2012). It has a 6 to 7-fold higher plasma concentration compared to amodiaquine. Desethyl-amodiaquine is thought to be responsible for antimalarial activity of amodiaquine given the long half-life and its propensity to be protein-bound in the red blood cells (Tarning et al., 2012). Amodiaquine elicits hepatotoxicity and neutropenia in prophylaxis (Nefitel et al., 1986, Adjei et al., 2008). This led to its withdrawal

for prophylaxis in 1990 but adverse effects when used in treatment are yet to be established. It is still used in Africa against chloroquine-resistant of uncomplicated malaria because it is inexpensive and selection for resistance is not particularly pronounced (Adjei et al., 2008, Faye et al., 2010).

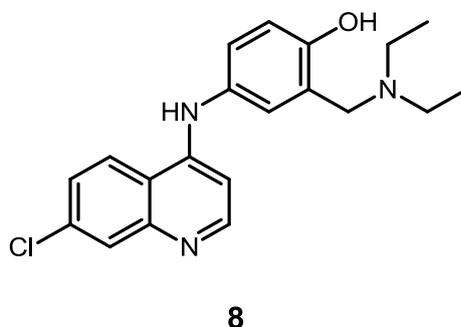


Figure 2.8: Amodiaquine **8**.

Treatment failures have been reported in some countries in Asia, Africa and South America. More pronounced is cross-resistance with chloroquine as established by *in vitro* studies, especially involving desethyl-amodiaquine (Le Bras, 1993, Childs et al., 1989, Sasi et al., 2009). In Africa and Asia it was found that resistance is due to *pfprt* SVMNT allele mutation at codons 72-76. It was also reported that with amodiaquine, like chloroquine, the *pfmdr1* mutation may also contribute to resistance. In the Asian study, however, the novel N86F mutation was found in some samples, suggesting a potentially new resistance marker (Sasi et al., 2009, Beshir et al., 2010).

Amodiaquine inhibits the proteolytic degradation of hemoglobin in the DV of the parasite and it also plays a role in destruction of free haem (Famin and Ginsburg, 2002). Although heme accumulates in the DV, it undergoes passive diffusion through the vacuolar membrane into the cytosol where it is degraded by glutathione. It has been found that amodiaquine inhibits this process as well (Ginsburg et al., 1998).

2.2.8.3. 8-Aminoquinolines

Primaquine

Primaquine **9** is a hypnozoitocidal 8-aminoquinolone active against dormant liver forms of *P. vivax* and *P. ovale* (Baird and Hoffman, 2004, Ashley et al., 2014b). This makes it the only drug clinically used to prevent transmission and relapse of malaria in the market to date. Primaquine is erythrocytotoxic against all the *Plasmodia* species except *P. falciparum*. It is gametocytocidal against mature or late stage *P. falciparum* gametocytes which makes it favourable for preventing gametocyte transmission (Peatey et al., 2009, Wilairatanam et al., 2010).

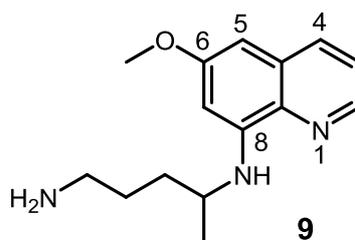


Figure 2.9: Primaquine **9**.

Primaquine is a fast acting antimalarial with a peak plasma concentration of 1-3 h and a half-life of 4-5 h (Potter et al., 2015). It is rapidly absorbed from the gastrointestinal tract and quickly concentrates in the liver, brain, lungs, heart and muscles. Its biotransformation in the liver is facilitated by CYP2D6 resulting in highly active metabolites, the main ones being the carboxyprimaquine and 5-hydroxyprimaquine (Vale et al., 2009). Given these pharmacokinetic considerations and the attendant toxicity of primaquine in monotherapy, the WHO recommends primaquine to be used in combination therapies. Of note is the use of primaquine in combination with artesunate for radical cure of *P. vivax* malaria (Pukrittayakamee et al., 2004, Baird and Hoffman, 2004).

Primaquine is well tolerated but can result in serious complications in some settings. The adverse effects associated with primaquine are vomiting, nausea, gastrointestinal upset, increased methemoglobinemia and in severe cases haemolysis due to G6PD deficiency. G6PD deficiency occurs in 3-30% of the population in endemic areas of the Mediterranean, Africa and Asia and this has resulted in reduced use of primaquine in these areas (Schlitzer, 2008). The lack of the at-point of care detection facilities for G6PD deficiency further adds to the reservations over use of primaquine in routine treatment. Primaquine is contra-indicated in pregnant women and in children under the age of five (Ashley et al., 2014b).

A few analogues of primaquine have been developed. Tafenoquine **10** is in phase III clinical stage and is developed for use in prophylaxis against malaria (Marcsisin et al., 2014). The other is bulaquine **11** which evidently had a better toxicity profile than that of primaquine in Phase II clinical trials; it induced less haemolysis in G6PD deficient individuals than primaquine (Valecha et al., 2001, Vale et al., 2009). NPC 1161 **12** is also in the late preclinical stages of development and has demonstrated long-term activity against the hypnozoites and erythrocytic forms of malaria (Dutta et al., 2011, Marcsisin et al., 2014). Success of these analogues will be alternatives for primaquine as they appear to improve on adverse effects associated with primaquine in malaria treatment.

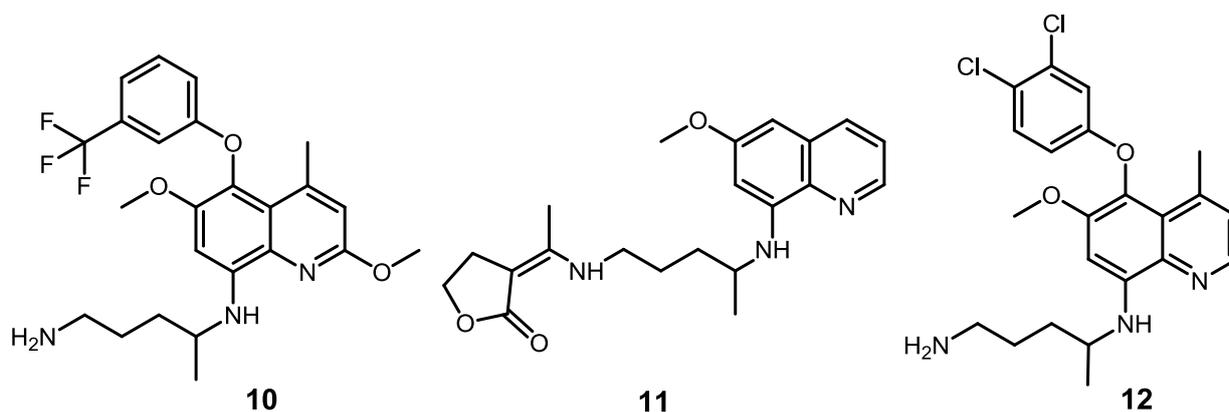


Figure 2.10: Tafenoquine **10**, bulaquine **11** and NPC 1161 **12**.

The MoA of primaquine is not yet elucidated. It may act by perturbing the normal functioning of the mitochondria as it induces formation of cristate mitochondria (Lang-Unnasch and Murphy, 1998). It is proposed that primaquine interferes with the ubiquinone function as an electron carrier and therefore impairs the electron transport chain. Primaquine metabolites are implicated in redox cycling and thus primaquine elevates oxidative stress following generation of reactive oxygen species (ROS) (Lang-Unnasch and Murphy, 1998, Vale et al., 2009).

There is currently no defined resistance against primaquine. However, there have been reports of reduced sensitivity in the treatment of *P. vivax* malaria. The broad spectrum of activity of primaquine may be responsible for the inability of the parasite to develop resistance against the agent (Ashley et al., 2014b, Vale et al., 2009).

2.2.8.4. Antifolates

Antifolates act by inhibiting enzymes involved in *de novo* folate synthesis (Figure 2.11). This perturbs the supply of tetrahydrofolate required for biosynthesis of DNA building blocks. DNA synthesis is integral to rapidly dividing cells of fast-growing organisms such as *Plasmodium* (Nzila et al., 2000, Yuthavong, 2002, Salcedo-Sora et al., 2011). Antifolates are therefore an important class of antimalarial drugs. Two groups are defined according to the enzymes they inhibit: Class I and Class II.

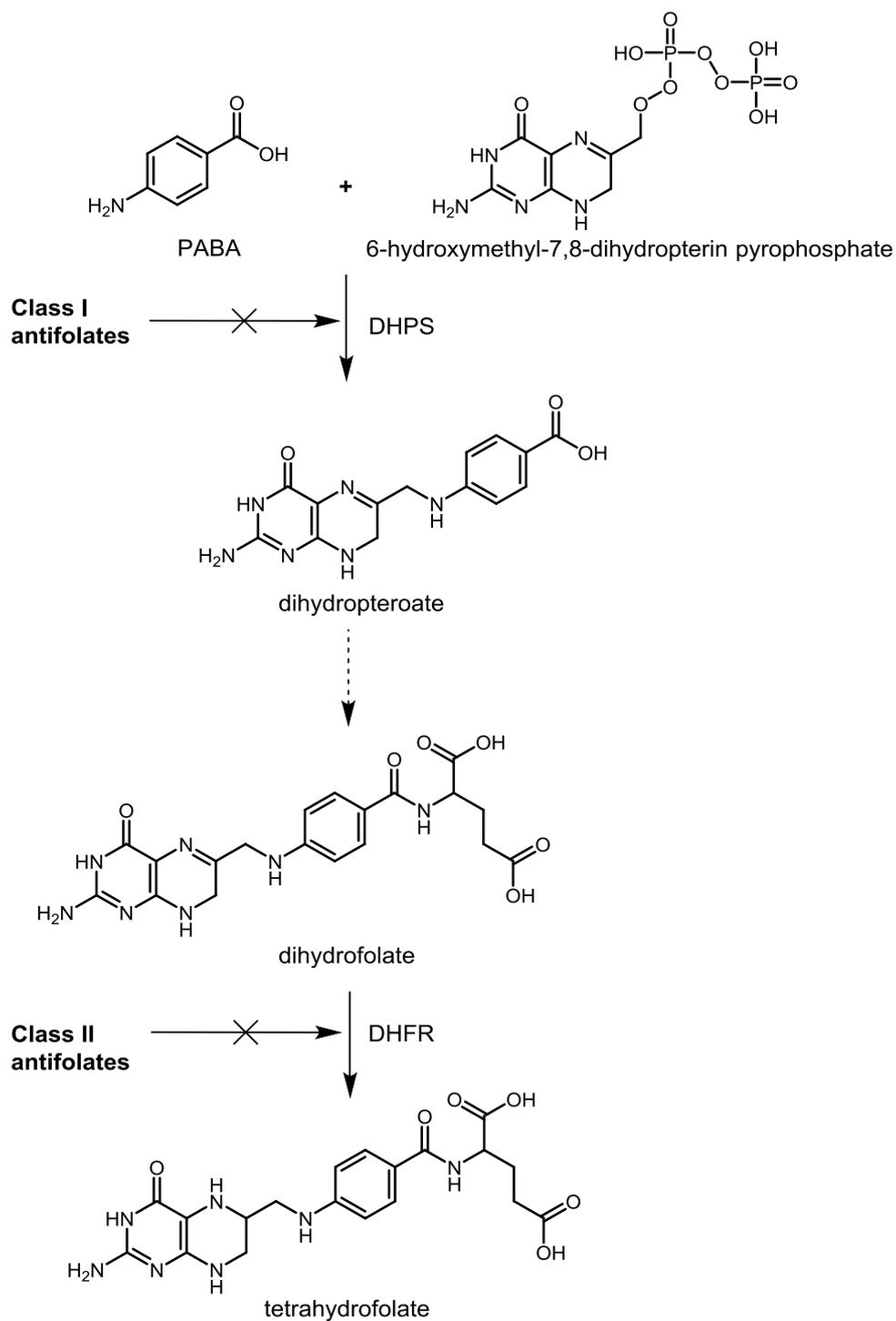


Figure 2. 11: Biosynthesis of tetrahydrofolate and the targets of Class I and Class II antifolates. Abbreviations: PABA, para-aminobenzoic acid; DHPS, dihydropteroate synthase; DHFR, dihydrofolate reductase (Zheng et al., 2013).

Class I Antifolates

These antimalarials inhibit dihydropteroate synthase (DHPS) of *P. falciparum* involved in the synthesis of dihydropteroate which is a precursor of dihydrofolate (DHF). This is an important step as failure of DHF synthesis leads to failure to synthesise DNA (Salcedo-Sora et al., 2011). The sulfa-based antifolates such as dapsone **13**, sulfadoxine **14** and related drugs act by competing with p-aminobenzoic acid (PABA) and dihydropteroate diphosphate for the active site of DHPS. These drugs are active only at micromolar concentration and are not given in monotherapy because of poor efficacy and high toxicity (Nzila, 2006). DHPS inhibitors have been combined with other drugs. Notable combinations include the use of pyrimethamine/dapsone (Maloprim), pyrimethamine/sulfadoxine (Fansidar), chlorproguanil/dapsone (Lapdap) and recently, chlorproguanil/dapsone with artesunate (CDA) (Sibley et al., 2001, Wootton et al., 2008, Luzzatto, 2010)

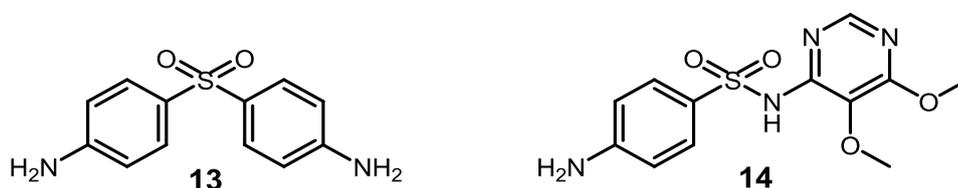


Figure 2.12: Dapsone **13** and sulfadoxine **14**.

Class II Antifolates

Like the Class I antifolates, Class II antifolates act on the folate synthesis pathway and here they inhibit dihydrofolate reductase (DHFR). These drugs act by inhibiting the reduction of DHF to tetrahydrofolate. DHFR inhibitors are active at nanomolar concentration and their activity is potentiated by reduced DHF following DHPS inhibitor activity (Nzila, 2006). This synergy led to the use of these antifolates in combination with DHPS inhibitors. Pyrimethamine **15**, proguanil **16** which is metabolised to cycloguanil **17** *in vivo* and chlorproguanil **18** which is metabolised to chlorcycloguanil **19** *in vivo* have been used in monotherapy but are widely used in combination therapies. The advantage of these antifolates, for instance pyrimethamine, when used in combination is that they have long half-lives and they balance fast-acting partner sulfa drugs and suppress development of resistant parasites (Nzila et al., 2000, Nzila, 2006).

P. falciparum developed resistance very quickly towards these drugs. Resistance to Lapdap and pyrimethamine is widespread. The mechanism of resistance is suggested to be due to point mutations on *dhfr* and *dhps* genes. The triple point mutation N51I, C59R and S108N on *dhfr* confers resistance to the Class II antifolates. A437G and K540E mutations on *dhps* lead to resistance to sulfa drugs (Sridaran et al., 2010, Gregson and Plowe, 2005).

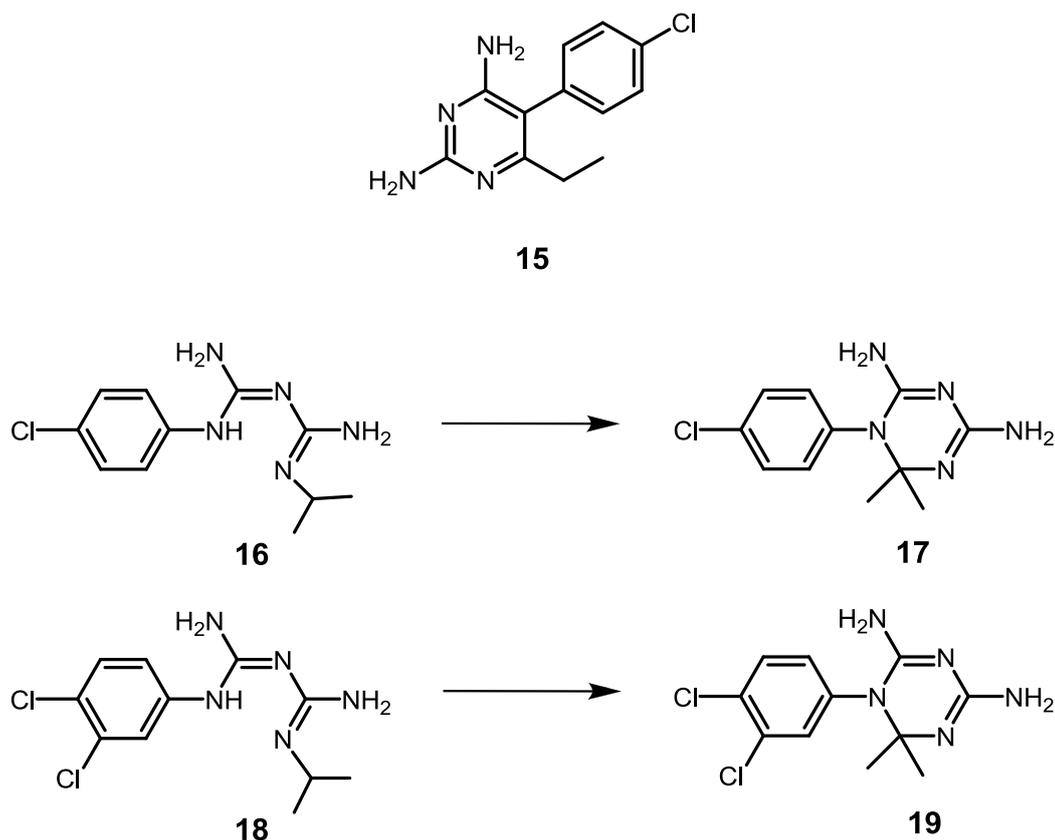
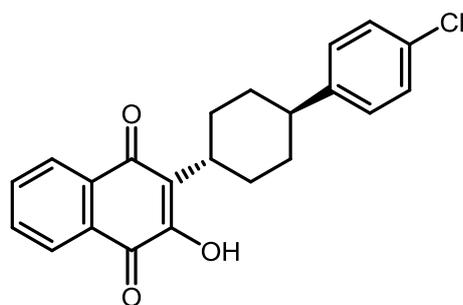


Figure 2.13: Pyrimethamine **15**, proguanil **16**, cycloguanil **17**, chlorproguanil **18** and chlorcycloguanil **19**.

2.2.8.5. Hydroxynaphthoquinones

Atovaquone

Atovaquone **20** is a hydroxynaphthoquinone used for treatment of uncomplicated *P. falciparum* and prophylaxis for travellers to endemic areas. Atovaquone alone faced resistance and it is mainly used in combination with proguanil (Malarone) in prophylaxis. The drug has a terminal half-life of over 87.5 h and has high affinity for plasma protein (99.5%) especially human serum albumin. It has limited absorption which is increased by administration with a high fat meal (Nixon et al., 2013). There is no evidence of metabolism or any enzyme systems that may be involved in its metabolism. Atovaquone is generally well-tolerated in the body with mild adverse events including diarrhoea, abdominal pain, skin disorders, headache and fever (Nixon et al., 2013).



20

Figure 2.14: Atovaquone **20**.

Atovaquone inhibits the mitochondrial electron transport chain of *P. falciparum* at the cytochrome bc₁ complex (Siregar et al., 2015). Atovaquone acts as a competitive inhibitor of ubiquinone at the ubiquinol oxidation site on the cytochrome bc₁. This results in collapse of mitochondrial membrane potential and the disruption of pyrimidine and purine biosynthetic pathways (Fry and Pudney, 1992, Bulusu et al., 2011). Resistance to atovaquone is proposed to be due to mutations Y268S and Y268N at position 268 of the cytochrome bc₁ complex of *P. falciparum* (Fisher et al., 2012).

Pyronaridine

Pyronaridine **21** was discovered in 1970 in China where it has since been used for treatment of malaria. It is a benzonaphthyridine derivative of mepacrine bearing an amodiaquine-like side group. This antimalarial drug showed potency against chloroquine resistant *P. falciparum* and has been in use against acute uncomplicated *P. falciparum* malaria. Pyronaridine is a blood-stage acting antimalarial drug with greater efficacy against the ring forms than trophozoites (Chen et al., 1987). It is a highly lipophilic drug with an elimination half-life of 6-9 days and poor oral bioavailability (Croft et al., 2012). It is efficacious both in monotherapy and in combination therapy, and is currently used in combination with artesunate (Pyramax). Pyramax has demonstrated outstanding clinical efficacy against *P. falciparum* and *P. vivax* malaria (Rueangweerayut et al., 2012, Poravuth et al., 2011). Pyronaridine is well tolerated with no serious toxicity (Croft et al., 2012). Common side effects after oral administration include abdominal discomfort, nausea, vomiting, dizziness and palpitations (Fu and Xiao, 1991, Chang et al., 1992).

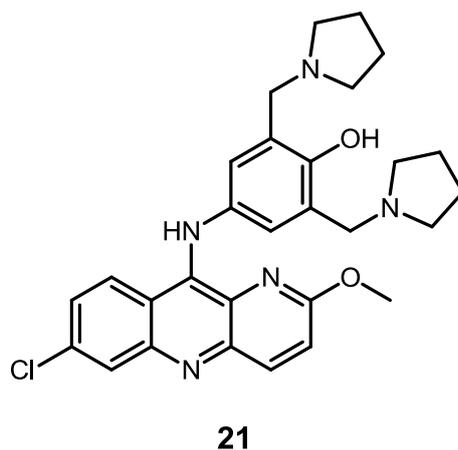


Figure 2.15: Pyronaridine **21**.

It has been reported that pyronaridine is both a blood schizonticidal and gametocytocidal antimalarial. It is presumed to act in the DV of the parasite by inhibiting haemozoin production leading to haem-induced erythrocyte lysis. In addition to this pyronaridine has been shown to bind haem *in vitro* thus enhancing haem-induced toxicity. It has also been shown that pyronaridine inhibits the glutathione-dependent haem degradation leading to ROS induced erythrocyte lysis (Auparakkitanon et al., 2006, Chavalitsheewinkoon-Petmitr et al., 2000).

The malaria parasite remains susceptible to pyronaridine. Reduced susceptibility reported in some areas was thought to have arisen as a result of cross-resistance with quinolones but recent work indicates otherwise. Common quinolone resistance markers such as *pfcr*, *pfmdr1*, *pfmrp* or *pfmhe-1* were not associated with decreased susceptibility of *P. falciparum* to pyronaridine *in vitro* (Pradines et al., 2010). The most recent *ex vivo* study, however, shows that the K76T mutation on the *pfcr* gene in *P. falciparum* isolates is associated with reduced efficacy of pyronaridine ($IC_{50} > 60$ nM) (Madamet et al., 2016). It is probable that resistance to pyronaridine is multigenic. It therefore remains to establish the mechanisms involved in reduced efficacy of therapies where pyronaridine is involved since the exact mechanism of resistance or biomarkers signalling resistance are currently not established.

2.2.8.6. Antibiotics

Antibiotics gained importance as antimalarials in the 1960s. Tetracyclines are effective bacteriostatic agents with a broad spectrum of activity. They are active against uncomplicated *P. falciparum* and have been largely used in prophylaxis for short-term travellers to endemic areas (Gaillard et al., 2015a). The commonly used antimalarial from this class is doxycycline **22** which is effective against erythrocyte stages of the parasite. Doxycycline is slow acting and can reach plasma concentration at 3 h with a terminal half-life of 14-24 h (Newton et al., 2005). It is not used in monotherapy due to its slow antimalarial activity. It is used in combination with fast

dihydroorotate dehydrogenase appears to be decreased. It was also found that doxycycline blocks the apicoplast and thus leads to reduced production of proteins (Lell and Kremsner, 2002, Dahl et al., 2006).

Treatment failures have been reported with antibiotics. In the case of doxycycline these failures have been attributed to the non-compliance to the treatment or wrong drug doses. However, resistance conferred by mutations on the *P. falciparum* metabolite drug transporter gene (*pfmdt*) and *P. falciparum* GTPase TetQ gene (*pfTetQ*) are responsible for reduced doxycycline susceptibility *in vitro*. The *pfmdt* encodes the membrane transporter that likely leads to drug efflux while the *pfTetQ* encodes TetA ribosomal protein that causes tetracycline resistance (Gaillard et al., 2015a, Gaillard et al., 2015b). Resistance to clindamycin has also been reported in the Amazon and this is said to be due to mutations on apicoplast 23S rRNA (Lell and Kremsner, 2002).

2.2.8.7. Artemisinins

The Chinese Project of the 1970s, project 523, resulted in the isolation of artemisinin **24** from *Artemisia annua* L, the plant that has been used for over 2000 years for treatment of febrile illnesses. Early work indicated 100% cure rates against rodent *P. berghei* malaria in 1972. Subsequent data showed that artemisinin was active against *in vitro*, and against animal and human forms of *P. vivax* and severe multidrug-resistant *P. falciparum*. Its structure was first reported in 1979 following X-ray diffraction and spectral analyses. It was shown that artemisinin is a sesquiterpene trioxane lactone (White et al., 2015).

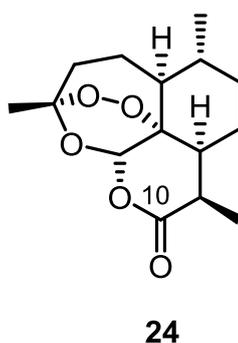


Figure 2.18: Artemisinin **24**.

Artemisinin was shown to be a fast-acting antimalarial. Although patients quickly recovered, relapse was a common problem. This indicated that artemisinin should be used in combination with longer-acting antimalarials. Dihydroartemisinin (DHA) **25** was prepared by reduction of artemisinin and soon after other derivatives were also synthesised. Amongst them, the oil-soluble ethers artemether **26** and arteether **27** and the water-soluble hemisuccinyl ester

artesunate **28** were found to be more active than artemisinin (Jansen, 2010, White, 2008). These antimalarials are blood schizonticides acting against ring and gametocyte stages of parasite development but not the tissue forms (Skinner et al., 1996, Price et al., 1996).

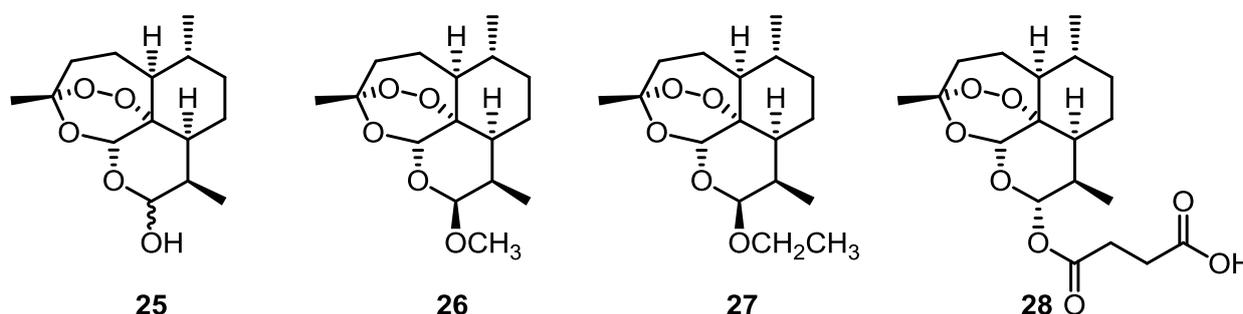


Figure 2.19: Dihydroartemisinin **25**, artemether **26**, arteether **27** and artesunate **28**.

Artemisinin and its derivatives (collectively called artemisinins) have short half-lives with elimination ranging between 1-3 h and peak plasma concentration between 1-2 h. Artemether, arteether and artesunate are metabolised to DHA *in vivo* following oral administration (Suputtamongkol et al., 2001, White, 2008). This is due to metabolism by cytochrome P450 enzymes in the liver, particularly CYP3A4, CYP2D6 and CYP2C19 (Van Agtmael et al., 1999). DHA has a short half-life and is quickly (≈ 1 h) eliminated by glucurodination *in vivo* (Balint, 2001). This short half-life limits antimalarial efficacy in that although there is rapid killing of malaria parasites, not all parasites are cleared. This is associated with high recrudescence in artemisinin monotherapy. These drugs are also implicated in induction parasite dormancy which is especially unfavourable if resistant strains are to be eradicated (Cheng et al., 2012, Codd et al., 2011).

All artemisinins have different bioavailability profiles. Artesunate is rapidly metabolised to DHA and has high bioavailability when administered orally but can be given rectally, intramuscularly or intravenously. Artemether can be administered intramuscularly or orally while arteether is administered intramuscularly. High first pass metabolism is seen with oral administration and the route of administration appears to affect the bioavailability and toxicological profile of each drug. As such arteether and artemether are associated with neurotoxicity in animal studies due to a depot effect when administered intravenously. Artesunate is not associated with such toxicity but repeated oral dosing result in neurotoxicity in rodents (LI et al., 1998, Navaratnam et al., 2000). Common side effects of artemisinin derivatives include nausea, vomiting and diarrhoea. With no human toxicity data and current safety profiles, artemisinins are still safe for use in treatment of malaria but pharmacovigilance is encouraged during pregnancy due to contradictory animal data implying developmental toxicity (Medhi et al., 2009).

Artemisinin Combination Therapy (ACT)

The WHO has recommended that artemisinins should be used in combination therapies with other antimalarials to prevent resistance to them. The rationale is that since artemisinins are fast acting and rapidly clear parasite biomass, the longer acting partner drug should be co-administered with an artemisinin derivative to clear the residual parasites. This will further ensure that artemisinin resistance is slowed as selection for resistant parasite will be reduced. Moreover, transmission of resistant strains will be prevented by the longer acting partner drug (WHO, 2005).

ACTs have been widely used as first-line treatment of uncomplicated *P. falciparum* malaria in most endemic areas of the world. This trend is continuing to increase in the treatment of febrile children and adults with *P. falciparum* malaria (WHO, 2015e). ACT is also effective, though equivalent to chloroquine, in treating other *Plasmodia* malaria such as *P. vivax* and *P. ovale* (Visser et al., 2014). ACTs have played a major role in the decline of malaria in areas where they have been applied. However, reports of the delayed parasite clearance in the Greater Mekong region following ACT are of great concern, as they imply that resistance to artemisinin derivatives has now become manifest (Dondorp et al., 2009, Mbengue et al., 2015, Ashley et al., 2014a). In African regions where this has not been noted so far, the close monitoring of parasite clearance is now recommended, and any report of increased times for parasite clearance must be reported (Talisuna et al., 2012). Currently used ACTs are artesunate-lumefantrine, artesunate-amodiaquine, dihydroartemisinin-piperaquine, artesunate-mefloquine, artesunate-sulfadoxine-pyrimethamine and dihydroartemisinin-naphthoquine-trimethoprim (Greenwood et al., 2005).

MoA of artemisinins

The MoA of artemisinins is yet to be elucidated. A number of theories exist that attempt to clarify the mechanism through which this drug class exerts its antimalarial activity. It is known that an endoperoxide on the artemisinin pharmacophore is important. It is likely that an endoperoxide, like most peroxides, induce reactive oxygen species and modulate the oxidative stress by countering the antioxidants in the parasite (Cui and Su, 2009). The most common theories include: (i) Reductive scission, (ii) Inhibition of PfATP6, (iii) Mitochondria electron transport chain disruption and (iv) Co-factor model. Each of these theories are briefly reviewed below.

(i) Reductive scission

Iron (II) or Fe^{2+} either in the free or bound form to the haem is essential for the antimalarial activity of artemisinins. It has been proposed that Fe^{2+} binds to the peroxide leading to formation of oxygen-centred radicals that rearrange to form carbon-centered radicals. These are held to

ultimately interact with vital biomolecules leading to death of the parasite. Two mechanisms for generation of the radicals are proposed. The first is that the Fe^{2+} binds to O1 of the peroxide resulting in the formation of the O2 radical which upon β -scission of C3-C4 results in formation of the primary carbon-centred radical **29** (Figure 2.20A). The second is that the Fe^{2+} binds to the O2 resulting in O1 radical which then abstracts a proton from C4 in a 1,5-H shift leading to secondary C4-centered radical **31** that also supposedly interact with the biomolecules (Figure 2.20B). The feasibility of these proposals is questionable since the C-centred radicals are required to migrate away from haem to react with vital biomolecules to exert their effect. They are too short-lived to survive this migration and therefore unlikely to interact with target biomolecules (Haynes et al., 2007, O'Neill et al., 2010). In addition, the Fe^{2+} is extruded resulting in inactive deoxyartemisinin adducts **30** and **32** (Haynes et al., 2013). It is also worth noting that artemisinins have some activity on early ring stages that do not depend on haem and they also exert effects on other parasites that do not form haem (Cui and Su, 2009).

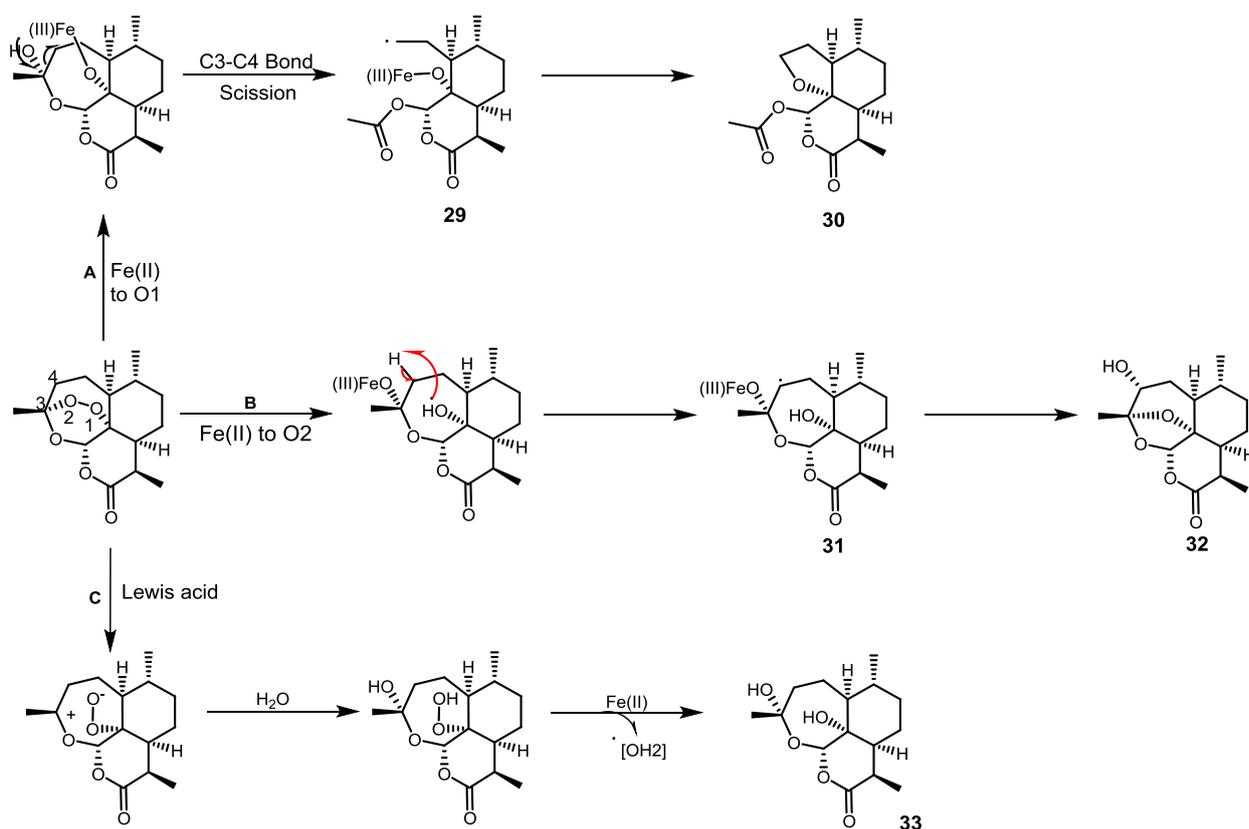


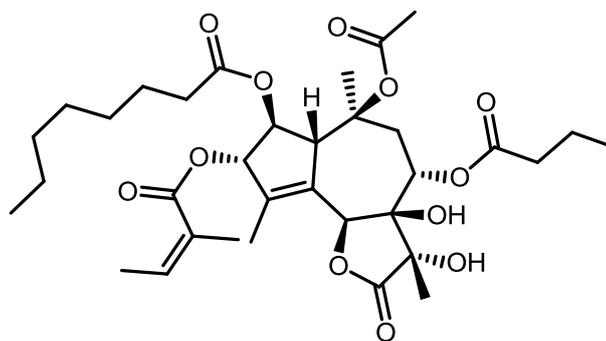
Figure 2.20: Proposed mechanism of action of artemisinins. A. C3-C4 bond scission; B. reductive scission of an endoperoxide (O'Neill et al., 2010); C. Lewis acid mediated ring opening of the peroxide to generate hydroperoxide (Haynes, Olliaro et al 2001).

An alternative mechanism involves ring opening (Figure 2.20C). This occurs through opening of the peroxide in the presence of Lewis acid (e.g. iron) forming a hydroperoxide (Olliaro et al.,

2001). Reduction of the hydroperoxide by Fe(II) releases a hydroxyl radical which does have sufficient activity for it to target biomolecules (O'Neill et al., 2010, Haynes et al., 1999). This mechanism also invokes involvement of the non-peroxide oxygen that stabilises the carbocation formed by the initial ring opening. In synthetic analogues with the non-peroxide oxygen substituted with carbon it has been shown that the antimalarial activity is significantly compromised. Thus for the artemisinins to exert their antimalarial activity the trioxane is important (Avery et al., 1996). The fate of the hydroxyl radical generated is likely hydroxylation of and/or proton abstraction from biomolecules (Olliaro et al., 2001).

(ii) Inhibition of PfATP6

Calcium (Ca^{2+}) plays an important role in malaria parasites as it is implicated in the egress from and invasion into the erythrocytes, parasite motility, intracellular development and cell signalling. As the parasite invades the erythrocyte a constant check is kept for optimal Ca^{2+} concentrations (Krishna et al., 2014). A mammalian Serca-endoplasmic reticulum Ca^{2+} ATPase (SERCA) homologue, PfATP6, of the *P. falciparum* is implicated to be fitted for maintaining the cytosolic Ca^{2+} homeostasis. It has been shown that artemisinin inhibits PfATP6 in the *Xenopus* oocytes expressing PfATP6. This was further supported by the ability of artemisinin to antagonise thapsigargin **34**, the known SERCA inhibitor. This study also showed that this antimalarial activity is dependent on Fe^{2+} artemisinin activation. In support of these it appeared the deoxyartemisinin is not effective and chelation of Fe^{2+} with desferrioxamine decrease the activity of artemisinin implying the importance of endoperoxide and interaction with Fe^{2+} (Eckstein-Ludwig et al., 2003). Recent docking study has also supported this model where it was shown that artemisinin and its Fe^{2+} adducts bind allosterically to PfATP6 thus changing the affinity for Ca^{2+} (Shandilya et al., 2013). However, Pandey *et al.* contradicts these proposals by showing that DHA and thapsigargin do not inhibit PfATP6 as they did not change the cytosolic Ca^{2+} concentration. Docking analysis also showed weak interaction between DHA and PfATP6 further supporting an idea that artemisinin do not affect Ca^{2+} homeostasis (Pandey et al., 2016). Endoperoxides have also not been found to bind to the PfATP6. It is therefore likely that the effect of the artemisinin on this transporter is just a downstream event induced by ROS (Haynes et al., 2012).



34

Figure 2.21: Thapsigargin **34**.

(iii) Mitochondrial electron transport chain disruption

Mitochondria play an important role in the parasite as they house dihydroorotate dehydrogenase (DHOD) which is involved in conversion of dihydroorotate to orotate. Orotate is involved in the synthesis of pyrimidines essential for DNA synthesis in the parasite (Painter et al., 2007). Artemisinins were shown to depolarise mitochondria and thus to disrupt its normal function (Antoine et al., 2014, Li et al., 2005). It is said that artemisinin does this through interference with the electron transport chain (ETC) resulting in ROS generation. In this instance it was proposed that artemisinin is activated through interaction with NADH (Wang et al., 2010). A recent study has shown that endoperoxides induce rapid loss of both mitochondrial and plasma membrane potential in an iron-dependent manner. Deoxyartemisinin did not induce any ETC loss indicating that endoperoxide is important for ETC loss. It could not be clarified whether iron was important for bioactivation of artemisinin or for mediation of the oxidative stress (Antoine et al., 2014). It is not yet fully understood how artemisinin acts on the ETC but this theory shows that ROS is involved in membrane damage.

(iv) Co-factor model

The maintenance of ROS homeostasis is central to survival of both mammalian cells and the malaria parasite. In order for this to happen, there are redox centres that are involved in homeostasis and their perturbation may lead to cellular death. A family of flavoenzymes is involved in controlling cellular ROS levels. These include glutathione reductase (GR) and thioredoxin reductase (TrxR) in which reduced flavin cofactors facilitate disulfide reduction. GR accepts electrons from NADPH provided through the rate limiting step of the glucose-6-phosphate dehydrogenase (G6PD)-catalysed hexose monophosphate shunt (HMS). The reduced flavin thereby generated within GR then transfer electrons to glutathione disulfide (GSSG) that is reduced to glutathione (GSH) which in turn reduces the ROS; thereby GSSG is regenerated that is again reduced by the reduced flavin cofactor in GR.

Artemisinins and related compounds are proposed to induce elevated ROS generation by oxidising the reduced flavin co-factors and/or related flavin co-factor precursors such as riboflavin (Figure 2.22) (Haynes et al., 2012). Artemisinin intercept transfer of electrons from the reduced flavin co-factor or dihydroflavin to GSSG and thus disable GSH generation and therefore ROS homeostasis. This process has two significant outcomes: (i) HMS is not able to keep up with the demands for NADPH from G6PD-catalysed step leading to failure to produce GSH and (ii) build-up of ROS which then lead to parasite death through adverse downstream events. Furthermore, under aerobic conditions artemisinin appears to induce autoxidation of dihydroflavin leading to increased demand for NADPH and enhanced abrogation of GSH activity.

Unlike other proposed MoAs, this model highlights the idea that two electrons are transferred, that artemisinins do not require iron(II) for bioactivation and that artemisinins exert their action in the cytosol and not in the DV (Haynes et al., 2013). Inhibition of haemozoin formation by chloroquine and quinine leads to an efflux of haem-iron(III) and chloroquine-quinine-heme-iron (quinoline haem-iron(III)) complexes from the DV into the cytosol. In the cytosol iron(III) is reduced to iron(II) by flavin co-factors. Iron(II) then is oxidised by oxygen resulting in ROS generation. Iron therefore appears to enhance artemisinins mechanism but does not chemically react with artemisinin. ROS generation according to this model may help to clarify effects of the PfATP6 model in that it is a downstream effect (Haynes et al., 2012).

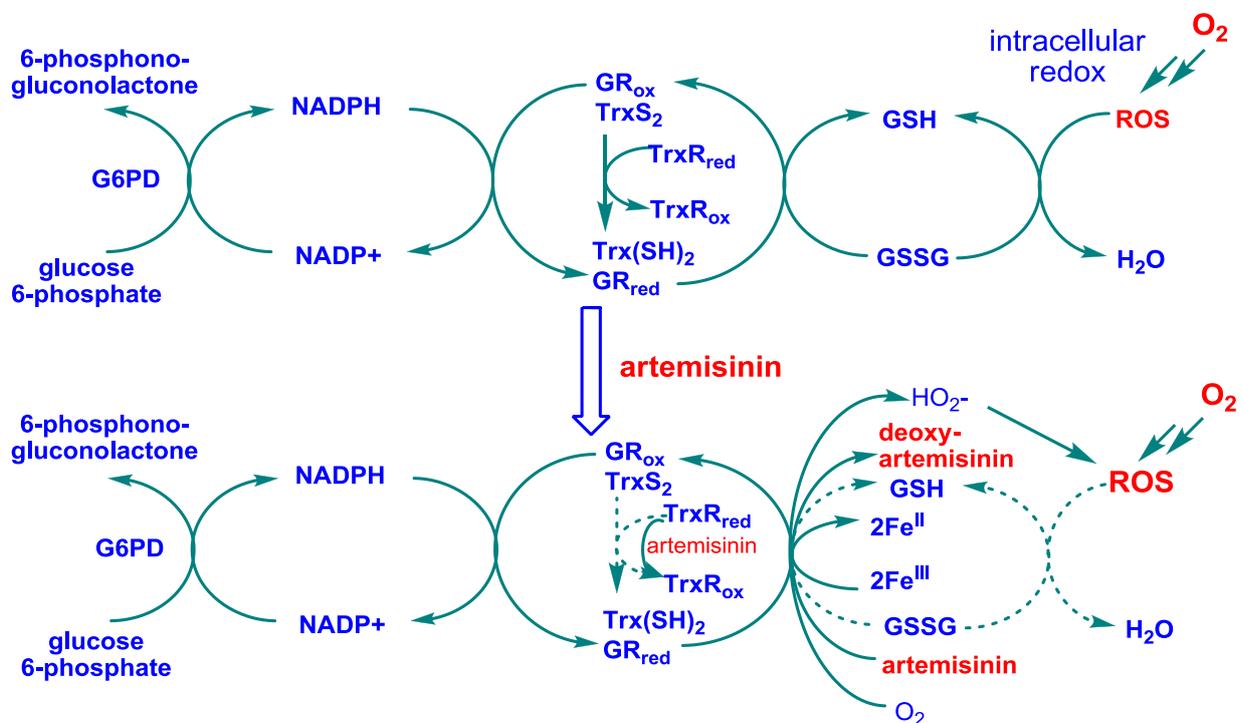


Figure 2.22: The co-factor model of the artemisinin mechanism of action (Haynes et al., 2012).

Resistance

Resistance to artemisinin was first reported in 2006 from Cambodia, the notorious focus of multidrug-resistant *P. falciparum*. Such resistance is now prevalent in South-East Asia but it has not been found in Africa (Paloque et al., 2016, Ménard et al., 2016). Current definition of resistance to artemisinin is based on delayed parasite clearance evidenced by prolonged half-lives ranging from 3.7 h to 7 h in Thai-Cambodia vs 1.9 h in Africa (Ashley et al., 2014a). Artemisinin resistance is now well established in South-East Asia and China and this is alarming as artemisinin are the only currently effective antimalarials available (Paloque et al., 2016). It is therefore crucial that such resistance is arrested and prevented from spreading to Africa where artemisinin are very effective and continue to save many lives.

An underlying mechanism of resistance has recently been described. The parasite enters a quiescent state in the young ring stages under pressure from the artemisinin drug. These dormant parasites are therefore able to resume to the next stage of development as soon as the artemisinin are removed. Though it is only a small number of these parasites that survive (<1%), their growth and spread proves fatal as the resistance to artemisinin is continuing to increase (Teuscher et al., 2010, Cheng et al., 2012).

The mechanisms of such resistance are linked primarily to the mutations on the *P. falciparum* Kelch13 (*PfKelch13*) gene encoding the PfK13 propeller domain. These mutations are found to occur after position 440 in the propeller domain and are prevalent in different parts of South-East Asia. The most common or currently identified mutations include the C580Y, R539T, I543T and Y493H mutation on chromosome 13 of the Kelch propeller gene. These mutations appear to interfere with the binding of important co-factors required for ubiquitinylation of proteins and this limit proteolysis. Parasites with resistance to artemisinin are therefore endowed with repair mechanisms which increase their survival (Paloque et al., 2016).

Another mode of resistance is said to be due to an upregulation of proteins involved in unfolded protein response (UPR). In resistant parasites, there is an elevated level of chaperones and major UPR proteins. Immunoglobulin-binding protein and cyclophilin B are elevated in resistant strains and these are important for protein folding and repair of the ER following oxidative stress in the parasite. Thus the upregulation of such proteins may bequeath the parasite with the ability to repair and degrade the misfolded proteins induced by the activity of artemisinin (Liu et al., 2010).

2.3. Tuberculosis

2.3.1. Epidemiology of tuberculosis (TB)

Recent figures for TB morbidity and mortality show a significant improvement compared to the levels of the 1990s. Although these figures are promising, incidence, prevalence and mortality are still inadmissibly high. The 2015 WHO Global Tuberculosis report indicates that there were an estimated 9.6 million new cases of TB globally with 12% of these cases in HIV-positive individuals in 2014. There were an estimated 1.5 million deaths due to TB the same year and 0.4 million of the deaths were among the HIV-positive individuals. Though this is still a high number, the report shows that mortality has decreased by 47% compared to 1990 and TB prevalence has been falling at 1.5% annually since 2000 (WHO, 2015f).

TB is the disease of poverty affecting resource-limited areas of the world with poor basic living conditions. The disease has a higher incidence in men especially at the productive age between 14-49 years than in women and children (Bruchfeld et al., 2015). This is evident in last year's report where it was reported that 56.3% men compared to only 33.3% women and 10.4% children were infected. Of the estimated incidences 58% were from South-East Asia and Western Pacific region and 28% were in the African region (Figure 2.23). The African region has the highest domestic incidences of any region in the world where 281 TB cases per 100,000 people present with TB compared to 133 TB cases per 100,000 people on a global basis. Plagued with high human immunodeficiency virus (HIV) infections, the African region accounts for 74% of global HIV/TB coinfections (WHO, 2015f). The cases of reactivated TB arising from latent TB are therefore high due to immune-compromised HIV-positive individuals. In addition to HIV, poor health care services in HIV endemic regions, clustered informal settlement in impoverished endemic regions and silicosis among miners and overpopulated public places like prisons play crucial roles in high TB infections in Africa.

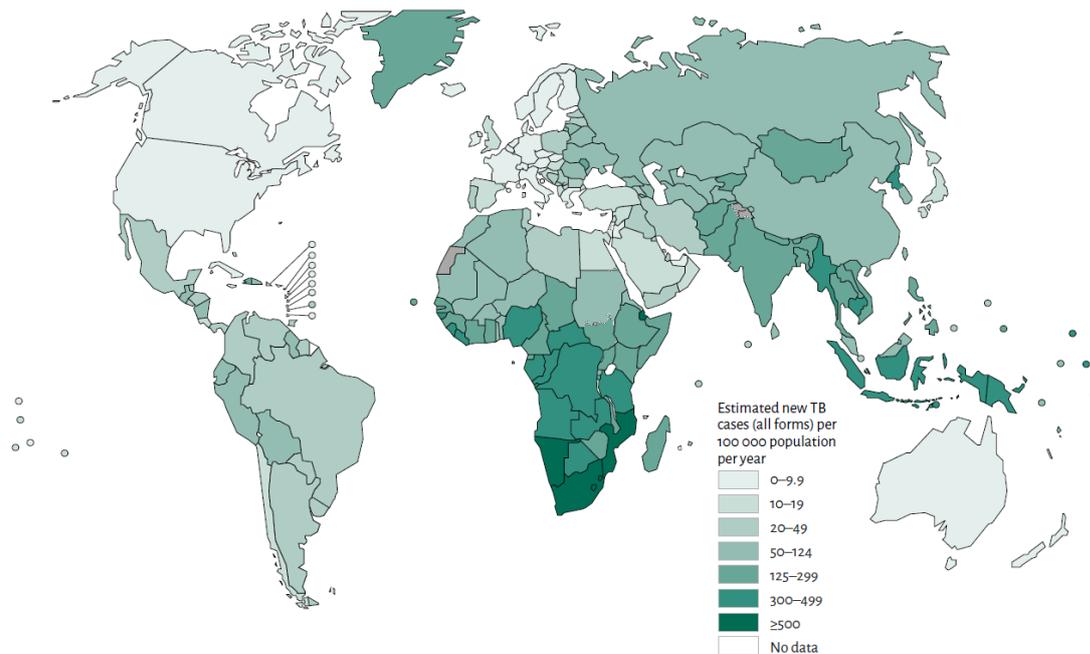


Figure 2.23: Global incidences of Tuberculosis (TB) in 2014 (WHO, 2015f).

2.3.2. Tuberculosis in South Africa

Sub-Saharan Africa accounts for most of the high-burden countries globally and for high HIV/TB coinfections. In South Africa (SA) the Kwa-Zulu Natal province has the highest number of coinfection cases due to high HIV cases, 16% vs 12% nationally (McGee et al., 2016). According to the 2013 WHO Global TB report, SA and Swaziland had the third highest incidences of TB at 1000 infections per 100,000 people (Loveday et al., 2013). SA still has an unacceptably high incidence and is amongst the top six most severely burdened countries globally. It is also in the region where 50% HIV/TB coinfections occur. The country's progress is impeded by paucity of quality healthcare services, increasingly drug-resistant TB strains, hyper-endemic HIV/TB coinfections, lack of proper diagnostic tools, poor retreatment outcomes, drug stock-outs and poor TB management policies (Loveday et al., 2013, Perumal et al., 2009).

2.3.3. Transmission and pathology of tuberculosis

2.3.3.1. Transmission

Tuberculosis is transmitted when an individual with active pulmonary or laryngeal TB coughs, sneezes, sings or speaks. Infection takes place when aerosolised droplets containing *Mycobacterium tuberculosis* (*Mtb*) bacilli are inhaled by a susceptible person. These small droplets are about 1-5 microns in diameter and can remain airborne for several hours. This is the main form of transmission but other forms such as transmission through contact with body fluids from infected individuals may occur (Frieden et al., 2003).

2.3.3.2. The life cycle of Tuberculosis

When the *Mtb* bacteria have been inhaled, they are attacked by the larynx macrophages but in most cases the bacilli travel to the alveoli (Figure 2.24). Here bacilli are recognised and phagocytised by alveolar macrophages which ultimately invade the subtending epithelium. This response leads to the recruitment of mononuclear cells from the surrounding blood vessels. The recruitment of these cells is the basis of granuloma development which is the hallmark of TB infection (Martin et al., 2016). Infected macrophages also differentiate into a number of cells including epithelioid cells, multinucleated giant cells and lipid-rich foamy macrophages. Other innate immunity cells like neutrophils and dendritic cells are also present at this stage. The periphery of the granuloma is made up of T-lymphocytes that have been recruited after initiation of acquired immune response at least 2-3 weeks after initial infection (Russell et al., 2010a). Eventually replication of the bacilli is brought to halt and the formation of granuloma contains and arrests the infected macrophages at the centre. The halt of replication renders the bacilli quiescent and it can remain in the lungs for decades without causing clinical disease (Russell et al., 2010b). Containment of bacilli is common in immunocompetent individuals. People who are immunocompromised or those who cannot control infection progress to TB disease from primary infection.

The granuloma starts to gain form as more cells accumulate and there is also a development of the fibrous cuff that further excludes the T-lymphocytes from the granuloma centre. Formation of the fibrous cuff also leads to the retraction of the blood vessels from the centre. Retraction of blood vessels from the centre of the granuloma limits oxygen supply which may also lead to aerobic bacilli quiescence (Russell et al., 2010a). The centre of the granuloma is also characterized by necrotic events and this leads to caseation (Kaplan et al., 2003). Caseum is a cheese-like coagulated tissue resulting from necrosis of foamy macrophages. Foamy macrophages are lipid rich and have been identified in lungs of necrotic granulomas. Thus metabolism of the macrophages may be the reason for caseation (Russell et al., 2009). Expansion and change from calcified form to the liquefied form results in the cavitation of the granuloma and thus the dissemination of the bacilli. It is at this phase that the bacilli can be transmitted from an infectious host (Russell et al., 2010a).

In 90-95% of infected individuals the granuloma remains intact and these people can live without progressing to the disease state. This form of TB is known as latent TB infection (LTBI) and about 2-3 billion of the world's population have this form of TB. Individuals with LTBI have about 5-15% chance of progressing to the disease state in their lifetime (WHO, 2015f). This happens when an individual becomes immunocompromised as in the case of HIV/AIDS patients. This form of the disease is termed reactivation or reinfection. LTBI is one of the major contributors to the delayed eradication of TB (Ai et al., 2016, Esmail et al., 2014).

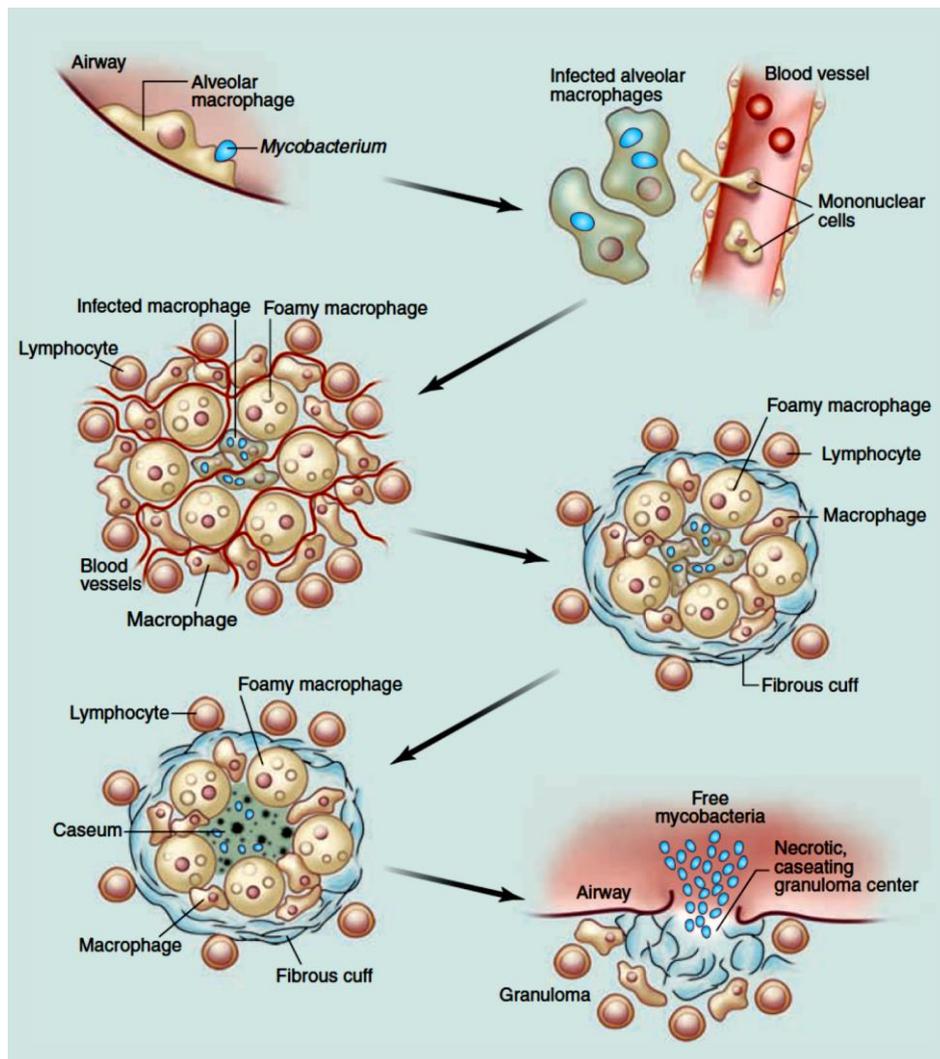


Figure 2.24: The life cycle of the tuberculosis bacteria (Russell et al., 2010a).

2.3.4. Clinical manifestation of tuberculosis

Clinical manifestations of TB differ from patient to patient depending on the form or the stage of TB. The most common symptoms of patients are cough, fever, weight loss, anemia, wheezing, sputum production, haemoptysis and night sweats (Knechel, 2009). Since about 90% of the primary infections do not lead to active disease, these symptoms are not generally noticed. It is in the immunocompromised individuals like HIV positive individuals or in children with underdeveloped immune system that the disease can follow. Due to a variety of TB forms, patients usually present with different symptoms. Patients may have pulmonary TB which occurs upon the infection of the lungs or may have extrapulmonary TB which will induce systemic symptoms. Brief descriptions of the different forms of TB and their accompanying symptoms are presented below.

2.3.4.1. Primary pulmonary TB

Two-thirds of primary infections are asymptomatic and only 5-15% of people progress to the

active disease state. Most immunocompetent individuals are able to contain bacilli and do not progress to the disease state. The only presenting features can be seen on the radiograph as the lungs have marked granulomas. The general symptoms of this TB form are nonspecific and include low-grade fever, malaise and progressive fatigue (Knechel, 2009, Hunter, 2011).

Over 90% of the adult TB cases are due to reactivation or reinfection. Reactivation occurs when an individual becomes immunocompromised such as in HIV positive people or when the granuloma undergoes necrosis and release the bacilli into air tracts. The common symptoms that are presented from this form include cavitation and abnormalities in the upper lobes of the lungs. The radiographic data of individuals with HIV are usually normal compared to those of immunocompetent individuals. The common symptoms of the disease are night sweats, fever, weight loss and fatigue (Sia and Wieland, 2011).

2.3.4.2. Miliary TB

Miliary TB is an acute and fatal form of TB most common in children and young adults. It usually occurs just after primary infection or may occur later with compromised immunity such as in late stages of HIV/AIDS individuals (Sharma and Mohan, 2004, Sharma et al., 2012). The mechanism by which this TB forms is not yet clear. One proposal suggests that it occurs as a result of bacilli eroding and evading the alveolar epithelium lining and then drenching into the systematic circulation. An alternative proposal suggests that bacilli are taken by phagocytic cells and disseminated from the lungs (Davis and Ramakrishnan, 2009, Krishnan et al., 2010). This allows for bacilli to be carried by lymph and blood and their spread to extrapulmonary sites becomes manifest in adverse TB symptoms. Autopsy findings show miliary TB is more common in organs with high blood flow such as the lungs, liver, kidneys, spleen and bone marrow. Choroid tubercles are early manifestations and may assist with early diagnosis (Sharma and Mohan, 2004). Clinical features are non-specific and include fevers, night sweats, anorexia, lassitude and cough. In adults, miliary TB also causes tuberculosis meningitis. Diagnosis is usually challenging due to high nonspecific manifestations but chest radiograph showing millet-like tiny spots on the lung can confirm miliary TB (Sharma et al., 2012).

2.3.4.3. Extrapulmonary TB (EPTB)

EPTB also includes miliary tuberculosis except that miliary TB can also occur in the lungs. EPTB specifically designates TB that occurs in sites of the body other than the lungs (Sharma and Mohan, 2004). Like miliary TB, EPTB is a result of acute lymphohaematogenous dissemination of bacilli throughout the body. A number of organs are affected and different TB and complications occur. This form of TB can manifest as skeletal TB which largely affects joints, leading to paraplegia, abdominal TB that causes pain in the ileum or caecum, or central nervous system (CNS) TB resulting in deadly meningitis TB which is fatal in most cases (Wani,

2013). Overall clinical features of EPTB include paraplegia, altered consciousness and common nonspecific fevers, headaches and meningismus in adults and seizures, nausea and vomiting occur in children (Rock et al., 2008).

2.3.5. Diagnosis of TB

Diagnosis of TB is still challenging and inadequate in most settings. The situation is further complicated by HIV/TB coinfections in most endemic settings and LTBI. Most of these challenges centre around (i) the delayed diagnosis leading to spread of infection, (ii) over-diagnosis leading to the use of already few resources and (iii) difficulty to diagnose HIV/TB coinfections (McNerney et al., 2012). Current TB diagnostics are briefly described below:

2.3.5.1. Sputum smear microscopy (SSM)

SSM is the most widely used TB diagnostic in the world and remains the cornerstone of diagnosis in the developing world (Singhal and Myneedu, 2015). This method requires that an individual with suspected TB infection to give sputum (two to three times) to the health-care center for microscopic analysis. The sputum is then stained with the Zeihl-Neelsen stain (ZNS) to prepare a sputum smear to visualise the presence of acid-fast bacteria. Preparation involves ZNS staining the sample red, decolorisation with an acid-alcohol and lastly staining with methylene blue. Mycobacteria will remain red and not be colour with methylene blue. Under the microscope the red coloured colonies will confirm the TB infection (Dezemon et al., 2014). Although widely used this diagnostic has several challenges. It requires that patients visit the health-care at least two or three times to give sputum, the sensitivity is less than 70%, it is labor intensive, cannot distinguish between *Mtb* complex species, and has reduced sensitivity in children, EPTB and HIV-positive individuals (WHO, 2015b).

2.3.5.2. Chest X-ray

This is the primary diagnostic of TB and it identifies lung lesions in the case of infection. Its usage is simple and available to most health-care centres even in the developing world. The major drawback with this diagnostic is its non-specificity for TB and other infections. TB can be misdiagnosed as pneumonia and this can lead to delayed treatment of TB. Lungs may even appear normal when a chest X-ray is used (Woodring et al., 1986, Ryu, 2015). To compensate for this, chest computed tomography may be used which is more sensitive and specific (Jeong and Lee, 2008). However, either the chest X-ray or the chest computed tomography still requires sputum smear confirmation and this may delay treatment as well.

2.3.5.3. TB Culture

Mycobacteria can be cultured in a liquid and solid media. This method is also further applied

when the drug sensitivity testing (DST) is performed. Mycobacteria culturing can be in a solid media such as Lowenstein-Jensen media or in a liquid media where mycobacteria can multiply and be assessed for infection. This is more sensitive than the sputum-smear microscopy but it does not have good turn-around time. Since mycobacteria grow very slowly, solid media culture results may be available in a month's time while for the liquid media culture may be available in only 2 weeks (WHO, 2007b). The other drawback with this diagnostic tool is the potential for contamination of the other microorganisms. This test is also expensive to perform and is not commonly used in developing countries (Ryu, 2015). However, it is more efficiently used with rapid immunochromatographic assays to distinguish TB from other species of the *Mtb* complex. Isolates from this test can be used to test for drug resistance by conducting the culture with an anti-TB drug added to the media (Považan et al., 2012, WHO, 2015b).

2.3.5.4. Molecular testing

At least two types of molecular testing are currently used:

A. Line-probe assay

Sample preparation for this assay involves isolation of the DNA and the amplification of the *rpoB* gene region by PCR followed by hybridisation of PCR products with specific oligonucleotide probes on the strip. Hybridisation forms colour bands on the strip and this can confirm whether there is infection. This test offers rapid detection (<48 h) of resistance and it also is not so labour intensive (Rossau et al., 1997, Morgan et al., 2005). The weaknesses of this method are the need for culture and DST as well as the SSM confirmation. Moreover, resistance can only be detected for first-line drugs (WHO, 2015b).

B. Xpert MTB/rifampicin

The use of this test is increasing worldwide and many countries have included it on their TB diagnostic policies (WHO, 2015f). Xpert MTB/rifampicin use automated, cartridge-based nucleic acid amplification with results available in <2 h (Helb et al., 2010). It is more sensitive than the culture method and has around 99% specificity. It can detect TB even in patients who have smear-negative culture-positive outcomes. The test can also detect most cases of rifampicin resistance with 98% specificity and 95% sensitivity (WHO, 2013a). Xpert MTB/rifampicin requires low levels of biosafety and no need for intensive personnel training. However, the cartridges need to be stored at 28 °C and the test be performed at 30 °C (WHO, 2015b).

2.3.5.5. Latent TB infection testing

This test measures memory T-cell response in individuals with latent TB infection (LTBI). Two

tests are recommended for LTBI: Tuberculin Skin Testing and Interferon gamma release assays. The former is the most common and the less expensive and easier to use. It is limited by the need for 2-3 visits to the health-care centre with the results are available in 2-3 days. It has poor sensitivity in immunocompromised individuals and has low specificity in BCG vaccinated people. The latter is fast and results can be available in 24-48 h but it is very expensive. It is not recommended for use in individuals with active TB (Schluger and Burzynski, 2010).

2.3.6. TB vaccine

Bacille Calmette-Gièrin (BCG) is a live-attenuated *Mycobacterium bovis* vaccine developed in 1921 and the only currently used vaccine against TB. BCG provides partial protection against severe TB in infants and children but does not offer protection in adults. This implies that as an individual grows the efficacy of the vaccine is decreased and the risk of TB infection increases during adolescence and adult years. There are currently about 15 vaccines in various phases of clinical development (Evans et al., 2016, Colditz et al., 1994). These vaccines are designed to provide, unlike BCG, prevention of TB infection, prevention of reactivation in LTBI individuals and prevention of primary infection progression to disease state in asymptomatic infected individuals who pose greater risk for transmission (Doherty and Andersen, 2002).

Most of the current candidates are aimed to stimulate CD4⁺ Th1 T-cell response. However, developing vaccines with one common target increases chances of inherent failure (Ginsberg et al., 2016, Kaufmann et al., 2015). These vaccines are developed in different ways including whole cell mycobacterium or lysates, viral-vectored, recombinant BCGs and adjuvant subunit vaccines. The most advanced of these vaccines is heat-killed *Mycobacterium vaccae* (*M. vaccae*) that was approved in China in for use together with TB drugs (Efremenko et al., 2013). *M. vaccae* is a whole cell mycobacterium which expresses antigens for a number of mycobacteria and it is thought to act by promoting the Th1 cell responses and to also restore host recognition of mycobacterial antigens (Johnson et al., 2000). *M. vaccae* has demonstrated potent adjuvant therapy to chemotherapy in a number of studies. It has a good safety profile and was found to be helpful to patients who were not previously treated (Weng et al., 2016, Yang et al., 2011). Furthermore, phase III studies are ongoing in China to show the efficacy of *M. vaccae* in latent TB individuals in order to reassess the potential for prevention of reactivation (Ginsberg et al., 2016).

2.3.7. Chemotherapy of tuberculosis

Chemotherapy plays an important role in the treatment and prevention of TB. There are a number of TB drugs currently in use. These drugs are effective but the rising multidrug-resistant TB (MDR-TB), extensively drug-resistant TB (XDR-TB) and recent totally drug-

resistant TB (TDR-TB) strains threaten their future use in treatment of TB. MDR-TB is TB where mycobacteria are resistant to rifampicin or isoniazid, whereas XDR-TB is MDR-TB with additional resistance to fluoroquinolones and one of the injectable aminoglycosides (Zumla et al., 2014). Rare but the threatening TDR-TB is MDR-TB resistant to all second line drugs (Velayati et al., 2009).

Chemotherapy of TB is protracted and requires the use of combination of drugs with different MoAs. The requirement for long-term treatment and many drugs lead to poor patient compliance. On the other hand drug discovery has been very slow (Ginsberg and Spigelman, 2007). Bedaquiline and delamanide are the only drugs which have succeeded in the clinical trials and received provisional approval after over 40 years. This section reviews the current chemotherapy of TB. Classes of anti-TB drugs are divided into first-line and second-line drugs and the latter is further divided into four groups (WHO, 2014). These classes are discussed in the following section.

2.3.8. First-line treatment drugs

Rifampicin

Rifampicin **35** is an antibiotic discovered in 1965 and is an important first-line anti-TB drug currently used in the clinical treatment of TB (Sensi, 1983). Rifampicin is used with isoniazid in a 6 months treatment regimen. This drug is lipid soluble and can penetrate the membranes to act on bacilli. Rifampicin is metabolised by the CYP450 enzyme system and has a half-life of 2-3 h. Over 60% of the drug is excreted via biliary tract and a small amount by urine. Food intake reduces uptake of this drug by 60% and therefore it is not recommended that rifampicin be taken with meals. Side effects associated with rifampicin include anaemia, vomiting, nausea, dizziness, thrombocytopenia and hepatotoxicity (Arbex et al., 2010, Sousa et al., 2008).

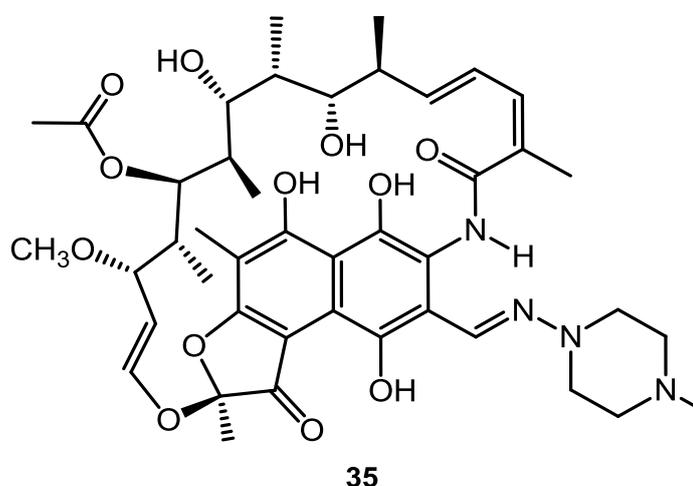


Figure 2.25: Rifampicin **35**.

Rifampicin is active against both growing and dormant or metabolically inactive bacilli. It acts by binding to the B-subunit of RNA polymerase and therefore it inhibits mRNA elongation. This leads to the failure to synthesise proteins that then results in cell death (Campbell et al., 2001).

Resistance to rifampicin has been associated with mutations on the 81 base pair region of the *rpoB* gene. This region is termed the hot-spot region of rifampicin resistance and includes codons from 507-533 of the *rpoB* gene (Telenti et al., 1993a). The most common mutations that account for majority of rifampicin-resistance cases are on condons 516, 526 and 533 (Nowshad et al., 2015, Hughes and Brandis, 2013). Additional mutations on the *rpoA* and *rpoC* genes have also been identified and these are reported to infer restored fitness of the resistant strains and increase their spread (De Vos et al., 2013). Genes *rpoA* and *rpoC* encode α - and β -subunits of RNA polymerase, respectively (Comas et al., 2012). The presence of these mutations changes the conformation of the RNA polymerase and lead to reduced affinity for the drug and therefore resistance (Telenti et al., 1993b).

Isoniazid

Isoniazid **36** was developed in 1952 and it forms part of the most important anti-tuberculosis first-line drugs. It is a nicotinamide containing a pyridine ring and hydrazine group active against growing bacilli and has some activity against the EPTB bacilli (Zhang et al., 1993, Shakya et al., 2012). The drug has short half-life of 1 h in patients with the acetylation phenotype and it is 2-5 h in deficient individuals. This phenotype is important for conversion of isoniazid into an active adduct (Arbex et al., 2010). Isoniazid is converted *in vivo* by activity of catalase-peroxidase enzyme *katG* to a hypothetical isonicotinoyl anion or radical which then reacts with the nicotinamide (NAD^+) to form an active isoniazid-NAD adduct. The isoniazid-NAD adduct covalently binds to an active site of NADH-dependent enoyl acyl carrier protein (ACP) reductase (*inhA*) and inhibits the elongation of fatty acid. Thus, this will inhibit the synthesis of mycolic acids which are essential for the cell wall of the mycobacterium and will then lead to cell death (Jena et al., 2015, Rozwarski et al., 1998).

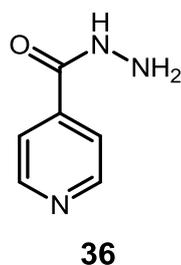


Figure 2.26: Isoniazid **36**.

Resistance to isoniazid is due to mutations on the *katG* and *inhA* genes. The former has been described as a S315T mutation which occurs in 64% of phenotypic isoniazid resistance and is frequent in MDR-TB clinical isolates. This mutation inhibits the conversion of isoniazid to its active adduct and makes it unable to bind the *inhA* (Zhang et al., 1993, Torres et al., 2015, Guo et al., 2006). The second common mutation, the *inhA* gene mutation, is associated with the overexpression of the *inhA* gene (Larsen et al., 2002). An additional mutation is a -15C/T on the promoter region of *inhA*. Together these mutations result in reduced affinity of the isoniazid-NAD adduct in the active site of *inhA* (Guo et al., 2006). Isoniazid is a well-tolerated drug with minor side effects including nausea, vomiting, headaches and major ones being psychosis, convulsions, mental confusion and coma. In patients who do not have liver and kidney disease isoniazid rarely causes side effects (Arbex et al., 2010).

Pyrazinamide

Pyrazinamide **37** is an important sterilising anti-TB drug that has played a role in reducing treatment from 1 year to 6 months in combination with isoniazid and rifampicin. It is a nicotinic acid derivative that was synthesised in 1936 and has been used in TB treatment since 1952. The sterilising effect of this drug is due to its ability to act on semi-dormant bacilli in the inflammatory zones of the lungs and thus prevent the relapse of such bacilli (Shakya et al., 2012). Pyrazinamide is converted to an active metabolite pyrazinoic acid by pyrazinamidase. Accumulation of pyrazinoic acid in the cytoplasm of the mycobacterium leads to an acidic pH environment and thus to inactivation of fatty acid synthase 1 (FAS1). FAS1 is important for the elongation of fatty acids and its inhibition leads to impaired mycolic acid biosynthesis and bacterium death (Zimhony et al., 2000). Pyrazinoic acid has also been reported to disrupt the membrane energetics of the mycobacterium and inhibits the efflux of pyrazinamide (Zhang et al., 2003). Pyrazinamide is well-absorbed and is distributed in the body reaching plasma concentration in 2 h. Pyrazinamide is metabolised in the liver with a half-life of 9-10 h and about 70% of the drug is excreted in the urine. Common side effects associated with pyrazinamide are nausea, vomiting, anorexia, hyperuricemia, photosensitivity dermatitis and hepatotoxicity, the last being more pronounced than for other first-line drugs (Arbex et al., 2010). Resistance to pyrazinamide is associated with mutations on the *pncA* gene which encodes pyrazinamidase

(Sreevatsan et al., 1997). Other mutations are associated with efflux of the active metabolite pyrazinoic acid to the extracellular environment (Sheen et al., 2009).

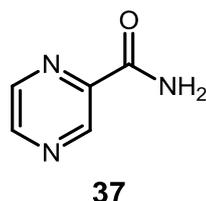


Figure 2.27: Pyrazinamide **37**.

Ethambutol

Ethambutol **38** was developed in 1961 and has been used in the first line treatment of TB, especially in the first two months of the treatment. It is a bacteriostatic agent active against both intracellular and extracellular growing bacilli (Shakya et al., 2012). Ethambutol acts by inhibiting the polymerisation of arabinose units into arabinogalactan and therefore formation of the cell wall. Arabinogalactan is an important component of the bacilli cell wall and its polymerisation is facilitated by arabinosyltransferase encoded by the *embB* gene. Ethambutol therefore inhibits the function of arabinosyltransferase (Mikusová et al., 1995). Most of the resistance to ethambutol has been associated with the mutations of the operon on the *embB* gene both *in vitro* and *in vivo* in clinical isolates (Plinke et al., 2011). Mutation on the *embB306* is the most prevalent and is found in most resistant strains. Some strains do not develop this mutation (Sreevatsan et al., 1997, Perdigão et al., 2009).

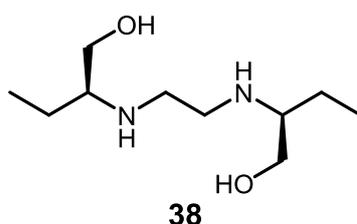


Figure 2.28: Ethambutol **38**.

Ethambutol is well-tolerated and the serum concentration reached at 2-4 h and the half-life is 3-4 h. Most of the drug is excreted in the urine (50-80%) with 20% unchanged. Side effects associated with ethambutol are dose- and time-dependent and occur at a dose >15 mg/kg. Most common side effects include red-green colour blindness, neuropathy, retrobulbar neuritis, fever and rash (Peloquin et al., 1999).

2.3.9. Second-line anti-TB drugs

2.3.9.1. Group 2 drugs

Aminoglycosides

The aminoglycosides play an important role for the treatment of tuberculosis especially the treatment of MDR-TB (Caminero and Scardigli, 2015). The aminoglycosides act by binding to the 30S ribosomal subunit and inhibit the translation of proteins. This class is based on aminoglycosides such as streptomycin **39**, amikacin **40**, kanamycin **41** and capreomycin **42**. Streptomycin, discovered in the 1940s, has the same effect as ethambutol when used in combination with other first-line drugs but because of its poor absorption and toxicity it is not commonly used (Kolyva and Karakousis, 2012). Also, streptomycin is rarely used for treatment of MDR-TB patients because of high resistance in some countries. Amikacin and kanamycin are therefore used in such cases. Capreomycin has been found to induce less ototoxicity than amikacin and kanamycin and it is used in preference to the latter drugs. Capreomycin is more expensive than amikacin and kanamycin and this may limit its use (Sturdy et al., 2011, WHO, 2014). Group 2 drugs are administered by intramuscular injection or by slow intravenous administration.

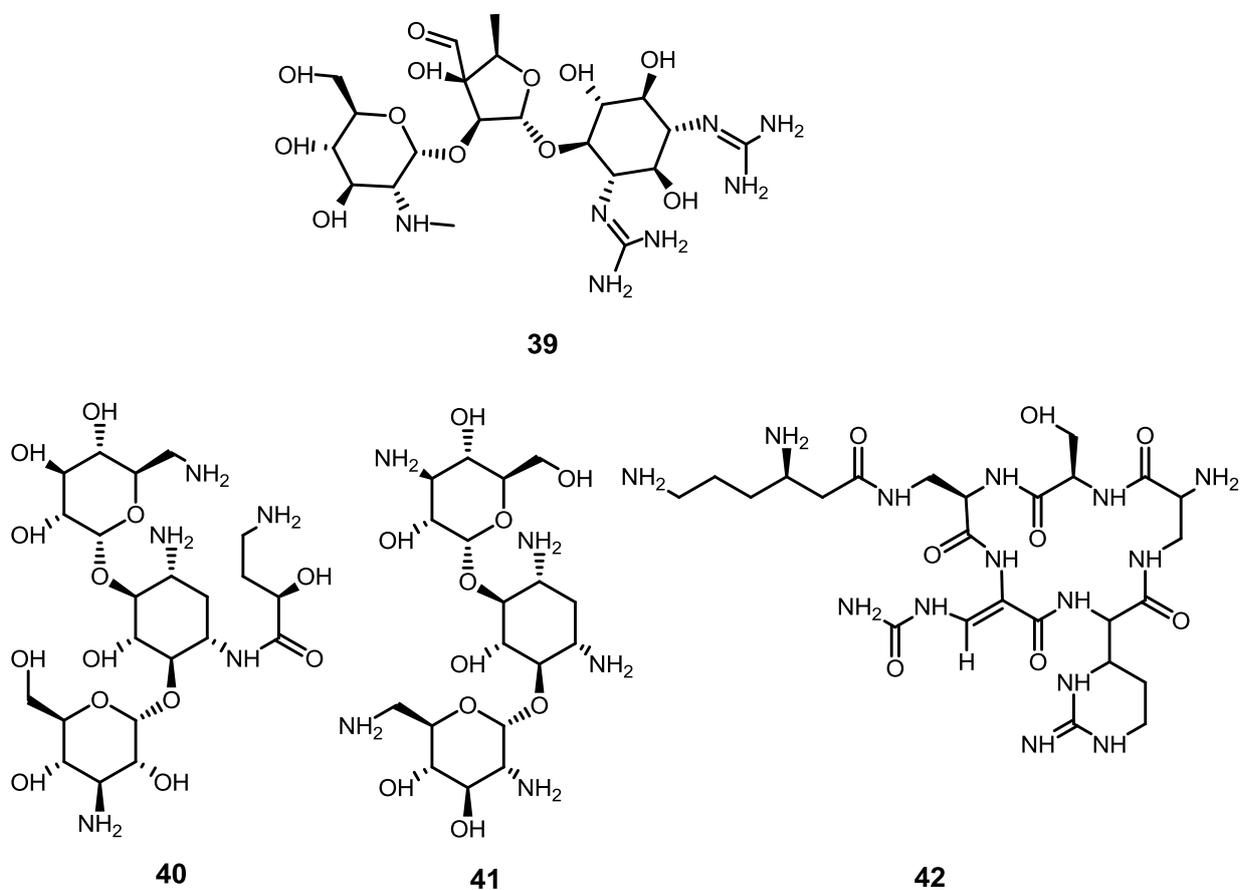


Figure 2.29: Streptomycin **39**, amikacin **40**, kanamycin **41** and capreomycin **42**.

Resistance to streptomycin is well documented and it is due to the mutation K43R on the *rpsL* gene which encodes the protein S12 and the *rrs* mutations around region 530 or 912 in 16S rRNA (Springer et al., 2001, Honore and Cole, 1994). Mutation on the *gidB* encoding 16S rRNA methyltransferase has also been implicated in low level resistance (Wong et al., 2011b). It has been found that there is no cross-resistance between streptomycin and other aminoglycosides in group 2. However, cross-resistance occurs for amikacin and kanamycin due to high structural similarities (Jugheli et al., 2009). Capreomycin does not appear to have cross-resistance with amikacin and kanamycin and its resistance is conferred by the *tylA* gene (Maus et al., 2005, WHO, 2014). Streptomycin is recommended for use in cases where resistance to amikacin and kanamycin occurs and capreomycin cannot be used. Aminoglycosides are known to induce ototoxicity and nephrotoxicity and their extended use requires monitoring (Sturdy et al., 2011, Mingeot-Leclercq and Tulkens, 1999).

2.3.9.2. Group 3 drugs

Fluoroquinolones

Fluoroquinolones are important bacteriostatic second-line drugs that act by inhibiting the activity of DNA gyrase and topoisomerase II in the *Mtb* by increasing DNA breaks. These drugs act by blocking the movement of forks and transcription complexes. The inability of the bacterium to synthesise DNA eventually leads to cell death (Blower et al., 2016). Fluoroquinolones currently used in the treatment of MDR-TB are ofloxacin **43**, levofloxacin **44**, gatifloxacin **45** and moxifloxacin **46**. Ofloxacin is a racemic mixture consisting of equal amounts of the (*R*)-enantiomer dextroflaxacin and the (*S*)-enantiomer levofloxacin **44**; it is the (*S*)-enantiomer levofloxacin that has the higher activity (Yew et al., 2003). For treatment of MDR-TB it is strongly advised that levofloxacin or moxifloxacin be used (WHO, 2014, Maruri et al., 2012, Alangaden et al., 1995).

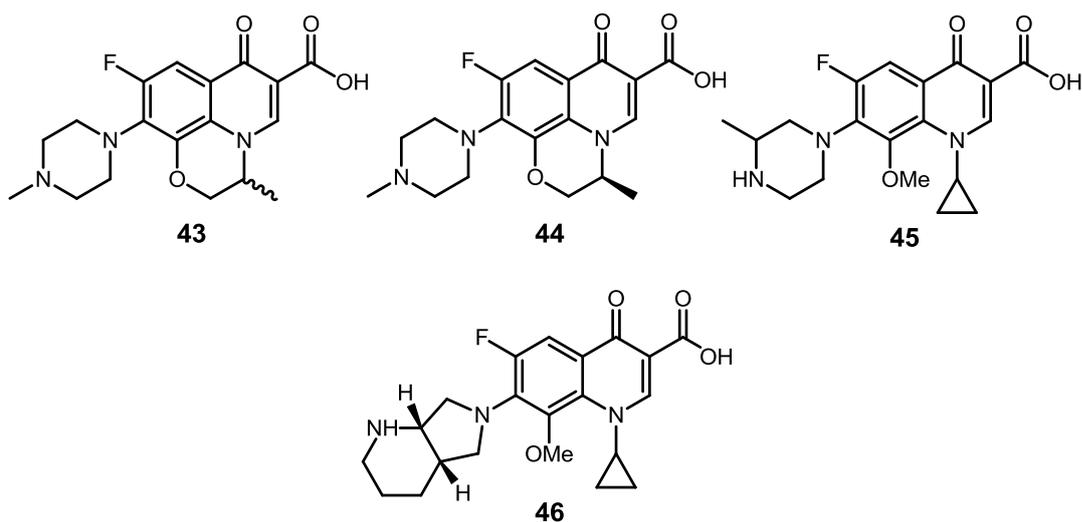


Figure 2.30: Ofloxacin **43**, levofloxacin **44**, gatifloxacin **45** and moxifloxacin **46**.

Most of the resistance to fluoroquinolones appear in the quinolone resistance-determining region (QRDR) on *gyrA* and *gyrB*. The QRDR is required for effective binding of the drug to the gyrase which, as recently shown, requires stability for translating the activity of fluoroquinolones. Instability leads to weakened drug-gyrase interaction. High-level resistance is mostly due to multiple mutations on gyrase A or concurrent mutations on gyrase A and gyrase B. The most common mutations from clinical isolates (42-85%) are on gyrase A in the region Ala-90 and Asp-94 (Maruri et al., 2012, Alangaden et al., 1995). Full cross-resistance exist among the group 2 fluoroquinolones (Kolyva and Karakousis, 2012). Fluoroquinolones induce prolonged QT (especially moxifloxacin and gatifloxacin), nephrotoxicity, and central nervous system toxicity in the elderly, tendonitis, phototoxicity and hepatotoxicity (Stahlmann and Lode, 2010). In children these adverse reactions are mild (Thee et al., 2015).

2.3.9.3. Group 4 drugs

Oral bacteriostatic

Thioamide drugs

Ethionamide **47** and prothionamide **48** are thioamide drugs that can be used interchangeably for the treatment of MDR-TB because of their structural similarity (WHO, 2014). They only differ by an additional methyl group in the 2 position of the pyridine ring of prothionamide. These compounds are structurally related to isoniazid and it has been found that they also act by inhibiting the *inhA* of the *Mtb*. They therefore interfere with the fatty acid synthesis in the *Mtb* and ultimately the mycolic acids synthesis. However, unlike isoniazid which is activated by KatG, these drugs are activated by a flavin-dependent monooxygenase EthA encoded by the *ethA* gene. The activated radicals of these drugs are proposed to form covalent adducts with

the NAD (ethionamide/prothionamide-NAD adducts) in *Mtb*. It is these adducts that bind to the inhA and therefore inhibit its activity (Mikusová et al., 1995).

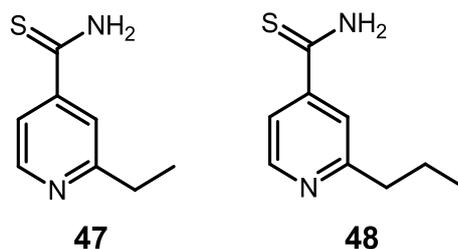


Figure 2.31: Ethionamide **47** and prothionamide **48**.

Thioamides are well-tolerated but the common adverse reactions following drug treatment are CNS toxicity, gastrointestinal intolerance and hepatitis. Cross-resistance with isoniazid has been established and it has been proposed to be largely due to the overexpression of the inhA and not mutations on the activating enzymes (Larsen et al., 2002). It has been observed that the C-15T mutation on the promoter region and the S94A as well as the I194T mutations confer cross-resistance to ethionamide and isoniazid. Implications of these mutations is that the ethionamide/prothionamide-NAD adducts have reduced affinity for the inhA and therefore fail to inhibit this enzyme (Machado et al., 2013). Furthermore, mutation in the *ethA* gene leads to failure to form the ethionamide metabolite required for binding NAD and therefore leads to resistance to the drug (Morlock et al., 2003).

Cycloserine

Cycloserine **49** is an analogue of D-alanine (D-Ala) **50** which is important for biosynthesis of peptidoglycan, an important cell wall building block of *Mtb*. Two molecules of D-Ala are ligated by D-alanine:D-alanine ligase (Ddl) in an ATP-driven ligation to form the D-alanyl:D-alanine dipeptide essential for peptidoglycan biosynthesis. Also important is the D-alanine racemase (AlrA) that converts L-alanine to D-alanine thus providing substrates for Ddl (Lambert and Neuhaus, 1972). Cycloserine is a competitive inhibitor of the Ddl, competing with D-Ala for binding site. Cycloserine therefore inhibits the function of Ddl and leads to the failure to synthesise peptidoglycan (Bruning et al., 2011). However, cycloserine has more adverse effects than other second-line drugs. To improve this, two cycloserine molecules have been linked together through terephthalaldehyde to form terizidone **51** which is better tolerated than cycloserine (Vora, 2010). Terizidone acts through the same mechanism as cycloserine but it has limited effectiveness to date. The adverse events associated with these drugs, especially cycloserine, are psychosis, CNS toxicity, and dizziness. Use of cycloserine is also associated

with suicidal ideations (Hwang et al., 2013). There is no data for definite resistant markers for cycloserine resistance in *Mtb*. However, studies in *M. smegmatis* and *M. bovis* BCG have shown that overexpression of AlrA confers resistance to cycloserine which is likely to cause resistance in *Mtb* (Caceres et al., 1997).

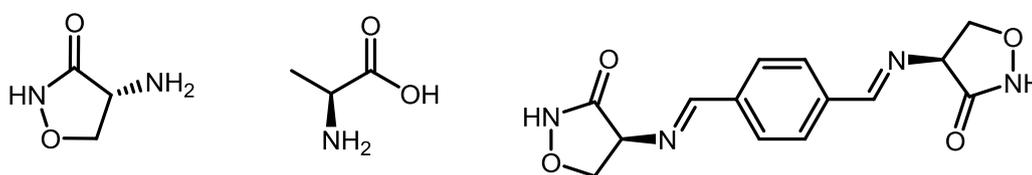


Figure 2.32: Cycloserine **49**, D-alanine **50** and terizidone **51**.

p-Aminosalicylic Acid

p-Aminosalicylic acid **52** was the second antibacterial to be incorporated into the treatment regimen of TB in 1946. It was used as first-line drug but was later used only as a second-line drug following availability of more effective anti-TB drugs in the 1960s (O'Connor, 1948). *p*-Aminosalicylic acid acts as an analogue of *p*-aminobenzoic acid in the folate metabolic pathway. Folate metabolism in *Mtb* yields tetrahydrofolate that plays a crucial role in the synthesis of formylmethionyl tRNA^{fMet} required for initiating protein synthesis (Zheng et al., 2013). *p*-Aminosalicylic acid interacts with the DHPS in this pathway to form hydroxy dihydrofolate. This ultimately leads to inhibition of enzymatic activity of DHFR (Figure 2.34).

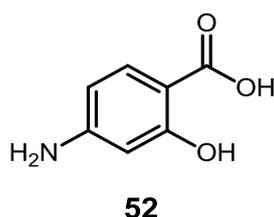


Figure 2.33: *p*-Aminosalicylic Acid **52**.

Resistance to *p*-aminosalicylic acid has been proposed to be due to the missense mutations in the *folC* gene encoding DHFS and this prevents bioactivation of *p*-aminosalicylic acid to hydroxy dihydrofolate (Zhao et al., 2014). Mutations on the *thyA* gene encoding thymidylate synthase required for thymine synthesis in the folate pathway are also implicated in *p*-aminosalicylic acid resistance. However, it has been found that there are mutations contributing to *p*-aminosalicylic acid resistance other than the *thyA* gene (Mathys et al., 2009). In a recent study, overexpression of RibD, the putative analogue of DHFS, was found to confer resistance to *p*-aminosalicylic acid both in the laboratory strains and clinical isolates (Zheng et al., 2013). *p*-

Aminosalicylic acid is recommended for treatment of MDR-TB with or without cycloserine because it does not have cross-resistance with other anti-TB drugs. However, *p*-aminosalicylic acid is not used with ethionamide or prothionamide because of high incidence of gastrointestinal intolerance and hypothyroidism (WHO, 2014).

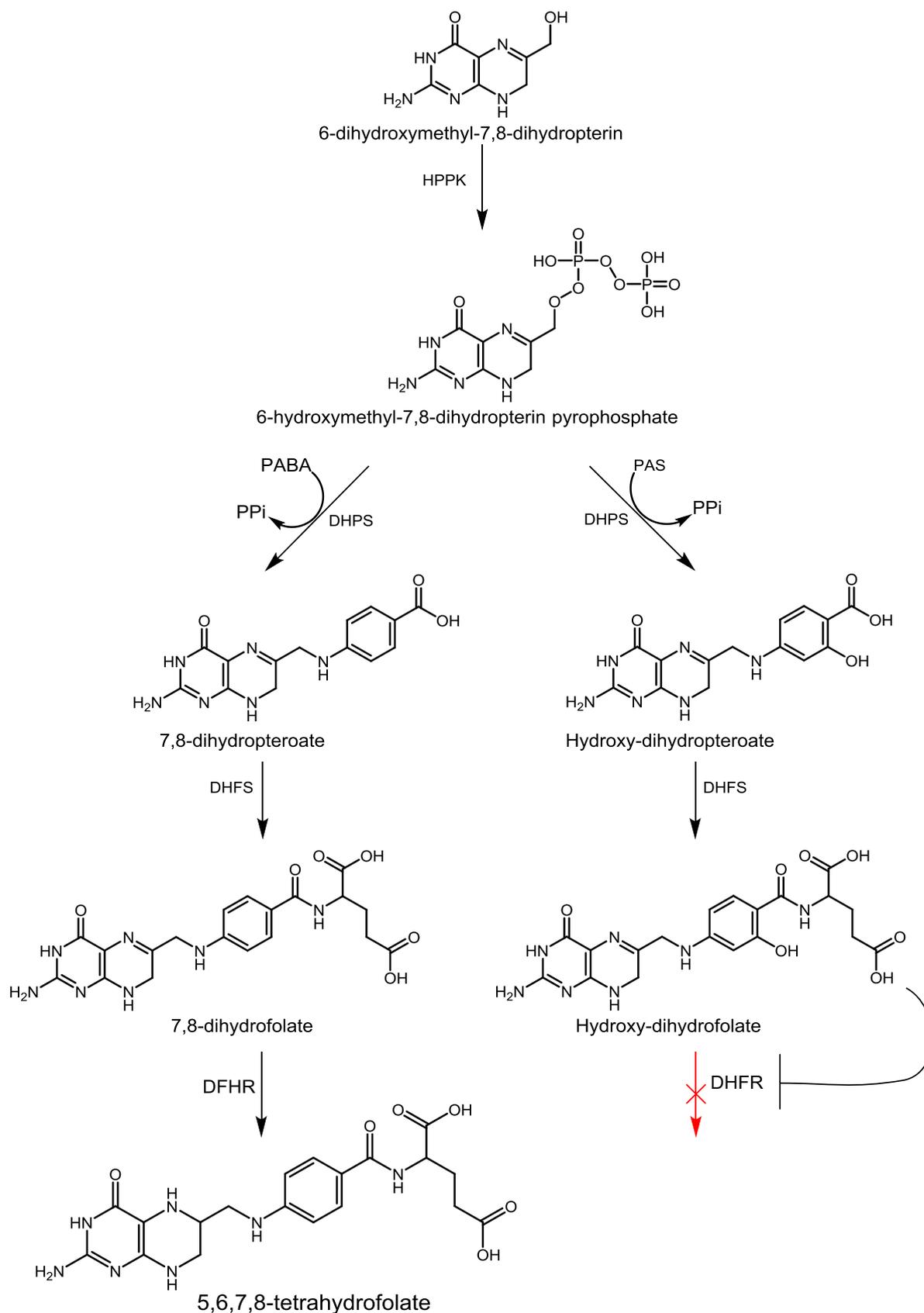


Figure 2.34: Biosynthesis of tetrahydrofolate and inhibition by *p*-aminosalicylic acid. Abbreviations: PABA, *p*-aminobenzoic acid; PAS, *p*-aminosalicylic acid; DHPS, dihydropteroate synthase; DHFS, dihydrofolate synthase; DFHR, dihydrofolate reductase (Zheng et al., 2013).

2.3.9.4. Group 5 drugs

Drugs in this group are either in their late clinical stages or have only received conditional approval for inclusion into the MDR-TB and XDR-TB treatment regimens. They are not recommended by the WHO but their inclusion into the treatment regimen requires expert recommendation. Their efficacy and safety data from different studies is varying and no concrete findings are available. Due to an urgent need for treatment of MDR-TB and XDR-TB, these drugs are used (Zhang et al., 2014). Some of them are expensive and have not been registered and are used “off-label” in treatment (WHO, 2014).

Linezolid

Linezolid **53** is an oxazolidinone used for treatment of MDR-TB and has shown activity against XDR-TB *in vitro* and *in vivo* (Grard et al., 2015). A study in XDR-TB infected patients showed that linezolid treatment improves the condition of this pulmonary infection (Zhang et al., 2014). Linezolid acts by inhibiting an initiation of protein synthesis by binding onto 23S rRNA on the 50S subunit. It is associated with irreversible side effects such as lactic acidosis, myelosuppression, neuropathy and hematological disturbances such as leukopenia and thrombocytopenia (Di Paolo et al., 2010). In the event of emergence of one or more of these side effects, it is recommended that an expert opinion be sought or treatment be stopped or the dose be reduced from 600 mg to 300 mg daily (WHO, 2014). Resistance has not fully been elucidated but *Mtb* strains with G2061S and G2576T mutations have demonstrated reduced susceptibility. It has also been found that the ribosomal L3 protein mutation due to mutation on the rplC gene at T460C is the dominant mutation conferring resistance to linezolid (Beckert et al., 2012).

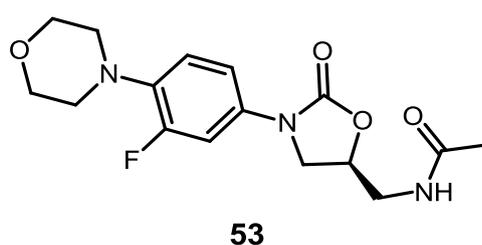


Figure 2.35: Linezolid **53**.

Clofazimine

Commonly used for treatment of leprosy, clofazimine **54** has long been known to possess anti-TB efficacy but was side-lined at a surge of potent antitubercular agents in the 1950s (Cholo et al., 2011). It is a riminophenazine that has been repurposed for treatment of MDR-TB (Tang et

al., 2015, Tyagi et al., 2015). Clofazimine acts by diverting electron supply to menaquinone in the electron transport chain involving the crucial respiratory flavoenzyme NADH dehydrogenase (NDH-2) of *Mtb*. Thereby the clofazimine is reduced. The reduced clofazimine is reoxidized to clofazimine by molecular oxygen thereby generating ROS that are also cytotoxic (Lechartier and Cole, 2015, Yano et al., 2011). Resistance to clofazimine is potentially conferred by mutations in the Rv0678 inducing upregulation of MmpL5. MmpL5 is a multisubstrate efflux pump that also confers resistance to bedaquiline. Therefore this suggests that cross-resistance between clofazimine and bedaquiline may exist (Hartkoorn et al., 2014). The most common adverse effect associated with clofazimine is skin colourisation occurring in 75-100% of patients with slow recovery requiring months or years after clofazimine treatment (WHO, 2014).

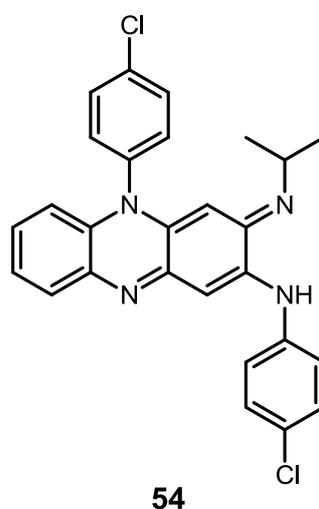


Figure 2.36: Clofazimine **54**.

Bedaquiline

Bedaquiline **55** is one of the two drugs that have recently been approved for TB therapy after over 40 years. It received USA Food and Drug Administration (FDA) accelerated approval in 2012 for use in the treatment of MDR-TB (Cohen, 2013). It was discovered from a whole-cell assay wherein it was found that diarylquinolines had activity against *M. smegmatis* and *M. tuberculosis* H37Rv (MIC < 0.5 µg/mL). It was found in this work that bedaquiline was more bactericidal than the first-line drugs rifampicin, isoniazid and pyrazinamide. *In vivo* studies in murine models revealed that bedaquiline was more effective than the combination of the first-line drugs and that its combination with these drugs led to an increased bactericidal effect. This was more pronounced in the combinations bedaquiline-isoniazid-pyrazinamide and rifampicin-bedaquiline-pyrazinamide with complete bactericidal activity achieved within 1 month of therapy. The pharmacokinetic studies revealed that bedaquiline is well distributed in tissues, lungs and spleen and, reached maximum plasma concentration in 5 h. Bedaquiline has a long half-life

likely due to slow release from tissue (Andries et al., 2005). This implies that the required doses of bedaquiline will be low. Human studies have shown that bedaquiline is well tolerated despite the phase II studies showing that there were more deaths in the bedaquiline treated individuals than those of the placebo control (Diacon et al., 2014).

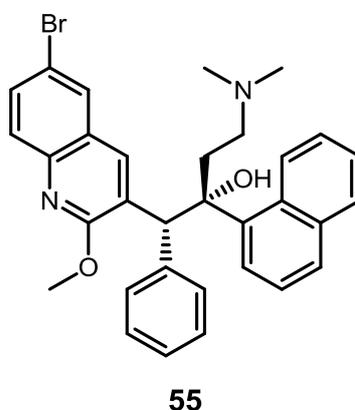


Figure 2.37: Bedaquiline **55**.

Bedaquiline has a different MoA compared to other anti-TB drugs with implications that this drug will not have cross-resistance. It has been shown that bedaquiline acts by inhibiting the proton pump of the ATP synthase. Detailed docking studies and biochemical assays have also shown that bedaquiline acts by interfering with the rotary movement of the subunit c of the ATP synthase. This is likely due to the possibility that bedaquiline mimics arginine 186 involved in the proton transfer chain, thus leading to inhibition of ATP synthase (Haagsma et al., 2011). Resistance to bedaquiline was proposed to be due to the mutation at A63P and I66M in the *atpE* gene encoding subunit C of ATP synthase (Haagsma et al., 2009). The clinical and safety data of bedaquiline are still to be completed, and this situation limits its potential use as a first-line treatment.

Delamanid

Delamanid **56**, like bedaquiline, has recently been approved for use in treatment of MDR-TB by the European Union in 2014 (Ryan and Lo, 2014). Delamanid shows good *in vitro* and *in vivo* anti-TB potency and current clinical data are promising. It is a nitro-dihydro-imidazo-oxazole which requires reduction of the nitro group by deazaflavin dependent nitroreductase (Rv3547) to form an active metabolite that release nitric oxide. As delamanid does not interact with CYP enzymes, it can be used effectively in combination therapies (Matsumoto et al., 2006). Moreover, the combination regimens with the first-line drugs appears to be synergistic both from the clinical and preclinical studies. These studies have also shown that delamanid combination therapies shorten the treatment period. Combination treatment with delamanid and the WHO background drug regimen induced significant and short sputum-culture clearance (2 months) in

treated patients than in the control group treated with conventional drugs (Gler et al., 2012). This confirmed preclinical study that showed rapid bacterial clearance in combination regimen with first-line drugs. Delamanid alone is more active than first-line drugs, and also has activity against intracellular bacilli with implications for treatment of LTBI (Matsumoto et al., 2006). Clinical studies involving XDR-TB patients also had high sputum-culture conversion compared to the control group. It was also established that a treatment of >6 months was more successful with less mortality compared to that of <2 months (Skripconoka et al., 2013, Gupta et al., 2015).

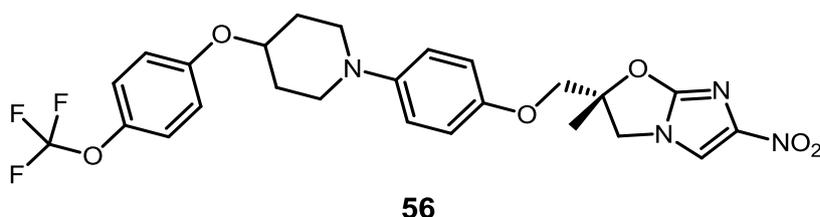


Figure 2.38: Delamanid **56**.

Delamanid is well-tolerated, although patients had more episodes of QT prolongation than placebo patients albeit in a dose dependent manner. An effect was more pronounced in the 200 mg/day group compared to the 100 mg/day group (Gler et al., 2012). Delamanid bioavailability is enhanced by food and it reaches plasma concentration within 4-5 h with a half-life of about 30-38 h. The currently recommended regimen is 100 mg x 2 daily for 24 months (Ryan and Lo, 2014). *In vivo* resistance will likely arise due to mutation in the Rv3547 as seen in an experimental model (Matsumoto et al., 2006). Significant safety and efficacy data are urgently required if delamanid is to be used for treatment of MDR-TB and XDR-TB.

SQ109

SQ109 **57** was discovered from a high-throughput screening of 63 238 compounds that was aimed to develop 1,2-ethylenediamine analogues of ethambutol. SQ109 is active against drug-sensitive and drug-resistant *Mtb* strains (Sequella Incorporated, 2016). SQ109 was the most active compound with a MIC range of 0.7-1.56 μ M against H37Rv *Mtb* strains. SQ109 was also 14-35 fold more active *in vitro* than ethambutol. It had 99% inhibition against intracellular bacilli and was also active *in vivo* in mice model, especially in the lungs (Lee et al., 2003, Protopopova et al., 2005). SQ109 acts by inhibiting the MmpL3 transmembrane transporter. MmpL3 transports trehalose monomycolate required for biosynthesis of trehalose dimycolate involved in mycolates and arabinogalactan assembly. Thus inhibition of this important step interferes with the biosynthesis of the cell wall (Tahlan et al., 2012, Li et al., 2014). SQ109 undergoes a first-pass effect and is metabolised by CYP2D6 and CYP2C19 through oxidation, epoxidation and *N*-dealkylation (Jia et al., 2006). It has been found that SQ109 act synergistically with isoniazid and rifampicin with the potential to shorten the treatment period (Chen et al., 2006, Sacksteder

et al., 2012). The Phase I and Phase II clinical studies have shown that SQ109 is safe and well-tolerated (Sacksteder et al., 2012). SQ109 received FDA fast-track approval in 2007 and phase III clinical studies are still in progress (Sequella Incorporated, 2016). SQ109 is aimed to replace ethambutol in the first-line drugs and is expected to be an efficient second-line drug for treatment of MDR-TB (Sacksteder et al., 2012).

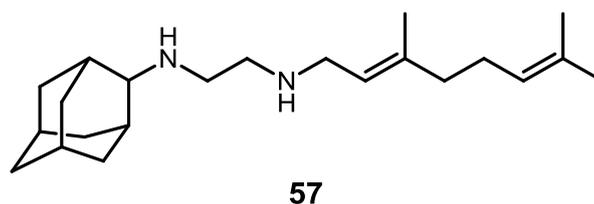


Figure 2.39: SQ109 **57**.

Pretomanid

Pretomanid or PA-824 **58** is a nitroimidazole in phase II clinical trials with potential to eliminate dormant bacilli in LTBI (Zumla et al., 2014). Its MoA resembles that of delamanid and it is also activated into an active metabolite by deazaflavin (cofactor F₄₂₀) dependent nitroreductase. The MoA proceeds via way of release of NO via reduction of the nitro group to nitronate that is hydrolysed to generate NO. Under hypoxic conditions PA-824 in releasing NO perturbs the respiratory complex as evidenced by reduced ATP production (Manjunatha et al., 2009, Lenaerts et al., 2005, Singh et al., 2008). *In vitro* and *in vivo* studies have shown that PA-824 is active against susceptible and multidrug-resistant *Mtb* strains (Somasundaram et al., 2013, Tyagi et al., 2005). Since PA-824 has a rapid bactericidal effect, it is not clear whether its combination with first-line drugs will shorten the treatment period or not (Nueremberger et al., 2006). However, a 14-days study in South African patients showed that PA-824 is highly active when combined with moxifloxacin and pyrazinamide. PA-824 was also found to be safe and well-tolerated in healthy volunteers (Diacon et al., 2012, Ginsberg et al., 2009). Not surprisingly, it has cross-resistance with delamanid and mutations *fbtA*, *fbtB* and *fbtC* affect cofactor F₄₂₀ synthesis and confer resistance (Haver et al., 2015, Manjunatha et al., 2006).

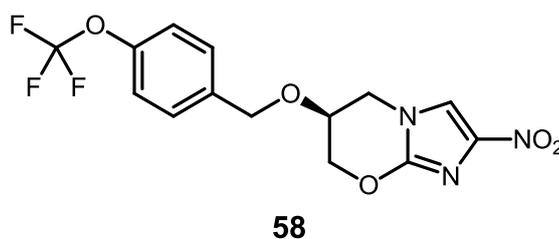


Figure 2.40: PA-824 **58**.

2.4. Rational design

Reports of decreasing artemisinin efficacy are daunting given that there are no new drugs against malaria and those recently discovered for TB lack compelling data for effective use. This implies that artemisinins need to be protected if their usefulness is to be preserved. Their limited efficacy against some stages of the malaria parasite requires improvement and activity against TB need thorough evaluation. The mycobactin-artemisinin conjugate **59** has been reported and this showed potency against both malaria and TB. The mycobactin portion of the conjugate chelates iron and transports it into the parasite where it supposedly bio-activate the artemisinin by interacting with the peroxide. This leads to the generation of oxygen-based free radicals which are proposed to be responsible for parasite death (Miller et al., 2011).

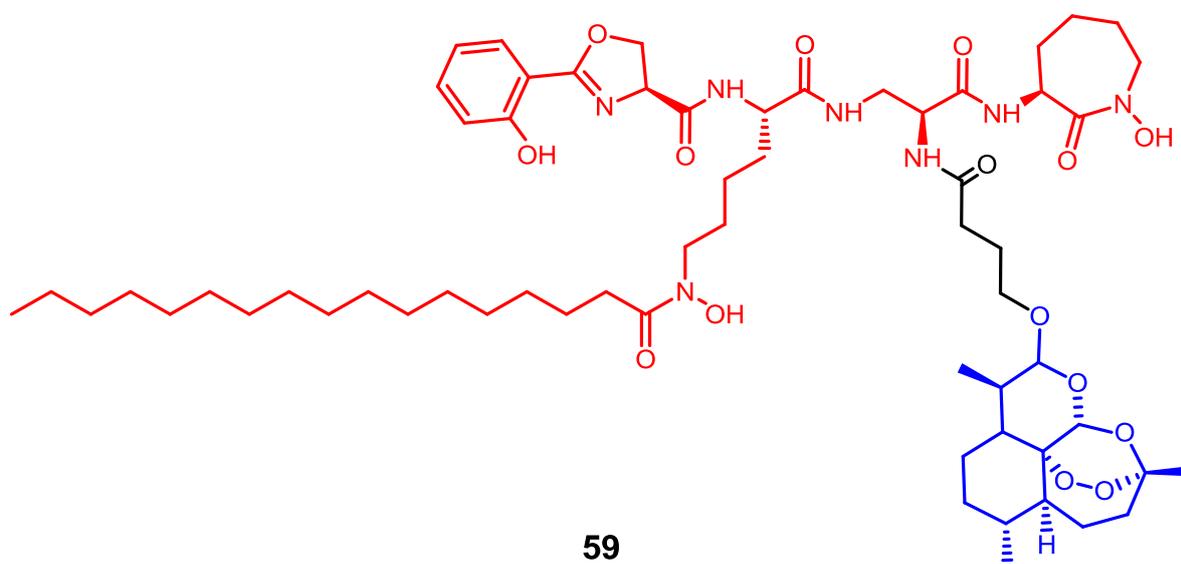


Figure 2.41: The mycobactin-artemisinin conjugate **59**. Mycobactin part (red) and artemisinin portion (blue).

Since cholesterol is needed by both malaria parasite and *Mtb* this molecule presents an important convergent point for drug development. The malaria parasite is a cholesterol auxotroph and continuously diverts newly synthesised cholesterol from the liver and LDL to support its development. It also takes up and incorporates cholesterol from the erythrocyte membrane into its parasitophorous vacuole membrane during invasion of erythrocytes in the blood stage of development (Tokumasu et al., 2014, Labaied et al., 2011, Bano et al., 2007). Cholesterol drives the formation of lipid rafts in the parasite and forms other channels vital for the passage of nutrients. On the other hand *Mtb* uses cholesterol as a carbon source during biosynthesis of molecules required for growth. The presence of the Mce4 transporter allows for efficient transport of cholesterol into the cell (Ouellet et al., 2011).

Given that *Mtb* also has an active redox homeostasis system, the lack of activity of artemisinins may be due to the fact that these drugs are not able to traverse the lipophilic *Mtb* cell wall.

Therefore enhancing the transport of these drugs into *Mtb* will likely improve their activity. Targeting Mce4 to transport artemisinin during cholesterol shuttling may improve the efficacy of artemisinins against *Mtb*. This will allow for the drug to interact with the intracellular redox homeostasis machinery in *Mtb*. In malaria, artemisinins act against all parasite forms during the erythrocyte stage of development. Enhancing their activity in the liver stage will greatly assist in halting early stage of parasite development. Therefore, artemisinin-cholesterol conjugates may be active at this stage as an uptake of cholesterol by the parasite will also incorporate the artemisinin. Furthermore, by enhancing their activity in the erythrocytes will prevent the development and transmission of gametocytes. Incorporation of artemisinin-bearing cholesterol molecules into the parasitophorous membrane will enhance the bio-availability of artemisinin for interaction with intracellular targets. This exposure will cause artemisinin to maintain elevated levels of ROS thus leading to parasite death. It is anticipated that these conjugates will remain intact throughout the circulation until they reach the site of action. Thus, it is hoped that the variation in linkers will assist in this regard. Therefore, for this work artemisinin-cholesterol conjugates incorporating linkers of varying chain lengths will be synthesised. The next chapter, chapter 3, is a manuscript for publication and covers the details of the synthetic, biological and analytical work of these conjugates. The chapter also elaborates on the major outcomes of the study and discusses them in detail. Proposed future work is also described.

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CHAPTER 3

ARTICLE FOR SUBMISSION

Chapter 3 contains the manuscript of an article to be submitted to the European Journal of Medicinal Chemistry. The article contains the background, aim, results, *in vitro* biological results and experimental details of synthesised compounds of this study. This article is prepared according to the author's guidelines available in the Author information pack at the journal homepage (Annexure B): <http://www.journals.elsevier.com/european-journal-of-medicinal-chemistry/>

Preliminary evaluation of artemisinin-cholesterol conjugates as potential drugs for treatment of intractable forms of malaria and tuberculosis

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Abstract

In order to evaluate the feasibility of developing drugs that may be active against both malaria and tuberculosis (TB) by utilising in part putative cholesterol transporters in the causative pathogens and through enhancement of passive diffusion in granulomatous TB, artemisinin-cholesterol conjugates were synthesised by connecting the component molecules through various linkers. The compounds were screened *in vitro* against *Plasmodium falciparum* (*Pf*) and *Mycobacterium tuberculosis* (*Mtb*). Antimalarial activities (IC_{50}) against *Pf* chloroquine (CQ) CQ-sensitive NF54, and CQ-resistant K1 and W2 strains ranged from 0.03 – 2.6, 0.03 – 1.9 and 0.02 – 1.7 μ M. Although the compounds are less active than the precursor artemisinin derivatives, the cholesterol moiety renders the compounds relatively insoluble in the culture medium, and variation in solubilities among the different compounds may reflect in the range of efficacies observed. Activities against *Mtb* H37Rv were assessed using a standardised CFU assay after 24 h pre-treatment of cultures with each of the compounds. Percentage inhibition ranged from 3 – 38% and 18 – 52% at 10 and 80 μ M, respectively. Thus, the conjugates do display appreciable activities against the respective pathogens. The immediate aims include the preparation of conjugates with enhanced aqueous solubilities, assays against malaria and TB *in vivo*, and for TB, assays using an infected macrophage model and assessment of granuloma influx.

Keywords: Malaria, tuberculosis, artemisinins, cholesterol, conjugates

Introduction

Malaria and tuberculosis (TB) are two of the most lethal infectious diseases of our time, and continue to overburden the resource-limited regions of the world. For malaria, in 2014 alone, 214 million cases and 438,000 deaths due to malaria are estimated to have occurred. The majority of cases (88%) and deaths (90%) occurred in the African region. In that same year, at least 306,000 children under the age of five died of malaria, of which 292,000 were in the African region [1]. Notwithstanding this, mortality appears to be decreasing given that in 2013 584,000 deaths were estimated compared to 438,000 in 2014. TB has recently surpassed human immunodeficiency virus (HIV) as a leading cause of death due to infectious disease [2]. In 2015, 1.4 million deaths due to TB and 10.4 million new TB cases were estimated to have occurred on a global scale. Most of the cases were in the South-East Asia (61%) and Africa (26%). Africa also accounted for most of the HIV/TB coinfections [3]. This region is also notorious for the relative number of TB cases with 288 TB cases recorded per 100,000 people compared to 133 TB cases per 100,000 people on a global basis in 2015 [4].

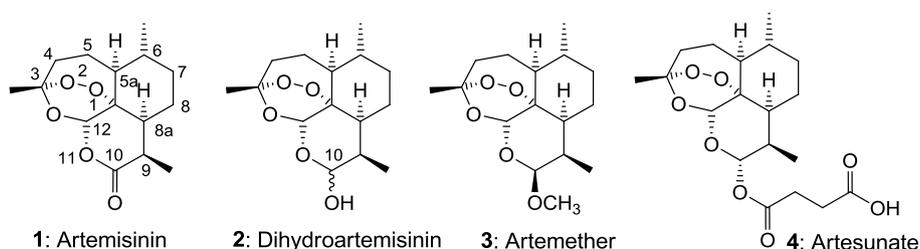


Figure 1: Artemisinin **1** and its current clinical derivatives, the hemiacetal dihydroartemisinin (DHA) **2**, the lactol ether artemether **3** and hemiester artesunate **4**.

Although the malaria parasite, in particular *P. falciparum* (*Pf*), has developed resistance to most antimalarial drugs [5, 6], reliance is now largely placed on the use of artemisinin and its derivatives (collectively called artemisinins) (Fig. 1) that are the most active antimalarial drugs currently available [7]. They are used in combination with other antimalarial drugs in artemisinin combination therapies (ACTs) [8]. ACTs were developed so as to allow the artemisinins to reduce parasite biomass while the long acting partner drug continues clearing parasites once levels of the short-lived artemisinins had dropped below therapeutically-effective levels. Nonetheless, *Pf* resistance involving ACTs is now established in South-East Asia; if the resistant phenotype enters other endemic regions, malaria control programs will be seriously compromised. Whilst TB chemotherapy usually employs drug combinations comprising rifampicin, isoniazid, pyrazinamide and ethambutol [8], multidrug-resistant (MDR-TB) strains of *Mtb* resistant to rifampicin and isoniazid have emerged. Extensively drug-resistant TB (XDR-TB) strains of *Mtb* additionally are resistant to fluoroquinolones and one or more aminoglycosides usually used as second-line drugs [4]. More alarming are recent reports of totally drug-resistant TB (TDR-TB) which is resistant to all second-line drugs [9-11]. The spread of these strains

hamper progress made thus far to combat TB. Although newer drugs such as bedaquiline and delamanid have recently received conditional approval for use in the treatment of MDR-TB [12], toxicity concerns are as yet unresolved and combination therapies are not yet available [12-14]. Overall, new TB drugs are urgently needed in order to eradicate these strains.

Artemisinin **1** appears to possess no activity against *Mtb*. However, in an elegant investigation, the dihydroartemisinin-mycobactin conjugate **5** (Fig. 2) was shown to be notably potent against *Mtb* including XDR-TB strains [15]. The lipophilic mycobactin moiety of the conjugate ligates Fe(III) which is then actively transported into the cell by the mycobactin transporter wherein the artemisinin exerts its cytotoxic effect; interestingly a strong ROS response was noted on administration, supporting the artemisinin oxidant hypothesis outlined below. Thus, provided that they can be transported into the cytosol, artemisinins are active against *Mtb*. The dissertation is supported by results of earlier work wherein it was shown that incorporation of the tetraoxane moiety into lipophilic steroidal moieties provides compounds such as **6** that is also strikingly active against *Mtb* (Fig. 2) [16]. However, cytotoxicity of the latter compound precluded its further development.

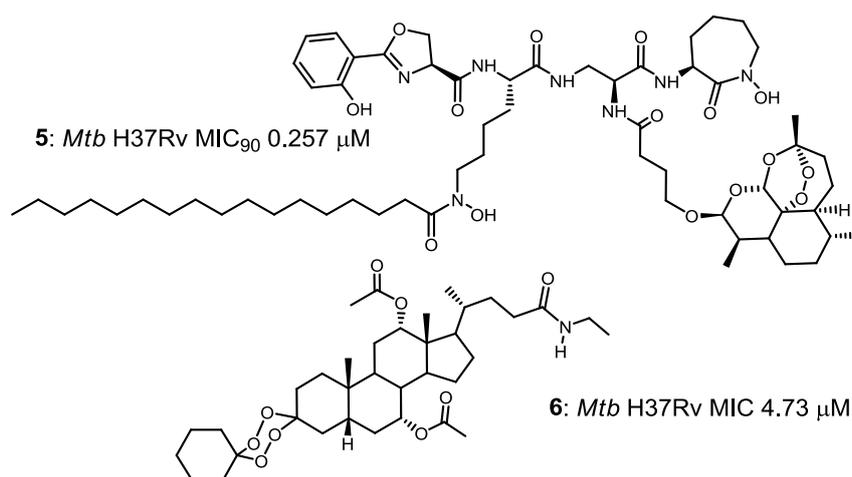


Figure 2: Artemisinins and analogues that are active against *Mtb in vitro*: the mycobactin-dihydroartemisinin conjugate **5** [15] and the steroidal tetraoxane **6** [16].

Under a programme designed to develop new triple drug combinations for the treatment of malaria, tuberculosis, and toxoplasmosis, we are preparing and evaluating efficacies of putative drug combinations which have oxidant and redox properties respectively [17] coupled with a third partner with a different mode of action. The precept is that artemisinins and other peroxides behave as *oxidants* in the intracellular milieu by rapidly oxidising reduced flavin cofactors such as FADH₂ of flavoenzyme disulfide reductases important for maintaining levels of GSH and other endogenous thiols required for intercepting reactive oxygen species (ROS) [18]. *Pf* as an aerobic organism experiences abnormally high oxidative stress within an erythrocyte [19, 20]. Thus, intraparasitic hexose monophosphate shunt (HMS) activity to generate NADPH required by flavin disulfide reductases, glutathione reductase (GR), thioredoxin reductase

(TrxR), lipoamide dehydrogenase and others is approximately 78 times higher than in normal erythrocytes. *Pf* utilises GR, TrxR and others for generation of GSH needed for detoxification of ROS. Thus, any drug that abruptly subverts redox homeostasis will have a cytotoxic effect. *Mtb* likewise is an aerobic organism and has to cope with high oxidative stress, particularly within alveolar macrophages [20, 21]. *Mtb* utilises mycothiol reductase (MR) whose substrate mycothiol (MSH) plays the same role in *Mtb* as GSH does in *Pf* [22]. In addition, ergothioneine (EGT) is an important redox-modulating molecule for *Mtb*, and indeed adequate levels of EGT appear essential to survival [22, 23]. Thus, *Pf* and *Mtb* will be susceptible to oxidant-redox active drug combinations. *Pf* and *Mtb* also contain other flavoenzymes (diaphorases) including the essential respiratory enzymes Type II NADH:quinone oxidoreductases (NDH-2) that induce electron transfer from NADH to the cofactor FAD to menaquinone in the respiratory chain [24]. These are potentially susceptible to electron-scavenging by the same redox-oxidant drug combinations. Additional drug components added to the drug mixtures are also required on the basis of enhancing efficacy through enabling activity against a different target, and suppressing emergence of resistant pathogens.

Our specific aim here is to enhance activity of the oxidant drug prior to combining it with the redox partner. Thus, we examine the effect of ligating the oxidant artemisinin to a group that assists in targeting selected stages of the malaria parasite, and in particular in the case of *Mtb* that assists in diffusion, either in a passive manner or by utilising a transporter in an active sense, across the mycolic acid barrier into the cytosol of the bacterium. In the latter case, the outer cell wall represents a formidable barrier to drug ingress, and the need to incorporate elements of structural design into drugs for penetration is well established.

Cholesterol is an important molecule for most organisms with roles such as acting as a carbon source, involvement in membrane or lipid raft formation, and in particular as a precursor for biosynthesis of vital molecules required by the organism [25, 26]. In the human liver, the malaria parasite associates with the endoplasmic reticulum (ER) in hepatocytes so that it may salvage newly synthesised lipids. The parasite also continuously diverts cholesterol from low-density lipoproteins and hepatocytes during the liver stage [27, 28]. During the intraerythrocytic stages, the cholesterol gradient is noted to decrease inwardly from the erythrocyte membrane to the parasitophorous vacuolar (PV) membrane of the parasite. Thus, the PV membrane contains cholesterol incorporated upon erythrocyte invasion. Overall, cholesterol plays a role in domain assemblies, lipid raft formation and protein trafficking in the malarial parasite [29, 30]. *Mtb* utilises cholesterol as a carbon source during its development [26, 31]. Studies with murine TB models have identified a cholesterol transporter called Mce4 which delivers cholesterol for metabolism into the bacterium. Cholesterol is initially acquired from the plasma membranes of the macrophages, and then from lipid-rich foamy macrophages. This molecule can also be acquired in insoluble crystal form from extracellular spaces [31]. One crucial issue is the ability of cholesterol to undergo passive diffusion through the granuloma into the core, wherein it is

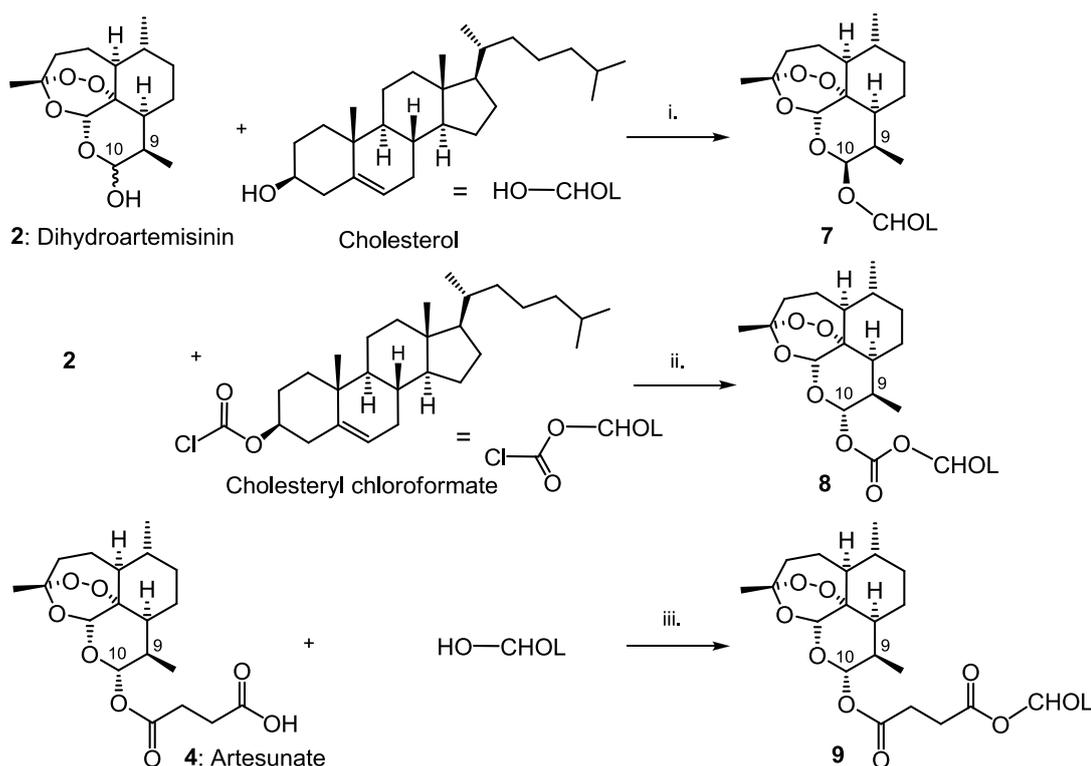
taken up by quiescent *Mtb* as an energy source *via* metabolic breakdown to methylmalonyl coenzyme A and eventual feed into the tricarboxylic acid cycle [26, 31, 32]. Overall, any drug which may penetrate a granuloma, and exert cytotoxic effects on quiescent bacteria is highly sought after, given the enormously aggregated epithelioid structure of the granuloma.

We describe here the preparation of artemisinin-cholesterol conjugates, and the results of preliminary screening *in vitro* against *Pf* and *Mtb*.

Results and Discussion

Chemistry

Three general coupling methods were used. Firstly, conjugates bearing an oxygen atom at C-10 of the artemisinin nucleus were prepared (Scheme 1). The cholesteryl lactol ether **7** was prepared by direct conjugation of cholesterol with DHA **2** according to the method used for preparing this compound and other acetals [33, 34] (Scheme 1).

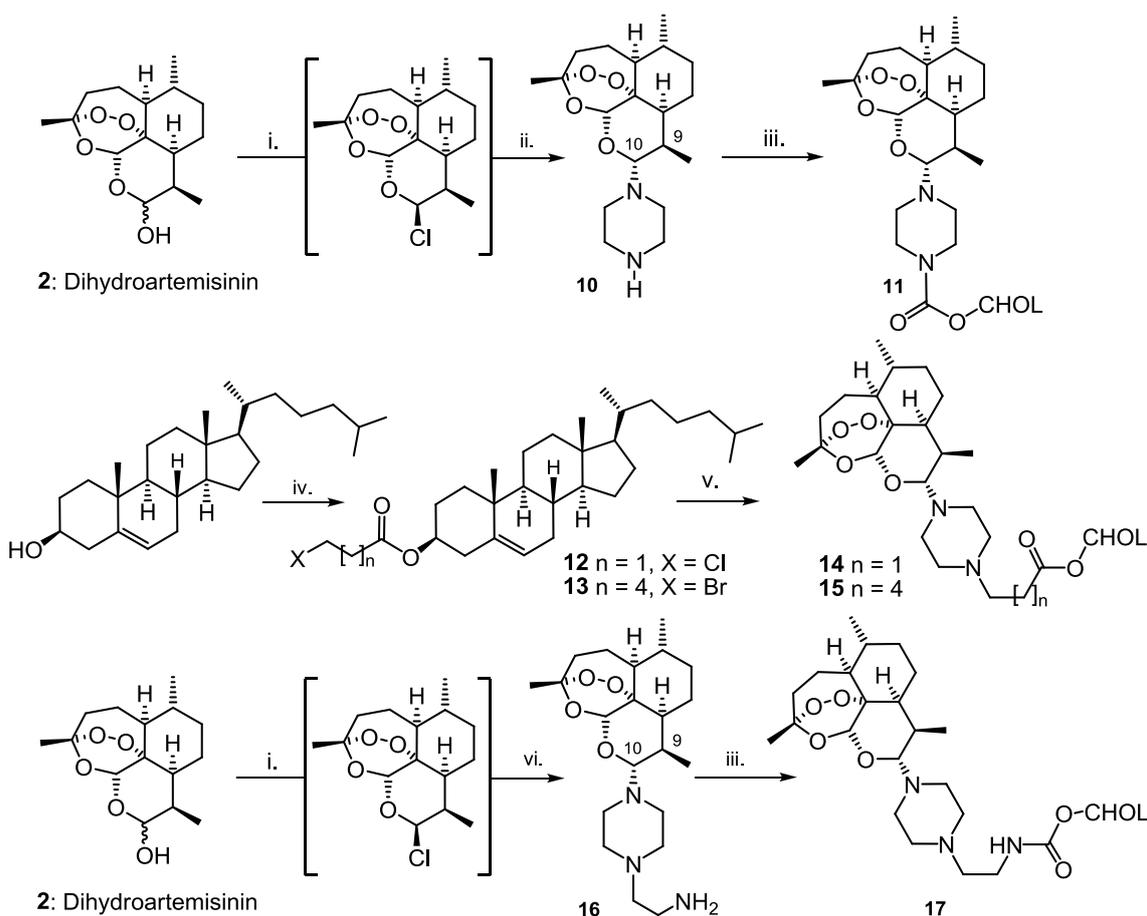


Scheme 1: Preparation dihydroartemisinin-cholesterol conjugates. i. Cholesterol (1.2 eq.), $\text{BF}_3 \cdot \text{OEt}_2$ (0.9 eq.), CH_2Cl_2 , 0°C to room temperature, overnight; ii. Cholesteryl chloroformate (1.2 eq.), 4-N,N-dimethylaminopyridine (0.1 eq.), triethylamine (1.1 eq.), CH_2Cl_2 , room temperature, 18 h; iii. Cholesteryl chloroformate (1 eq.), DCC (1 eq.), 4-N,N-dimethylaminopyridine (0.2 eq.), room temperature, 4 h.

The β -configuration at C-10 in **7** is secured through the ^1H NMR spectrum displaying a vicinal

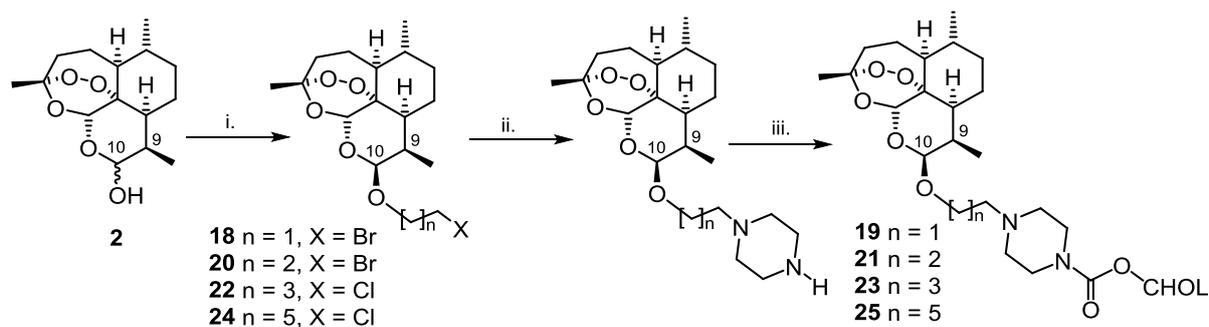
coupling of 5.1 Hz between H-9 and H-10 indicating syn axial-equatorial coupling. The carbonate **8** was obtained in moderate yield (45%) following esterification of cholesteryl chloroformate with **2** under standard basic conditions. Given that DHA acts as the nucleophile, the equilibrating mixture of DHA epimers react only via the α -epimer, as noted previously [34]; the α -configuration at C-10 is secured by the trans-diaxial coupling of 9.8 Hz between H-9 and H-10. The diester **9** was prepared in moderate yield (35%) by activation of the free carboxyl group of artesunate with dicyclohexyl carbodiimide and treatment with cholesterol. As in the foregoing case, the product has the α -configuration at C-10 ($J_{9,10}$ 9.9 Hz).

We have previously noted that artemisinin derivatives bearing an amino group at C-10 tend to display superior antimalarial activities with respect to the O- or C-substituted counterparts. This is ascribed to facilitated hydride transfer from the reduced flavin cofactors to the peroxide of the artemisinin driven by protonation of the amino group at C-10 under intracellular conditions [35]. Accordingly, DHA **2** was converted into the piperazine derivative **10** by treatment with oxalyl chloride in the presence of DMSO followed by quenching of the reaction mixture by addition to an excess of piperazine (Scheme 2) [36]. The reaction, which proceeds *via* the intermediate β -chloride works well with cyclic secondary amines. Notably, in attempts to vary the C-10 amino group attached to the DHA, it was found here that the use of primary alkyl amines did not return aminated products. The α -configuration on the C-10 is secured by the trans-diaxial coupling of 10.2 Hz between H-9 and H-10. Subsequent treatment of **10** with cholesteryl chloroformate provided the amide derivative **11** in a moderate yield (42.5%). Cholesterol was esterified with ω -haloalkyl acid chlorides to provide the haloesters **12** and **13** [37, 38] that upon treatment with the artemisinin-piperazine derivative **10** gave the corresponding cholesterol conjugates **14** and **15** in 32 and 6% yields, respectively. The same idea was applied to the synthesis of compound **17** (Scheme 2) wherein 1-(2'-aminoethyl)-piperazine was used instead of piperazine to convert DHA into the artemisinin-piperazine derivative **16** in low yield (35.9%). Interestingly, the preference is for this bifunctional amine to react through the secondary amine site with the β -chloride formed *in situ*. The product arising from reaction through the exocyclic primary amino group was not observed. Treatment of the compound **16** with cholesteryl chloroformate provided compound **17** in low overall yield (14%).



Scheme 2: Preparation of artemisinin-piperazine cholesterol conjugates. Reagents and conditions: i. DMSO (0.1 eq.), oxalyl chloride (1.13 eq.), toluene, room temperature, N₂ atmosphere; ii. Direct addition of reaction mixture from i. to piperazine (5 eq.) in CH₂Cl₂, overnight; iii. Cholesteryl chloroformate (1.2 eq.), triethylamine (1.2 eq.), CH₂Cl₂, overnight; iv. Cholesterol, ω-haloalkyl acid chloride (2 eq.), CH₂Cl₂, room temperature, 24 h; v. Compound **10** (1 eq.), triethylamine (1 eq.), THF, 65 °C reflux, 24 h; i. as above; vi. Direct addition of reaction mixture from i. to 1-(2'-aminoethyl)piperazine (5 eq.) in CH₂Cl₂, overnight; iii. as above.

Lastly, ethers **18**, **20**, **22** and **24** were prepared through BF₃-catalysed condensation of DHA **2** with halohydrins following the procedure generally used for coupling DHA with alcohols [33, 34, 39]. These products were treated with excess piperazine and the crude amines so obtained were coupled with cholesteryl chloroformate in the next step. These reactions provided the carbamates **19** (40%), **21** (16%), **23** (7%) and **25** (10%) (Scheme 3). All the compounds were obtained as stereochemically pure isomers; although the intermediate **22** appeared to be a mixture according to its ¹H and ¹³C NMR spectra, the final product obtained after reaction of the derived amine with cholesteryl chloroformate and purification by chromatography was the pure β-isomer **23**. The vicinal coupling ranging from 3.2 – 3.5 Hz between H-9 and H-10 in the ¹H NMR spectra indicates the β-configuration at C-10 was secured for all the compounds.



Scheme 3: Preparation of carboxylates. Reagents and conditions: i. Halohydrin (2 eq.), $\text{BF}_3 \cdot \text{Et}_2\text{O}$ (0.9 eq.), CH_2Cl_2 , 0 °C – room temperature, overnight; ii. Piperazine (5 eq.), tetrahydrofuran, DMF (catalytic, 0.1 eq.), 65 °C reflux, 24 h; iii. Cholesteryl chloroformate (1.2 eq.), triethylamine (1.2 eq.), CH_2Cl_2 , 0 °C – room temperature, overnight.

Biological activity

Antimalarial activity *in vitro* was first assessed using a dual point assay by adding compounds at concentrations of 5 and 1 μM to asexual blood stages of the chloroquine (CQ) CQ-sensitive *Pf* NF54 strain. Those compounds which showed >70% inhibition at 5 μM and >50% inhibition at 1 μM were carried forward for IC_{50} (the concentration that inhibits 50% parasite proliferation at 96 h) determination against *Pf* CQ-sensitive NF54, and CQ-resistant *Pf* K1 and W2 strains. Compounds **8**, **11**, **14**, **15**, **17** and **21** were thereby carried forward for screening using the dihydroartemisinin derivatives artemether **3** and artesunate **4** as comparator compounds. Compounds **14** and **15** were the most active against NF54 with IC_{50} values of 0.03 and 0.078 μM , respectively. Compound **21** was the least active with an IC_{50} of 2.60 μM . A similar trend was observed for drug resistant K1 and W2. The most active compounds were **14** and **15** with IC_{50} values of 0.03 and 0.05 μM against K1 and against W2 0.024 and 0.07 μM , respectively. Compounds **8**, **11** and **17** had IC_{50} values of 0.32, 0.16 and 0.20 μM against K1 and 0.36, 0.20 and 0.18 μM against W2, respectively. Compound **21** remained the least active of the compounds with IC_{50} values of 1.9 and 1.7 μM against K1 and W2, respectively. Resistance index (RI) values between the strains varied but all compounds displayed values less than 1 between CQ-sensitive NF54, and CQ-resistant K1 and W2 (Table 1); that is, these compounds show no cross-resistance. Cytotoxicity of the compounds was determined *in vitro* against the HEK293 cell line and did not show toxicity towards these cells but had selectivity towards parasitic cells given the high selectivity indices (Table 1). Compound **21** showed a low selectivity index (SI) with implications that this compound may have exerted its antimalarial activity through toxicity.

Overall, activities were some threefold lower than the comparator artesunate against the drug resistant K1 and W2 for the most active compound **14**. Nevertheless, the data is encouraging, given that all the compounds, with the exception of **14**, had notably poor solubility

at 10 mM concentration in 100% DMSO stored at 4 °C. Poor solubility may be the reason for compounds **7**, **9**, **19**, **23** and **25** not being active even during the dual-point dilution assay as this lack of activity according to structure-activity considerations *vis-à-vis* compound **14** is not at all apparent. All compounds are highly lipophilic with cLogP values ranging from 13.7 to 15.3 (Table 1). The high lipophilicity likely accounts for solubility problems encountered when dissolving these compounds in the dipolar aprotic solvent DMSO. It is not uncommon to encounter solubility problems with new chemical entities and this affects the quality of both *in vitro* and *in vivo* biological assays and consequently the interpretation of results [40]. A compound may appear to be less active than it really is because of low solubility. Inability of DMSO to solvate the compounds is ascribed to lipophilicity as expressed in poorly solvent-accessible surface areas and rotatable bonds [41]. The compounds synthesised in this work have high molecular weights and compounds **15** (6C linker), **23** (4C linker) and **25** (6C linker) have highly flexible linkers. Overall, in conjunction with evolving the synthetic routes here to generate more soluble artemisinin-cholesterol conjugates, resort will also be made to generating lipophilic formulations of the current compounds in order to facilitate administration [42].

Table 1. IC₅₀ values of compounds against asexual blood stages of NF54, K1 and W2 strains of *Plasmodium falciparum* and their cytotoxicity against WI-38 HFLF cells. Data are averaged from n=3 independent biological repeats, performed in triplicate ± SEM. Cells were incubated with compounds at various concentrations for 96 h; antimalarial activities and cytotoxicities were determined using SYBR Green I and SRB assays, respectively.

Compound	cLog P ^a	Antimalarial activity IC ₅₀ ±SEM, μM ^b			Resistance Index		Cytotoxicity IC ₅₀ , μM	Selectivity Index
		NF54	K1	W2	RI ^c	RI ^d	HEK293 ^e	SI ^f
2	nd	nd	nd	nd	nd	nd	0.05	
3	3.07	0.002 ± 0.00	0.009 ± 0.00	0.007 ± 0.00	4.8	3.8	nd	nd
4	2.94	0.003 ± 0.00	0.004 ± 0.00	0.002 ± 0.00	1.3	0.8	nd	nd
8	13.9	0.429 ± 0.04	0.315 ± 0.037	0.364 ± 0.05	0.73	0.85	83.3	194
11	14.6	0.213 ± 0.004	0.164 ± 0.065	0.209 ± 0.04	0.77	0.98	243	1141
14	14.7	0.031 ± 0.005	0.029 ± 0.005	0.024 ± 0.04	0.92	0.78	121	3903
15	15.3	0.078 ± 0.003	0.052 ± 0.008	0.070 ± 0.01	0.67	0.89	142	1821
17	14.3	0.267 ± 0.01	0.201 ± 0.010	0.176 ± 0.077	0.75	0.66	82.6	309
21	13.7	2.569 ± 0.046	1.946 ± 0.047	1.680 ± 0.657	0.76	0.65	74.2	28.9

^a cLogP values calculated with ACD/ChemSketch Version 14.02; ^bData from at least three independent biological repeats, each performed in triplicate; CQ = chloroquine; nd = not determined; ^cResistance index (RI) = IC₅₀ K1/IC₅₀ NF54; ^dResistance index (RI) = IC₅₀ W2/IC₅₀ NF54; ^eHEK293 cell line of normal human embryonic kidney cell line; ^fSelectivity index (SI) = IC₅₀ HEK293/IC₅₀ NF54.

The antimycobacterial activity of the compounds against *Mtb* H37Rv was determined by CFU enumeration following plating of CFU on 7H11 agar for 12 – 15 days. *Mtb* H37Rv cultures were treated with compounds at 10 and 80 μ M concentrations for 24 h. Data are presented in Table 2. Compound **19** was the least active compound at both concentrations with 3 and 18% inhibition at 10 μ M and 80 μ M, respectively. Most of the compounds, except **15** and **23**, showed low inhibition at 10 μ M (13 - 27%) and low to moderate activity at 80 μ M (28 - 51%). On the other hand, compound **15** and **23** had moderate activity at both concentrations with 38 and 31% inhibition at 10 μ M and 52 and 47% inhibition at 80 μ M, respectively. The artemisinin-piperazine derivative **10** (Scheme 2) was used as a reference; it is soluble in DMSO. It was also moderately active with 23 and 43% inhibition for 10 μ M and 80 μ M, respectively. However, the compound is less active than the artemisinin-cholesterol conjugates **15** and **23**. As in the malaria assays, the nature of the linker or the conjugating group, e.g. piperazine or ester, did not impact on the efficacy of the compounds. However, compounds with longer alkyl chain linkers appear to have better activity than those with shorter linkers. Compound **15** with 6 carbon chain linker and **23** with 4 carbon chain were the most active compounds which may suggest the possibility that increased lipophilicity enhances the passage through the lipophilic wall of the *Mtb*. Compounds bearing linkers with <4 carbon atoms had less than 22% inhibition at low concentration (10 μ M) although it varied at high concentration (18 – 51% inhibition at 80 μ M). Compounds **7** and **11** were comparable to that of **15** at 80 μ M.

Table 2. The antimycobacterial activity of compounds against *Mtb* H37Rv determined by CFU enumeration. Data are averaged from n=3 independent biological repeats, performed in triplicate \pm SEM. The *Mtb* H37Rv cultures were treated with the desired drug (10 and 80 μ M) for 24 h following which 200 μ L was centrifuged, re-suspended and washed twice in 7H9 and CFU were plated on 7H11 agar. CFUs were counted after 12-15 days.

Compound	Antimycobacterial activity, μ M			
	^a Av CFU/mL \pm STDEV (10 μ M)	% inhibition	^a Av CFU/mL \pm STDEV (80 μ M)	% inhibition
7	^b 1.18 \pm 4.4	22	1.3 \pm 3.2	51
8	1.15 \pm 2.1	14	1.48 \pm 5.1	44
9	1.62 \pm 3.3	15	1.43 \pm 2.2	46
10	1.92 \pm 5	23	1.52 \pm 3.9	43
11	1.52 \pm 3.1	19	1.3 \pm 3.2	51
14	1.42 \pm 2.4	13	1.62 \pm 2.8	39
15	1.55 \pm 1.4	38	1.28 \pm 4.1	52
17	1.55 \pm 2.3	18	1.77 \pm 3.3	33
19	1.97 \pm 2.4	3	2.18 \pm 4.5	18
21	1.13 \pm 2.5	14	1.92 \pm 5	28
23	1.50 \pm 2.5	31	1.42 \pm 4.6	47
25	1.82 \pm 3.7	27	1.63 \pm 4.9	38
DMSO	2.65 \pm 4.4	0	2.48 \pm 3.2	0

^aAv CFU/ml \pm STDEV = Average Colony Forming Units/mL \pm STANDARD DEVIATION

^bAll values = CFU/mL \times 10⁸

Conclusion

Dihydroartemisinin was conjugated either *via* O- or N-linked functional groups to cholesterol to generate ether, ester and carbamate linked artemisinin-cholesterol conjugates. Antimalarial activity against *Pf* NF54, K1 and W2 strains did not show improved activity compared to artemether and artesunate, but results are encouraging in the context of the future evaluation of the cholesteryl moiety in enhancing targeting of parasite stages in an *in vivo* situation, in particular against liver stage parasites. Compound **14** and **15** were the most active compounds. These represent hit compounds that will not only be used as a platform to design newer conjugates with enhanced polarity so as to improve solubility, but also in the development of lipophilic formulations employing liposomes and others to enhance activities.

The antimycobacterial activity was determined by CFU enumeration following treatment of *Mtb* H37Rv culture with compounds. Compounds **15** and **23** displayed moderate antimycobacterial activities at both 10 μ M and 80 μ M. The others displayed low antimycobacterial activity but compound **25** was comparable to the reference compound **10** in terms of activity. Compound **15** and **23** will be taken forward for further screening both in macrophages and through alternative *in vitro* screens, employing for example the mutant H37RvMA::*gfp* strain. If these compounds retain activities in these screens, their ability to target quiescent *Mtb* and to penetrate the granuloma will also be determined.

Experimental Section

Materials and methods

Reagents were purchased from Sigma Aldrich, Johannesburg, South Africa and used as supplied. Bulk solvents, magnesium sulfate and sodium hydrogen carbonate were purchased from ACE Chemicals, Johannesburg, South Africa. Dichloromethane was distilled, dried over calcium carbonate and stored over 3 Å molecular sieves, diethyl ether and tetrahydrofuran (THF) were dried over sodium, and distilled before use. Dihydroartemisinin was purchased from Changzhou Kaixuan Chemical Co, Chunjiang, China.

^1H and ^{13}C NMR spectra were recorded on a Bruker AvanceTM III spectrometers as solutions in chloroform-d (CDCl_3). Chemical shifts (δ) are reported in parts per million (ppm) and the ^1H chemical shifts are reported downfield of tetramethylsilane (TMS) with internal reference to the residual proton in CDCl_3 (δ 7.25 ppm). ^{13}C chemical shifts were internally referenced to the CDCl_3 resonances (δ 77.00 ppm). The splitting patterns are abbreviated as follows: s (singlet), d (doublet), dd (doublet of doublets), t (triplet) and m (multiplet). The coupling constant J are reported in Hz. Spectra were analysed with MestReNova Software, version 5.3.2-4936. High resolution mass spectrometry (HRMS) was recorded on a Bruker MicroTOF Q II mass spectrometer, equipped with an ESI source set at 180 °C using Bruker Compass DataAnalysis 4.0 software. A full scan from m/z 50 to 1500 was performed at a capillary voltage of 4500 V, an end plate offset voltage of -500 V, with the nebuliser set at 0.4 Bar, r, and a collision cell RF voltage of 100 Vpp. Infrared (IR) spectra were recorded on a Bruker Alpha-P FTIR (ATR) instrument. Melting points (mp) were determined with a Büchi melting point B-545 instrument and were uncorrected. Column chromatography was performed using high-purity grade silica gel (pore size 60 Å, 70-230 mesh, 63-200 μm) from Sigma Aldrich and thin layer chromatography was performed with silica gel plates (60F₂₅₄) from Merck.

Syntheses

(1*S*,5*R*,9*R*,10*S*,12*R*,13*R*)-10-[[*(2R*,5*S*,15*R*)-2,15-dimethyl-14-[(*2R*)-6-ethylheptan-2-yl]tetracyclo[8.7.0.0^{2,7}.0^{11,15}]heptadec-7-en-5-yl]oxy}-1,5,9-trimethyl-11,14,15,16-tetraoxatetracyclo[10.3.1.0^{4,13}.0^{8,13}]hexadecane **7**. A solution of dihydroartemisinin (DHA, **2**) (0.5 g, 1.76 mmol) and cholesterol (1360 mg, 3.52 mmol, 2 eq.) was prepared in dichloromethane (20 mL) and cooled to 0 °C in an ice-bath. To this solution BF₃·OEt₂ (200 μL, 0.9 eq.) was added dropwise and the reaction mixture was left to stir in the dark for 4 h as the temperature increased from 0 °C to room temperature. The reaction mixture was quenched with saturated aqueous NaHCO₃ (20 mL), extracted with dichloromethane (3 x 20 mL) and the extracts were combined and dried over MgSO₄. Following filtration to remove MgSO₄, the filtrate was evaporated under reduced pressure and the residue was submitted to column chromatography on silica gel. Elution with ethyl acetate-hexane (1:9 v/v) gave the product that was obtained after evaporation of the eluate as a white powder (114 mg, 10%), mp: the product started softening at 166 °C and was completely molten at 178 °C ; literature 168.3 – 168.8 °C [34]; ; ¹H NMR (600 MHz, CDCl₃) δ (ppm): 5.41 (s, 1H, 12-H), 5.30 (d, 1H, *J* = 5.1 Hz, 10-H), 4.88 (t, 1H, *J* = 5.1 Hz, 6'-H), 3.48 - 3.56 (m, 1H, 3'-H), 2.56 - 2.53 (m, 1H, H-4'), 1.40 (s, 3H, H-14), 0.96 (s, 3H, H-19'), 0.91 (d, *J* = 6.5 Hz, 3H, H-15), 0.87 (d, *J* = 6.5 Hz, 3H, H-16), 0.84 (s, 3H, 21'-H), 0.82 (dd, *J* = 6.5, 2.7 Hz, 6H, H-26', H-27'), 0.63 (s, 3H, H-18'); ¹³C NMR (151 MHz, CDCl₃): 141.04 (C-5'), 122.21 (C-6'), 104.21 (C-3), 99.84 (C-10), 88.52 (C-12), 81.60 (C-12a), 76.64 (C-3'), 57.05 (C-14'), 56.49 (C-17'), 52.97 (C-5a), 50.44 (C-9'), 44.87 (C-8a), 42.65 (C-13'), 23.18 (C-26',27), 14.48 (C-16), 13.56 (C-18'); IR ν_{\max} cm⁻¹: 2936, 2868, 2850, 1464, 1439, 1375, 1362, 1137, 1099, in agreement with the data reported in the literature [34].

(*2R*,4*R*,15*R*)-2,15-dimethyl-14-[(*2R*)-6-methylheptan-2-yl]tetracyclo[8.7.0.0^{2,7}.0^{11,15}]heptadec-7-en-4-yl(1*S*,5*R*,9*R*,10*S*,12*R*,13*R*)-1,5,9-trimethyl-11,14,15,16-tetraoxatetracyclo[10.3.1.0^{4,13}.0^{8,13}]hexadecan-10-yl carbonate, **8**. A solution of DHA (1000 mg, 3.52 mmol) in dichloromethane (7.5 mL) containing a catalytic amount of 4-*N,N*-dimethylaminopyridine (100 mg) and triethylamine (539 μL, 3.87 mmol, 1.1 eq.) was treated with cholesteryl chloroformate (1630 mg, 3.87 mmol, 1.1 eq.) in dichloromethane (7.5 mL). The mixture was stirred for 18 h at room temperature, and then quenched with saturated NaHCO₃ (15 mL) and extracted with dichloromethane (3 x 15 mL). The extracts were combined, and dried over MgSO₄. After filtration, the solvent was evaporated under reduced pressure to leave a residue that was submitted to chromatography on silica gel. Elution with ethyl acetate-hexane (3:7, v/v) gave after evaporation of the eluate the product as a white solid (1109 mg, 45%), mp: the product started softening at 160 °C and was completely molten at 185 °C; ¹H NMR (600 MHz, CDCl₃) δ (ppm): 5.56 (d, *J* = 9.8 Hz, 1H, H-10), 5.42 (s, 1H, H-12), 5.36 (d, *J* = 4.8 Hz, 1H, 6'-H), 4.46 – 4.51 (m, 1H, 3'-H), 2.59 – 2.53 (m, 1H, 8a), 2.35 (d, *J* = 2.9 Hz, 2H, 4'-H), 1.41 (s, 3H, H-14), 1.40 (s, 3H, H-16), 0.98 (s, 3H,

H-15), 0.93 (d, $J = 6.1$ Hz, 3H, H-19'), 0.88 (d, $J = 1.8$ Hz, 3H, H-21'), 0.84 (dd, $J = 6.6$ Hz, 2.6 Hz, 6H, H-26', H-27'), 0.65 (s, 3H, H-18'); ^{13}C NMR (151 MHz, CDCl_3): 153.56 (C-18), 139.37 (C-5'), 122.88 (C-6'), 104.43 (C-3), 95.53 (C-10), 91.43 (C-12), 79.90 (C-12a), 78.20 (C-3'), 56.66 (C-14'), 56.08 (C-17'), 51.47 (C-5a), 49.94 (C-9'), 45.19 (C-8a), 42.28 (C-13'), 39.67 (C-24'), 22.80 (C-25'+C-26'), 19.25 (C-19'), 18.68 (C-21'), 12.09 (C-16), 11.83 (C-18'); IR ν_{max} cm^{-1} : 2934, 2867, 1750, 1456, 1375, 1250, 1204, 1182, 1170; HRMS-ESI m/z $[\text{M}+\text{Na}]^+$ 719.4774 (calcd for $\text{C}_{43}\text{H}_{68}\text{O}_7\text{Na}$: 719.4863).

(2*R*,5*S*,15*R*)-2,15-dimethyl-14-[(2*R*)-6-methylheptan-2-yl]tetracyclo[8.7.0.0^{2,7}.0^{11,15}]heptadec-7-en-5-yl (1*S*,5*R*,9*R*,10*S*,12*R*,13*R*)-1,5,9-trimethyl-11,14,15,16-tetraoxatetracyclo [10.3.1.0^{4,13}.0^{8,13}]hexadecan-10-yl butanedioate, **9**. To a solution of artesunate (1000 mg, 2.60 mmol) in dichloromethane (10 mL) was added *N,N*-dicyclohexylcarbodiimide (540 mg, 2.60 mmol, 1 eq.) and 4-*N,N*-dimethylaminopyridine (64 mg, 0.52 mmol, 0.2 eq.). To this mixture was then added a solution of cholesterol (1010 mg, 2.60 mmol, 1 eq.) in dichloromethane (10 mL) and the resulting mixture was stirred at room temperature for 4 h. The white precipitate that formed was removed by filtration, and the filtrate was washed with deionized water (20 mL) and the organic phase was dried over MgSO_4 . Following filtration of MgSO_4 the solvent was evaporated from the filtrate under reduced pressure. The residue was submitted to column chromatography over silica gel. Evaporation of the eluate obtained with ethyl acetate-hexane (3:7 v/v) left a white powder (689 mg, 35%), mp: the product started softening at 152 °C and was completely molten at 185 °C; ^1H NMR (600 MHz, CDCl_3) δ (ppm): 5.77 (d, $J = 9.9$ Hz, 1H, H-10), 5.41 (s, 1H, H-12), 5.34 (d, $J = 4.1$ Hz, 1H, 6'-H), 4.62 - 4.57 (m, 1H, H-3'), 2.74 - 2.50 (m, 4H, H-19, H-20), 2.37 - 2.32 (m, $J = 3.2$ Hz, 1H, H-14'), 2.29 - 2.28 (m, $J = 7.1$ Hz, 1H, H-8a), 1.41 (s, 3H, H-14), 0.98 (s, 3H, H-19'), 0.94 (d, $J = 6.1$ Hz, 3H, H-15), 0.88 (d, $J = 6.5$ Hz, 3H, H-16), 0.85 (d, $J = 1.8$ Hz, 3H, H-21'), 0.82 (dd, $J = 2.6, 2.9$ Hz, 6H, H-26', H-27'), 0.65 (s, 1H, H-18'); ^{13}C NMR (151 MHz, CDCl_3): 171.47 (C-21), 171.19 (C-18), 139.57 (C-5'), 122.65 (C-6'), 104.44 (C-3), 92.08 (C-10), 91.47 (C-12), 80.10 (C-12a), 74.33 (C-3'), 51.53 (C-5a), 49.97 (C-9'), 45.20 (C-8a), 42.27 (C-13'), 39.69 (C-24'), 31.86 (C-6), 18.68 (C-21'), 12.06 (C-16), 11.82 (C-18'); IR ν_{max} cm^{-1} : 2949, 2895, 2867, 2847, 1761, 1714, 1467, 1458, 1374; HRMS-ESI m/z $[\text{M}+\text{Na}]^+$ 774.5047 (calcd for $\text{C}_{46}\text{H}_{72}\text{O}_8\text{Na}$: 774.5047).

Preparation of Artemisinin-piperazine conjugates

1-[(1*S*,5*R*,9*R*,10*R*,12*R*,13*R*)-1,5,9-trimethyl-11,14,15,16-tetraoxatetracyclo [10.3.1.0^{4,13}.0^{8,13}]hexadecan-10-yl]piperazine, **10**.

To a solution of DHA (1000 mg, 3.52 mmol) in toluene (10 mL) under a nitrogen atmosphere was added DMSO (25.1 μL , 0.35 mmol, 0.1 eq.) and this was followed by dropwise addition of oxalyl chloride (350 μL , 1.13 eq.). The reaction mixture was left to stir for 1 h at room temperature and

then was added directly to a solution of piperazine (1500 mg, 5 eq.) in dichloromethane (10 mL). The resulting mixture was stirred overnight at room temperature under nitrogen, and then quenched with saturated aqueous NaHCO₃ (20 mL), and extracted with ethyl acetate (3 x 20 mL). The extracts were combined and dried over MgSO₄. After filtration, the solvent was removed by evaporation under reduced pressure to leave the crude product that was submitted to column chromatography. Elution with methanol-dichloromethane (1:9 v/v) and evaporation of the eluate gave the intermediate **10** as cream white solid (743 mg, 60%), mp: 163.6 – 163.7 °C; ¹H NMR (600 MHz, CDCl₃) δ (ppm): 5.20 (s, 1H, H-12), 3.92 (d, *J* = 10.2 Hz, 1H, H-10), 3.07 - 2.80 (m, 4H, H-19, H-21), 2.68 - 2.61 (m, 4H, 18-H, 22-H), 2.55 - 2.46 (m, 1H, H-8a), 2.27 (td, *J* = 14.0, 3.9 Hz, 2H, H-4), 1.98 - 1.89 (m, 2H, H-7), 1.32 (s, 3H, H-14), 0.88 (d, *J* = 6.3 Hz, 3H, H-16), 0.73 (d, *J* = 7.3 Hz, 3H, H-15); ¹³C NMR (151 MHz, CDCl₃) δ (ppm): 103.96 (C-3), 91.64 (C-10), 91.06 (C-12), 80.34 (C-12a), 51.74 (C-5a), 50.70 (C-18, C-22), 45.84 - 45.69 (C-19, C-22), 26.02 (C-14), 24.78 (C-5), 21.67 (C-7), 20.31 (C-16), 13.45 (C-15); IR ν_{\max} cm⁻¹: 3261, 2926, 2869, 2839, 1738, 1453, 1408, 1375, 1349; HRMS-ESI *m/z* [M+H]⁺ 353.2469 (calcd for C₁₉H₃₂N₂O₄H: 353.2440).

(2*R*,5*S*,15*R*)-2,15-dimethyl-14-[(2*R*)-6-methylheptan-2-yl]tetracyclo[8.7.0.0^{2.7}.0^{11,15}]heptadec-7-en-5-yl4-[(1*S*,5*R*,9*R*,10*R*,12*R*,13*R*)-1,5,9-trimethyl-11,14,15,16-tetraoxatetracyclo[10.3.1.0^{4,13}.0^{8,13}]hexadecan-10-yl]piperazine-1-carboxylate, **11**. To a stirred solution of **10** (740 mg, 2.09 mmol) and triethylamine (344 μ l, 2.72 mmol, 1.2 eq.) in dichloromethane (10 mL) under a nitrogen atmosphere was added a solution of cholesteryl chloroformate (1030 mg, 2.72 mmol, 1.2 eq.) in dichloromethane (5 mL). The reaction mixture was stirred overnight and was then quenched with saturated aqueous NaHCO₃ (20 mL). The crude mixture was extracted with dichloromethane (3 x 20 mL) and the extracts were combined and dried over MgSO₄. After filtration to remove MgSO₄ the filtrate was concentrated under reduced pressure to leave the crude product that was submitted to column chromatography over silica gel. Elution with ethyl acetate-hexane (2:8 v/v) gave the product as a white powder (688 mg, 43%), mp: 169 – 172 °C; ¹H NMR (600 MHz, CDCl₃) δ (ppm): 5.34 (d, *J* = 2.3 Hz, 1H, 6'-H), 5.25 (s, 1H, 12-H), 4.45 – 4.48 (m, 1H, 3'-H), 4.00 (d, *J* = 8.5 Hz, 1H, H-10), 3.44 - 3.40 (m, 2H, H-19), 2.88 – 2.96 (m, 2H, 21-H), 2.63 – 2.52 (m, 4H, 18-H, 22-H), 1.62 (s, 3H, H-14), 1.36 (s, 3H, H-16), 0.99 (s, 3H, H-19'), 0.92 (d, *J* = 6.1 Hz, 3H, H-15), 0.89 (d, *J* = 6.5 Hz, 3H, H-21'), 0.84 (dd, *J* = 6.6, 2.8 Hz, 6H, H-26', H-27'), 0.65 (s, 3H, H-18'); ¹³C NMR (151 MHz, CDCl₃) δ (ppm): 154.97 (C-23), 140.07 (C-5'), 122.36 (C-6'), 103.84 (C-3), 91.79 (C-10), 90.75 (C-12), 80.26 (C-12a), 74.64 (C-3'), 56.65 (C-14'), 56.08 (C-17'), 45.80 (C-19, C-21), 44.05 (C-8a), 42.28 (C-13'), 20.25 (C-15), 19.34 (C-19'), 18.68 (C-21'), 13.46 (C-16), 11.82 (C-18'); IR (ATR) ν_{\max} cm⁻¹: 2930, 2864, 1690, 1429, 1379, 1246, 1195, 1127, 1110; HRMS-ESI *m/z* [M+H]⁺ 765.5678 (calcd for C₄₇H₇₆N₂O₆H: 765.5782).

Preparation of artemisinin-piperazine cholesterol conjugates

Cholesterol (1000 mg, 2.59 mmol) was dissolved in dichloromethane (20 mL) and triethylamine (541 μ L, 5.18 mmol, 2 eq.) was added. To this mixture acid chloride (5.18 mmol, 2 eq.) was added and the reaction mixture was left to stir at room temperature overnight [Scheme 3, step (iii)]. The reaction mixture was quenched with saturated NaHCO₃ (20 mL) and the crude mixture extracted with dichloromethane (3 x 20 mL). The extracts were combined and dried over MgSO₄. After filtration to remove MgSO₄ the filtrate was concentrated under reduced pressure to leave the crude product. To a solution of **10** (1005 mg, 2.98 mmol) in THF (10 mL) under reflux (65 °C) was added triethylamine (286 μ L, 2.05 mmol, 1.3 eq.) followed by a solution of the crude product from the previous step in THF (10 mL). The reaction mixture was left to stir for 24 h and was quenched with saturated aqueous NaHCO₃ (20 mL). The crude mixture was extracted with diethyl ether (3 x 20 mL) and combined extracts were dried over MgSO₄. Following filtration to remove MgSO₄ the filtrate was concentrated under reduced pressure and submitted to column chromatography over silica gel. Elution with ethyl acetate-hexane (v/v) column chromatography gave pure product. This method was used for synthesis of the conjugates **14**, **15** and **17** as described below.

*(2R,5S,15R)-2,15-dimethyl-14-[(2R)-6-methylheptan-2-yl]tetracyclo[8.7.0.0^{2,7}.0^{11,15}] heptadec-7-en-5-yl-4-[(1S,5R,9R,10R,12R,13R)-1,5,9-trimethyl-11,14,15,16-tetraoxa tetracyclo[10.3.1.0^{4,13}.0^{8,13}]hexadecan-10-yl]piperazine-1-carboxylate, **14**.*

The product obtained from the reaction of chloroacetyl chloride with cholesterol was treated with compound **10** according to the method described above. The product was purified by chromatography with ethyl acetate-hexane (2.5:7.5, v/v) to give a light-purple solid (743 mg, 32.0%), mp: the product started to soften at 145 °C and was completely molten at 203 °C; ¹H NMR (600 MHz, CDCl₃) δ (ppm): 5.34 (d, J = 3.7 Hz, 1H, H-6'), 5.24 (s, 1H, H-12), 4.64 (s, 1H, H-3'), 4.03 (d, J = 9.9 Hz, 1H, H-10), 3.29 (s, 2H, H-18), 3.21 (s, 2H, H-23), 3.15 – 3.05 (m, 2H, H-19, H-21), 2.90 – 2.73 (m, 2H, H-22), 1.34 (s, 3H, H-14), 0.98 (s, 3H, H-19'), 0.91 (d, J = 6.2 Hz, 3H, H-15), 0.88 (d, J = 6.5 Hz, 3H, H-16), 0.83 (dd, J = 6.6, 2.7 Hz, 6H, H-26', H-27'), 0.79 (d, J = 6.6 Hz, 3H, H-21'), 0.64 (s, 3H, H-18'); ¹³C NMR (151 MHz, CDCl₃) δ (ppm): 139.37 (C-5'), 122.84 (C-6'), 103.94 (C-3), 91.51 (C-10), 90.41 (C-12), 80.25 (C-12a), 74.82 (C-3'), 56.63 (C-14'), 56.08 (C-17'), 51.62 (C-5a), 49.94 (C-18, C-22), 45.79 (C-13'), 23.79 (C-23'), 22.79 (C-26', C-27'), 20.26 (C-15), 19.27 (C-19'), 18.68 (C-21'), 13.39 (C-16), 11.82 (C-18'); IR (ATR) ν_{max} cm⁻¹: 3422, 2931, 2867, 1743, 1450, 1376, 1208, 1176, 1128; HRMS-ESI m/z [M+H]⁺ 779.5897 (calcd for C₄₈H₇₈N₂O₆H: 779.5938).

(2R,5S,15R)-2,15-dimethyl-14-[(2R)-6-methylheptan-2-yl]tetracyclo[8.7.0.0^{2,7}.0^{11,15}] heptadec-7-

*en-5-yl*6-{4-[(1*S*,5*R*,9*R*,10*R*,12*R*,13*R*)-1,5,9-trimethyl-11,14,15,16-tetraoxa
tetracyclo[10.3.1.0^{4,13}.0^{8,13}]hexadecan-10-yl]piperazin-1-yl}hexanoate, **15**.

The product obtained from the reaction of bromohexanoyl chloride with cholesterol was treated with compound **10** as described above. The crude product was purified by chromatography with ethyl acetate-hexane (1:9, v/v) to give a cream-white solid (144 mg, 5.79%), mp: the product started softening at 121 °C and was completely molten at 171 °C; ¹H NMR (600 MHz, CDCl₃) δ (ppm): 5.34 (d, *J* = 4.0 Hz, 1H, H-6'), 5.24 (s, 1H, H-12), 4.61 – 4.54 (m, 1H, H-3'), 4.06 (d, *J* = 9.4 Hz, 1H, H-10), 2.54 – 2.51 (m, 2H, H-23), 2.33 – 2.24 (m, 2H, H-27), 1.33 (s, 3H, H-14), 1.25 – 1.20 (m, 2H, H-8), 0.99 (s, 3H, H-16), 0.92 (d, *J* = 6.3 Hz, 3H, H-15), 0.88 (d, *J* = 6.5 Hz, 3H, H-19'), 0.83 (dd, *J* = 6.6, 2.7 Hz, 6H, H-26', H-27'), 0.76 (d, *J* = 7.1 Hz, 3H, H-21'), 0.65 (s, 3H, H-18'); ¹³C NMR (151 MHz, CDCl₃) δ (ppm): 139.64 (C-5'), 122.59 (C-6'), 103.95 (C-3), 91.40 (C-10), 80.28 (C-12a), 73.83 (C-3'), 56.65 (C-23), 56.08 (C-17'), 51.58 (C-5a), 49.98 (C-18), 45.72 (C-8a), 42.27 (C-13'), 22.53 (C-26', C-27'), 20.25 (C-15), 19.29 (C-19'), 18.68 (C-16), 13.39 (C-21'), 11.82 (C-18'); IR (ATR) ν_{\max} cm⁻¹: 2927, 2864, 1732, 1460, 1444, 1375, 1322, 1236, 1188, 1173; HRMS-ESI *m/z* [M+H]⁺ 835.6573 (calcd for C₅₂H₈₆N₂O₆H: 835.6564).

(2*R*,5*S*,15*R*)-2,15-Dimethyl-14-[(2*R*)-6-methylheptan-2-yl]tetracyclo[8.7.0.0^{2,7}.0^{11,15}]heptadec-7-
*en-5-yl*N-(2-{4-[(1*S*,5*R*,9*R*,10*R*,12*R*,13*R*)-1,5,9-trimethyl-11,14,15,16-
tetraoxatetracyclo[10.3.1.0^{4,13}.0^{8,13}]hexadecan-10-yl]piperazin-1-yl}ethyl)carbamate, **17**. DMSO (25.1 μL, 0.35 mmol, 0.1 eq.) was added to a solution of DHA (1000 mg, 3.52 mmol) in toluene (10 mL) under an inert atmosphere at room temperature. To the resulting solution was added dropwise oxalyl chloride (350 μL, 1.13 eq.). The resulting reaction mixture was left to stir for 1 h at room temperature and then transferred into a solution of 1-(2-amino-ethyl)piperazine (1500 mg, 5 eq. of **2**) in dichloromethane (10 mL). The reaction mixture was stirred overnight at room temperature under nitrogen, and was then quenched with saturated aqueous NaHCO₃ (20 mL). The crude mixture was extracted with ethyl acetate (3 x 20 mL) and the combined extracts were dried over MgSO₄. After filtration to remove MgSO₄, the filtrate was concentrated by evaporation under reduced pressure to give a yellow oil. This was dissolved in dichloromethane (5 mL) without further purification and triethylamine (588 μL, 4.2 mmol, 1.2 eq. to **2**) was added. This was followed by a solution of cholesteryl chloroformate (1900 mg, 4.2 mmol, 1.2 eq. to **2**) in dichloromethane (5 mL). The reaction was stirred overnight at room temperature and quenched with saturated NaHCO₃ (20 mL). The crude mixture was extracted with dichloromethane (3 x 20 mL) and dried on MgSO₄. MgSO₄ was removed by filtration and the filtrate was submitted to column chromatography on silica gel. Elution with ethyl acetate-hexane (3:7, v/v) column chromatography gave product as a cream-white solid (141 mg, 4.96%), mp: the product started softening at 150 °C and was completely molten at 170 °C; ¹H NMR (600 MHz, CDCl₃) δ (ppm):

5.33 (d, $J = 4.8$ Hz, 1H, H-6'), 5.25 (s, 1H, H-12), 4.45 (s, 1H, H-3'), 4.09 (d, $J = 7.2$ Hz, 1H, H-10), 3.52 – 3.43 (m, 2H, H-23), 2.55 – 2.49 (m, 1H, H-8a), 2.35 – 2.28 (m, 4H, H-18, H-22), 1.35 (s, 3H, H-14), 0.92 (d, $J = 6.3$ Hz, 3H, H-16), 0.89 (d, $J = 6.5$ Hz, 3H, H-15), 0.84 (dd, $J = 6.6, 2.7$ Hz, 6H, H-26', H-27'), 0.76 (d, $J = 7.1$ Hz, 3H, H-21'), 0.65 (s, 3H, H-18'); ^{13}C NMR (151 MHz, CDCl_3) δ (ppm): 156.49 (C-26), 122.39 (C-6'), 103.86 (C-3), 91.42 (C-12), 80.27 (C-12a), 74.53 (C-3'), 56.69 (C-23), 56.10 (C-17'), 50.00 (C-22), 42.28 (C-13'), 27.98 (C-25'), 22.80 (C-26', C-27'), 20.24 (C-15), 19.29 (C-19'), 18.68 (C-16), 13.39 (C-21'), 11.82 (C-18'); IR (ATR) ν_{max} cm^{-1} : 3344, 2931, 2866, 1701, 1460, 1434, 1376, 1333, 1303; HRMS-ESI m/z $[\text{M}+\text{H}]^+$ 808.6201 (calcd for $\text{C}_{49}\text{H}_{81}\text{N}_3\text{O}_6\text{H}$: 808.6204).

Preparation of Carboxylates

A solution of DHA (1000 mg, 3.52 mmol) was prepared in dichloromethane (20 mL) and the halohydrin (7.04 mmol, 2 eq.) was added at 0 °C in an ice-bath. To this solution $\text{BF}_3 \cdot \text{EtO}_2$ (390.7 μL , 3.17 mmol, 0.9 eq.) was added and the reaction flask was covered with aluminium foil. The reaction mixture was stirred overnight with gradual increase of temperature from 0 °C to room temperature and then quenched with saturated aqueous NaHCO_3 (20 mL), followed by extraction with dichloromethane (3 x 20 mL). The extracts were combined and dried over MgSO_4 . The MgSO_4 was removed by filtration and the filtrate was concentrated by evaporation under reduced pressure. The resulting concentrate was either submitted to column chromatography on silica gel to give the intermediates **22** and **24** or direct recrystallisation to give **18** and **20**. To the solution of piperazine (5 eq.) and triethylamine (5 eq.) in THF (8 mL) was added the appropriate intermediate in THF (8 mL). This mixture was stirred for 24 h under reflux at 65 °C and was then washed with deionised water (16 mL). The solution was dried over MgSO_4 . Following filtration to remove MgSO_4 , the filtrate was concentrated under reduced pressure and the crude product was used in the next step without further purification. To a cooled solution of the crude product in dichloromethane (10 mL) was added triethylamine (1.2 eq.) and then a solution of cholesteryl chloroformate (1.2 eq.) in dichloromethane (10 mL). The reaction mixture was stirred overnight as the temperature increased from 0 °C to room temperature and then washed with deionised water (3 x 20 mL), the crude mixture was extracted with diethyl ether (3 x 20 mL) and the combined extracts were dried over MgSO_4 . After removing the MgSO_4 by filtration, the filtrate was concentrated under reduced pressure and the products **19**, **21**, **23** and **25** were obtained by column chromatography and elution with ethyl acetate-hexane (v/v).

(1S,5R,9R,10S,12R,13R)-10-(2-Bromoethoxy)-1,5,9-trimethyl-11,14,15,16-tetraoxa tetracyclo[10.3.1.0^{4,13}.0^{8,13}]hexadecane, 18.

1-Bromoethanol was used and the pure product was obtained as a white solid by recrystallisation from methanol (867 mg, 63%), mp: the product started softening at 160 °C and was completely

molten at 169 °C; ¹H NMR (600 MHz, CDCl₃) δ (ppm): 5.51 (s, 1H, H-12), 4.86 (d, *J* = 3.4 Hz, 1H, H-10), 4.19 – 4.09 (m, 1H, H-19), 3.80 (t, *J* = 5.5 Hz, 1H), 3.58 – 3.50 (m, 2H, H-18), 2.70 – 2.63 (m, 1H, H-8a), 1.45 (s, 3H, H-14), 0.97 (d, *J* = 6.4 Hz, 3H, H-16), 0.95 (d, *J* = 7.4 Hz, 3H, H-15); ¹³C NMR (151 MHz, CDCl₃) δ (ppm): 104.14 (C-3), 102.07 (C-10), 88.17 (C-12), 81.12 (C-12a), 68.20 (C-19), 52.60 (C-5a), 44.38 (C-8a), 37.42 (C-9), 36.42 (C-4), 34.69 (C-7), 31.46 (C-19), 30.92 (C-6), 26.18 (C-14), 24.67 (C-5), 24.39 (C-8), 20.40 (C-16), 13.01 (C-15); IR (ATR) ν_{\max} cm⁻¹: 2953, 2921, 2889, 2868, 1464, 1422, 1373, 1343, 1270 in agreement with the literature [43].

(2R,5S,15R)-2,15-Dimethyl-14-[(2*R*)-6-methylheptan-2-yl]tetracyclo[8.7.0.0^{2,7}.0^{11,15}]heptadec-7-en-5-yl4-(2-[[*(1S,5R,9R,10S,12R,13R)*-1,5,9-trimethyl-11,14,15,16-tetraoxa tetracyclo[10.3.1.0^{4,13}.0^{8,13}]hexadecan-10-yl]oxy}ethyl)piperazine-1-carboxylate, **19**.

The product **18** (850 mg, 2.17 mmol) from the previous step was used and the final product was obtained by chromatography and eluting with ethyl acetate-hexane (6:4, v/v) as white solid (695 mg, 39.5%), mp: the product started softening at 120 °C and was completely molten at 175 °C; ¹H NMR (600 MHz, CDCl₃) δ (ppm): 5.41 (s, 1H, H-12), 5.35 (t, *J* = 4.8 Hz, 1H, H-6'), 4.78 (d, *J* = 3.3 Hz, 1H, H-10), 4.52 – 4.46 (m, 1H, H-3'), 4.00 (s, 2H, H-18), 3.64 – 3.45 (m, 4H, H-22, H-25), 2.65 – 2.57 (m, 4H, H-21, H-26), 2.36 – 2.25 (m, 2H, H-4'), 1.40 (s, 3H, H-14), 0.99 (s, 3H, H-19'), 0.93 (d, *J* = 6.3 Hz, 3H, H-16), 0.88 (d, *J* = 7.0 Hz, 3H, H-15), 0.86 (s, 3H, H-21'), 0.83 (dd, *J* = 6.6, 2.7 Hz, 6H, H-26',27'), 0.65 (s, 3H, H-18'); ¹³C NMR (151 MHz, CDCl₃) δ (ppm): 139.89 (C-5'), 122.55 (C-6'), 104.10 (C-3), 102.00 (C-10), 87.90 (C-12), 80.99 (C-12a), 56.63 (C-23), 56.08 (C-17'), 52.96 (C-19, C-21), 52.50 (C-5a), 49.95 (C-18, C-22), 44.27 (C-8a), 42.27 (C-13'), 31.87 (C-6), 28.17 (C-16'), 24.67 (C-14), 22.79 (C-26', C-27'), 20.34 (C-15), 19.33 (C-19'), 18.68 (C-21'), 13.06 (C-16), 11.82 (C-18'); IR (ATR) ν_{\max} cm⁻¹: 2934, 2866, 1699, 1459, 1431, 1376, 1235; HRMS-ESI *m/z* [M+H]⁺ 809.6034 (calcd for C₄₉H₈₀N₂O₇H: 809.6044).

(1S,5R,9R,10S,12R,13R)-10-(3-Bromopropoxy)-1,5,9-trimethyl-11,14,15,16-tetraoxa tetracyclo[10.3.1.0^{4,13}.0^{8,13}]hexadecane, **20**.

3-Bromo-1-propanol was used and the pure product was obtained by recrystallization from methanol as a white solid (576 mg, 40%), mp: 88 – 91 °C; ¹H NMR (600 MHz, CDCl₃) δ (ppm): 5.44 (s, 1H, H-12), 4.82 (d, *J* = 3.5 Hz, 1H, H-10), 4.02 (ddd, *J* = 10.2, 6.2, 5.4 Hz, 1H, H-18), 3.55 – 3.47 (m, 3H, H-20), 2.66 (dt, *J* = 7.7, 3.5 Hz, 1H, H-8a), 2.39 (td, *J* = 14.0, 4.0 Hz, 1H, H-19), 1.46 (s, 3H, H-16), 0.97 (d, *J* = 6.4 Hz, 3H, H-16), 0.93 (d, *J* = 7.3 Hz, 3H, H-15); ¹³C NMR (151 MHz, CDCl₃) δ (ppm): 104.13 (C-3), 102.12 (C-10), 87.95 (C-12), 81.07 (C-12a), 65.71 (C-18), 52.29 (C-5a), 44.40 (C-8a), 37.47 (C-9), 36.44 (C-4), 34.65 (C-7), 32.56 (C-19), 30.91 (C-6), 26.21 (C-20), 24.68 (C-14), 24.54 (C-8), 20.39 (C-16), 13.00 (C-15); ν_{\max} cm⁻¹: 2990, 2945, 2928, 2865, 1480, 1452, 1384, 1373, 1361, 1250, 1011, in agreement with the literature [34].

(2R,5S,15R)-2,15-Dimethyl-14-[(2*R*)-6-methylheptan-2-yl]tetracyclo[8.7.0.0^{2,7}.0^{11,15}] heptadec-7-

en-5-yl-4-(3-[(1S,5R,9R,10S,12R,13R)-1,5,9-trimethyl-11,14,15,16-tetraoxatetracyclo[10.3.1.0^{4,13}.0^{8,13}]hexadecan-10-yl]oxy}propyl)piperazine-1-carboxylate **21**.

The product **20** (550 mg, 1.36 mmol) from the previous step was used and the final product was obtained by chromatography and eluting with ethyl acetate-hexane (8:2, v/v) as a light yellow powder (181 mg, 16%), mp: the product started softening at 142 °C and was completely molten at 184 °C; ¹H NMR (600 MHz, CDCl₃) δ (ppm): 5.34 (s, 1H, H-6'), 5.27 (s, 1H, H-12), 4.74 (d, *J* = 3.3 Hz, 1H, H-10), 4.52 – 4.46 (m, 1H, H-3'), 3.88 (dd, *J* = 15.9, 5.9 Hz, 2H, H-23), 3.62 (s, 2H, H-18), 3.40 (dd, *J* = 16.0, 6.1 Hz, 2H, H-24), 2.63 – 2.58 (m, 4H, H-22, H-25), 2.31 - 2.25 (m, 5H, H-8a, H-20, H-4'), 1.40 (s, 3H, H-14), 0.99 (s, 3H, H-19'), 0.93 (d, *J* = 6.3 Hz, 3H, H-15), 0.88 (d, *J* = 6.5 Hz, 3H, H-16), 0.86 (d, *J* = 7.4 Hz, 3H, H-21'), 0.83 (dd, *J* = 6.6, 2.7 Hz, 6H, H-26', 27'), 0.65 (s, 3H, H-18'); ¹³C NMR (151 MHz, CDCl₃) δ (ppm): 139.68 (C-5'), 122.62 (C-6'), 104.12 (C-3), 102.06 (C-10), 87.88 (C-12), 80.99 (C-12a), 75.32 (C-3'), 66.02 (C-18), 53.41 (C-22, C-26), 52.48 (C-5a, C-20), 44.29 (C-8a, C-23, C-25), 30.81 (C-19), 24.64 (C-14), 22.80 (C-26', C-27'), 20.32 (C-15), 19.32 (C-19'), 18.68 (C-21'), 13.02 (C-16), 11.82 (C-18'); IR (ATR) ν_{\max} cm⁻¹: 2935, 2866, 1699, 1461, 1431, 1376, 1230; HRMS-ESI *m/z* [M+H]⁺ 823.6207 (calcd for C₅₀H₈₂N₂O₇H: 823.6200).

(1S,5R,9R,10S,12R,13R)-10-(4-Chlorobutoxy)-1,5,9-trimethyl-11,14,15,16-tetraoxatetracyclo[10.3.1.0^{4,13}.0^{8,13}]hexadecane, **22**.

4-Chloro-1-butanol was used and the final product was obtained by chromatography and eluting with ethyl acetate-hexane (1:9, v/v) as a yellow oil (748 mg, 57%); ¹H NMR (600 MHz, CDCl₃) δ (ppm): 5.40 (s, 1H, H-12A), 5.35 (s, 1H, H-12B), 4.79 (dd, *J* = 16.6, 5.9 Hz, 1H, H-10), 4.45 (t, *J* = 9.4 Hz, 1H, H-10B), 3.95 – 3.86 (m, 1H, H-18), 3.60 (dt, *J* = 13.0, 4.9 Hz, 3H, H-18, H-21), 3.43 (ddd, *J* = 9.8, 8.0, 3.1 Hz, 1H), 2.69 – 2.60 (m, 1H, H-8a), 2.53 – 2.27 (m, 2H, H-12), 1.46 (s, 3H, H-14), 0.97 (d, *J* = 6.4 Hz, 3H, H-16), 0.92 (d, *J* = 7.4 Hz, 3H, H-15); ¹³C NMR (151 MHz, CDCl₃) δ (ppm): 104.28 (C-3A), 104.12 (C-3B), 102.03 (C-10A), 100.07 (C-10B), 91.21 (C-12B), 87.93 (C-12A), 81.12 (C-12aA), 80.36 (C-12aB), 67.96 (C-18A), 67.61 (C-18B), 52.59 (C-5aA), 51.67 (C-5aB), 45.34 (C-8aA), 45.02 (C-8aB), 20.39 (C-16A), 20.31 (C-16B), 13.05 (C-15A), 12.66 (C-15B); IR (ATR) ν_{\max} cm⁻¹: 2922, 2871, 1447, 1375, 1279, 1251, 1226, 1193, 1175, 1155.

(2R,5S,15R)-2,15-dimethyl-14-[(2R)-6-methylheptan-2-yl]tetracyclo[8.7.0.0^{2,7}.0^{11,15}]heptadec-7-en-5-yl-4-(4-[(1S,5R,9R,10S,12R,13R)-1,5,9-trimethyl-11,14,15,16-tetraoxatetracyclo[10.3.1.0^{4,13}.0^{8,13}]hexadecan-10-yl]oxy}butyl)piperazine-1-carboxylate, **23**.

22 (700 mg, 1.87 mmol) was used and the final product was obtained by chromatography and elution with ethyl acetate-hexane (8:2, v/v) as cream-white solid (109 mg, 7%), mp: the product started softening at 117 °C and was completely molten at 191 °C; ¹H NMR (600 MHz, CDCl₃) δ

(ppm): 5.35 (s, 1H, H-12), 5.28 (s, 1H, H-6'), 4.74 (d, $J = 3.3$ Hz, 1H, H-10), 4.49 (s, 1H, H-3'), 3.83 (dt, $J = 9.9, 6.1$ Hz, 4H, H-18, H-26), 3.65 (s, 2H, H-24), 3.37 (t, $J = 6.2$ Hz, 2H, H-21), 2.63 – 2.58 (m, 4H, H-23, H-27), 2.31 (dq, $J = 26.5, 13.8$ Hz, 1H, H-8'), 1.41 (s, 3H, H-14), 0.99 (s, 3H, H-19'), 0.93 (d, $J = 6.3$ Hz, 3H, H-15), 0.88 (d, $J = 7.2$ Hz, 3H, H-16), 0.87 (d, $J = 7.4$ Hz, 3H, H-21'), 0.84 (dd, $J = 6.6, 2.8$ Hz, 6H, H-26', H-27'), 0.65 (s, 3H, H-18'); ^{13}C NMR (151 MHz, CDCl_3) δ (ppm): 122.68 (C-6'), 104.11 (C-3), 102.03 (C-10), 95.27 (C-12), 87.89 (C-12a), 81.04 (C-3'), 67.74 (C-18), 56.63 (C-21), 56.08 (C-17'), 49.95 (C-9'), 44.32 (C-24, C-26), 24.65 (C-14), 22.80 (C-26', 27'), 22.54 (C-8), 21.01 (C-11'), 20.33 (C-15), 19.32 (C-19'), 18.68 (C-21'), 13.08 (C-16), 11.82 (C-18'); IR (ATR) ν_{max} cm^{-1} : 2936, 2869, 2851, 1695, 1463, 1432, 1415, 1376; HRMS-ESI m/z $[\text{M}+\text{H}]^+$ 837.6288 (calcd for $\text{C}_{51}\text{H}_{84}\text{N}_2\text{O}_7\text{H}$: 837.6357).

(1S,5R,9R,10S,12R,13R)-10-[(6-Chlorohexyl)oxy]-1,5,9-trimethyl-11,14,15,16-tetraoxatetracyclo[10.3.1.0^{4,13}.0^{8,13}]hexadecane, 24.

6-Chloro-1-hexanol was used and the final product was obtained by chromatography and elution with ethyl acetate-hexane (3:7, v/v) as a colourless oil (573 mg, 40%). ^1H NMR (600 MHz, CDCl_3) δ (ppm): 5.40 (s, 1H, H-12), 4.79 (d, $J = 3.4$ Hz, 6H, H-10), 3.86 (dt, $J = 9.6, 6.5$ Hz, 1H, H-18), 3.84 – 3.52 (m, 3H, H-18, H-23), 3.43 – 3.35 (m, 6H), 2.80 – 2.59 (m, 1H, H-8a), 2.49 – 2.34 (m, 2H, H-4), 2.08 – 1.90 (m, 2H, H-8), 1.46 (s, 3H, H-14), 0.97 (d, $J = 6.4$ Hz, 3H, H-16), 0.92 (d, $J = 7.4$ Hz, 3H, H-15); ^{13}C NMR (151 MHz, CDCl_3) δ (ppm): 104.08 (C-3), 102.00 (C-10), 87.93 (C-12), 81.16 (C-12a), 68.26 (C-18), 52.61 (C-5a), 45.03 (C-23), 44.49 (C-8a), 37.52 (C-9), 36.47 (C-4), 34.69 (C-7), 32.57 (C-6), 30.96 (C-19, C-22), 29.53 (C-21), 26.25 (C-20), 25.60 (C-14), 20.40 (C-16), 13.06 (C-15); IR (ATR) ν_{max} cm^{-1} : 2923, 2865, 1448, 1375, 1343, 1251, 1226, 1193, 1176.

(2R,5S,15R)-2,15-dimethyl-14-[(2R)-6-methylheptan-2-yl]tetracyclo[8.7.0.0^{2,7}.0^{11,15}]heptadec-7-en-5-yl4-(6-[(1S,5R,9R,10S,12R,13R)-1,5,9-trimethyl-11,14,15,16-tetraoxatetracyclo[10.3.1.0^{4,13}.0^{8,13}]hexadecan-10-yl]oxy)hexyl)piperazine-1-carboxylate 25.

The foregoing product **24** (500 mg, 1.24 mmol) was used and the final product was obtained by chromatography and elution with ethyl acetate-hexane (4:6 v/v) as cream-white powder (54.0 mg, 9.5%), mp: the product started softening at 126 °C and was completely molten at 162 °C; ^1H NMR (600 MHz, CDCl_3) δ (ppm): 5.34 (d, $J = 6.7$ Hz, 1H, H-12), 5.34 (s, 1H, H-6'), 4.74 (d, $J = 3.2$ Hz, 1H, H-10), 4.52 – 4.45 (m, 1H, H-3'), 3.79 (dd, $J = 16.1, 6.5$ Hz, 2H, H-18), 3.58 (s, 2H, H-28), 3.33 (dd, $J = 16.1, 6.5$ Hz, 2H, H-26), 2.61 – 2.56 (m, 2H, H-25), 2.34 (dt, $J = 14.1, 3.8$ Hz, 4H, H-23, H-29), 2.26 (d, $J = 12.3$ Hz, 2H, H-4'), 2.02 – 1.92 (m, 2H, H-8), 1.41 (s, 3H, H-14), 0.99 (s, 3H, H-19'), 0.93 (d, $J = 6.3$ Hz, 3H, H-15), 0.88 (d, $J = 6.6$ Hz, 3H, H-16), 0.86 (d, $J = 7.4$ Hz, 3H, H-21'), 0.83 (dd, $J = 6.6, 2.7$ Hz, 6H, H-26', H-27'), 0.64 (s, 3H, H-18'); ^{13}C NMR (151 MHz, CDCl_3) δ (ppm): 154.77 (C-30), 139.72 (C-5'), 122.55 (C-6'), 104.03 (C-3), 101.94 (C-10), 87.87

(C-12), 81.10 (C-12a), 75.05 (C-3'), 68.25 (C-18), 58.41 (C-14'), 56.63 (C-23), 52.54 (C-25, C-29), 49.94 (C-9'), 44.42 (C-26, C-28), 30.89 (C-19), 27.98 (C-21), 27.09 (C-22), 26.20 (C-20), 26.03 (C-14), 22.79 (C-26', C-27'), 22.53 (C-11'), 20.36 (C-15), 19.32 (C-19'), 18.68 (C-21'), 13.01 (C-16), 11.82 (C-18'); IR (ATR) ν_{\max} cm^{-1} 2927, 2851, 1698, 1431, 1234, 1225; HRMS-ESI m/z $[\text{M}+\text{H}]^+$ 865.6639 (calcd for $\text{C}_{53}\text{H}_{88}\text{N}_2\text{O}_7\text{H}$: 864.6670).

Antimalarial assays

Malaria parasite proliferation can be directly monitored in their intraerythrocytic environment through detecting and monitoring DNA replication in the absence of interference from erythrocytes, which lack DNA [44]. SYBR Green I is a fluorescent dye that interacts with DNA. The correlation between DNA content (SYBR Green I signal) and parasitaemia can be used to monitor the decrease in parasitaemia as a measurement of inhibition of parasite proliferation [44, 45]. *P. falciparum* parasites were maintained at 37 °C in human erythrocytes (O⁺) suspended in complete culture medium 1640 medium (RPMI) (Sigma-Aldrich) supplemented with 25 mM HEPES (Sigma-Aldrich), 20 mM D-glucose (Sigma-Aldrich), 200 μM hypoxanthine (Sigma-Aldrich), 0.2% sodium bicarbonate, 24 $\mu\text{g}/\text{ml}$ gentamycin (Sigma-Aldrich) and 0.5% AlbuMAX II in an atmosphere consisting of a 90% N_2 , 5% O_2 , and 5% CO_2 [46]. *In vitro* ring stage intraerythrocytic *P. falciparum* parasite cultures genotyped according to drug-sensitive or resistant strains; W2 (chloroquine, quinine, pyrimethamine and cycloguanil resistant), K1 (chloroquine, pyrimethamine, mefloquine and cycloguanil resistant) and NF54 (drug-sensitive) (200 μL at 1% haematocrit, 1% parasitaemia) were treated with the compounds. The controls for this assay included chloroquine disulfate (1 μM , as positive control) and complete Roswell Park Memorial Institute (RPMI) media (as negative control) and grown for 96 h at 37 °C under a 90% N_2 , 5% O_2 , and 5% CO_2 gas mixture in 96-well plates. At the conclusion of the 96 h growth period, equal volumes (100 μL each) of the *P. falciparum* parasite cultures were combined with SYBR Green I lysis buffer (0.2 $\mu\text{L}/\text{mL}$ 10000 x SYBR Green I, Invitrogen 20 mM Tris, pH 7.5, 5 mM EDTA, 0.008% (w/v) saponin, 0.08% (v/v) Triton X-100). The samples were incubated at 37 °C for 1 h after which the fluorescence was measured using a Fluoroskan Ascent FL microplate fluorometer (Thermo Scientific, excitation at 485 nm and emission at 538 nm). The 'background' fluorescence (i.e. that measured in the samples derived from chloroquine-treated iRBC samples in which parasite proliferation was completely inhibited) was subtracted from the total fluorescence measured for each sample to provide a measure of parasite proliferation. Data obtained were analysed in Excel and sigmoidal dose-response curves were plotted using GraphPad 5.0. Experiments were performed in triplicate, and repeated mainly 3 times.

Cytotoxicity assay

HEK293 cells (ATCC[®] CRL-1573[™]) were cultured in Dulbecco's modified essential medium (DMEM; Hyclone, GE healthcare, South Logan, UT, USA) containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1% 200 mM L-glutamine and 1% non-essential amino acids (Lonza, Basel, Switzerland). Cells were cultured at 37 °C in a humidified atmosphere of 5% CO₂. For compound treatment, cells were seeded into a 96 well plate and cultured until 80-90% confluent. Stock solutions for compounds were prepared in ethanol preheated to 40 °C. All subsequent dilutions were prepared in serum free DMEM and vehicle controls were included in all experiments.

Following this, *in vitro* cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma-Aldrich). The HEK293 cells were seeded in a 96-well plate and incubated until cells were ~90% confluent. After 24 hours exposure to the compounds (12 -1800 µM) the growth medium was removed, cells rinsed twice with 1 x phosphate buffered saline (PBS) and 100 µL fresh serum free medium containing 5 mg/mL MTT solution was added. Cells were then incubated for 4 hours at 37 °C, after which the MTT was carefully removed and replaced with 100 µL DMSO. After 1 hour of incubation at 37 °C, cell viability was determined using a microplate reader (SpextraMac Paradigm) at an excitation wavelength of 550 nm and emission wavelength of 630 nm with DMSO measured as a blank. Cell viability is expressed as a percentage relative to the untreated control, which is assumed to be 100% viable. As a positive control, cells were treated with 0.01% Triton-X 100 (Sigma-Aldrich, St Louis, MO, USA) for 4 hours. Using the MTT assay data, IC₅₀ values were calculated using GraphPad Prism 5. In brief, data was normalised to the negative controls (presumed to be 100% viable), followed by the log-transformation of the concentration values. The curve was fitted using the log (inhibitor) vs. response function and the IC₅₀ values calculated. Experiments were performed at least in triplicate.

Antituberculosis assay

Mtb H37Rv from stock cultures were initially inoculated for 1:10 dilution in a 25 cm² tissue flasks for 4 days and the OD₆₀₀ reached 0.6. These cultures were sub-cultured at 1:5 dilutions into 75 cm² until OD₆₀₀ of 0.3 was reached for the experiment. Aliquots of 10 mL from a single culture were transferred into 25 cm² tissue flasks. Each culture flask was treated with the desired amount of drug (80 and 10 µM) for 24 hours and one control flask with no drugs. Two hundred µL of the untreated culture flasks was used to count CFU/ml at the time of treatment (T-zero). After 24 hours of incubation at 37 °C, aliquots of 200 µL from each culture flask was centrifuged, re-suspended and washed twice in 7H9 medium and CFU were plated on 7H11 agar. CFUs were counted after 12-15 days.

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CHAPTER 4

SUMMARY AND CONCLUSION

Malaria and tuberculosis (TB) continue to degrade and destroy many lives in most resource limited regions of the world. These diseases are most prevalent in Africa and South-East Asia. Malaria is estimated to have killed at least 438,000 people and infected 214 million people in 2014. The majority of the cases come from the African region (88%) which also accounted for 90% of the estimated deaths. That year alone at least 306,000 children under the age of five died of malaria and 292,000 of these lived in the African region (WHO, 2015a).

Malaria treatment still largely depends on chemotherapy for which artemisinins remain the most effective drugs. The mechanism of action of artemisinins is not fully elucidated. It has long been suggested that this involves the generation of carbon-centred radicals which interact with vital biomolecules and lead to parasite death (O'Neill et al., 2010). Inconsistencies with this model are highlighted by Haynes *et al.* (Haynes et al., 2013). Alternatively artemisinins are proposed to act by oxidising reduced cofactors such as FADH₂ in redox homeostasis pathway of the malaria parasite. This leads to the buildup of reactive oxygen species (ROS) which ultimately kill the parasite (Haynes et al., 2012).

To safeguard artemisinins against resistance, these drugs are used in artemisinin combination therapies (ACTs) where they are partnered with longer acting antimalarial drugs. However, there are now incontrovertible signs that resistance by the most important malaria *Plasmodium falciparum* (*Pf*) to artemisinins is now emerging. The underlying mechanisms of resistance have been explained and it appears that antimalarial efficacy of artemisinins is endangered (Paloque et al., 2016). Therefore, new and effective antimalarials are urgently needed.

More alarming are the 1.4 million deaths and 10.4 million cases due to tuberculosis (TB) estimated to have occurred globally in 2015. As in the malaria report, the majority of cases are from the African (26%) and South-East Asian (61%) regions (WHO, 2016). The incidence of TB in the African region is higher than the global average: there are 281 TB cases per 100,000 people in Africa compared to 133 TB cases per 100,000 people on a global basis. The African region is also noted for the prevalence of human immunodeficiency virus (HIV) and TB coinfections (HIV/TB). In 2014 it is estimated that 400,000 of the TB deaths and 12% of the TB cases were due to HIV/TB coinfections (WHO, 2015b).

The Bacille Calmette-Gièrin (BCG) TB vaccine is currently the only one used and this offers minimal protection in children (Sterne et al., 1998). There are currently over 16 candidate

vaccines in various stages of clinical development (Ginsberg et al., 2016). Therefore, chemotherapy remains the vital prevention and treatment method. This is used despite its drawback of protracted treatment regimens which lead to non-compliance and thus treatment failures (Nimmo et al., 2015). This is one of the factors that contribute to drug resistance. No new TB drugs have been discovered for over 50 years. It is only recently that bedaquiline and delamanid have received conditional approval (Brigden et al., 2015). These drugs are used in cases of multidrug-resistant TB (MDR-TB) treatments but have some shortcomings. In a phase II placebo-controlled study it was found there were more deaths in bedaquiline treated groups than the placebo groups (Diacon et al., 2014). On the other hand, delamanid has been found to induce hepatotoxicity and QT prolongation (Gler et al., 2012). Therefore, these drugs cannot be effectively used until all these safety concerns have been cleared.

Currently used anti-TB first-line drugs are rifampicin, isoniazid, pyrazinamide and ethambutol. Resistance against rifampicin and isoniazid has arisen and this is defined as MDR-TB. MDR-TB with additional resistance to fluoroquinolones and injectable aminoglycosides amikacin, capreomycin or kanamycin is called extensively drug-resistant TB (XDR-TB) (Zumla et al., 2015, Zumla et al., 2014). There are also sporadic cases of TB resistant to all second-line antimalarials called totally drug-resistant (TDR-TB) in India and Italy (Maeurer et al., 2014, Migliori et al., 2007, Velayati et al., 2009). The spread of such strains will be cataclysmic to the control of TB. As such, new TB drugs are urgently needed if TB is to be entirely eradicated.

Despite obvious differences between pathogens responsible for malaria and TB, these organisms appear to converge on the need for cholesterol for growth. The malaria parasite diverts and salvages cholesterol from low density lipoproteins and *de novo* synthesized lipids from the endoplasmic reticulum in liver cells (Grellier et al., 1991, Labaied et al., 2011, Bano et al., 2007). During the erythrocyte stages it also appears that cholesterol is incorporated into the parasitophorous vacuolar membrane and forges nutrient trafficking pathways (Tokumasu et al., 2014). Notably, *Mycobacterium tuberculosis (Mtb)* utilizes cholesterol as a carbon source and this is important for driving its virulence. There is also a putative cholesterol transporter known as Mce4 which may enhance an uptake of this molecule into the bacterium (Brzostek et al., 2009, Ouellet et al., 2011, Pandey and Sasseti, 2008).

With the above considerations, new artemisinin derivatives with activity against both malaria and TB are in principle attainable. In fact, a mycobactin-artemisinin conjugate has been synthesized and this was highly potent against both malaria and TB. This conjugate was also active against some XDR-TB strains. This highlights the fact that artemisinins are potent agents against TB (Miller et al., 2011).

The aims of this study were to synthesise and assess *in vitro* activities of a series of artemisinin-cholesterol conjugates against CQ-sensitive and CQ-resistant strains of *P. falciparum* and against *Mtb*. A selected group of these compounds was also to be assessed for antimalarial activity against the liver stage forms of the malaria parasite. For TB, the H37Rv strain was available for screening. It was anticipated that the artemisinin-cholesterol conjugates initially shown to be active against *Mtb* would be assessed for their ability to penetrate infected macrophages and kill *Mtb* within the macrophage.

A series of novel artemisinin-cholesterol conjugates were prepared. The compounds **7**, **18**, **20**, **22** and **24** were prepared by a modified etherification method described by Li *et al.* (Li *et al.*, 2000). Reaction of these intermediates with piperazine and subsequently cholesteryl chloroformate afforded compounds **19**, **21**, **23** and **25** in low to moderate yields (7 – 45%). The compounds **11** – **15** and **17** were synthesized through the modification of the method described by Haynes *et al* (Haynes, 2010). Compound **8** was obtained by direct conjugation of dihydroartemisinin with cholesteryl chloroformate. Compound **9** was synthesised from dihydroartemisinin and artesunate through a coupling reaction using *N,N'*-dicyclohexylcarbodiimide. The structures of these compounds were elucidated by the use of ¹H nuclear magnetic resonance (NMR), ¹³C NMR, and infra-red (IR) spectroscopies, and high resolution mass spectrometry. The cLogP values (ACD/Chemsketch v 14.02) for the compounds ranged from 13.9 – 15.3; thus the compounds are highly lipophilic.

Antimalarial activity (IC₅₀) of the novel artemisinin-cholesterol conjugates was determined against *Pf* chloroquine (CQ) CQ-sensitive NF54, and CQ-resistant K1 and W2 strains. The antimycobacterial activities were determined from colony forming units (CFU/ml) resulting from treatment of *Mtb* H37Rv cultures with each of the compounds at 10 and 80 µM concentrations. The cytotoxicities were determined from *in vitro* cell viability of the HEK293 cells using the synthesised compounds in conjunction with the 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT) assay.

All the compounds were initially evaluated for *in vitro* activity against *Pf* NF54 asexual stage parasites in dual point screening. The compounds **7**, **8**, **9**, **19**, **21**, **23** and **25** had minimal to no activity in dual point assay studies and were not considered for determination of the IC₅₀ values. However, compounds **11** – **15** and **16** showed moderate to good activity in this screening and were taken forward for determination of IC₅₀ values. The *in vitro* antimalarial activities against *Pf* NF54, K1 and W2 were evaluated alongside the comparator drugs artemether and artesunate. The compounds did not outperform the comparator drugs but showed appreciable activity with IC₅₀ values ranging from 0.03 – 2.6, 0.03 – 1.9, 0.02 – 1.7 µM for *Pf* NF54, K1 and W2 respectively. Compounds **14** and **15** were consistently the most active compounds across all

strains.

All compounds were insoluble in 100% DMSO. According to the calculated cLogP values, the compounds are highly lipophilic (13.9 – 15.3). This characteristic may explain the solubility problems observed for these compounds. It is therefore likely that low antimalarial activity is due to the compounds not fully dissolving and therefore being taken by the parasite. This further prevented the assessment of these compounds for their antimalarial activities against the early and late stage gametocytes.

The resistance index (RI) with respect to the CQ-sensitive and –resistant strains ranged from 0.67 – 0.92 and 0.65 – 0.98 for RI (K1/NF54) and RI (W2/NF54) respectively. This implies that there was no cross-resistance with these compounds. Although compound **21** had the least antimalarial activity, the RI (W2/NF54) value, 0.65, was the lowest in the series. Compounds were not cytotoxic *in vitro* towards normal HEK293 cell line as they displayed selectivity towards parasitic cells given high selectivity indices. It is only compound **21** which showed a low selectivity index with implications that this compound may have exerted its antimalarial activity through toxicity.

Antimycobacterial activity determined by CFU enumeration of *Mtb* H37Rv cultures showed percentage inhibition ranging from 3 – 38% and 18 – 52% at 10 and 80 μ M respectively. Compound **15** and **23** showed moderate activities 38 and 31% at 10 μ M respectively. At 80 μ M the percentage inhibition was 52 and 47% for **15** and **23** respectively. These compounds have been selected for more advanced screening against *Mtb*.

Although the artemisinin-cholesterol compounds herein reported did not show improved antimalarial activity as compared to artemether and artesunate, the data encourages future evaluation of these compounds in *in vivo* models, particularly liver stages. It is only compound **14** – **15** that show appreciable activity against *Pf* strains used in this study. Although antimycobacterial activity of artemisinins in general is not established, the data obtained in the current study supports findings that artemisinins do indeed possess activity against *Mtb*. This is in line with findings by Miller *et al.* that dihydroartemisinin once it is enabled to penetrate the mycolic acid wall of *Mtb* through attachment of a ligand that facilitates transport into the cytosol is highly potent (Miller *et al.*, 2011). Structural modification of the compounds evaluated in the current study may improve both antimalarial and antimycobacterial activities. The assessment of these compounds in the liver stages of malaria will greatly assist in understanding the role of cholesterol and/or its relevance thereof in malaria parasite growth. Assessment of activities of compounds **15** and **23** in infected macrophage assays *in vitro* will be undertaken. If these compounds are active in the macrophage assays their antimycobacterial activity against quiescent *Mtb* and penetration into the granuloma will be determined.

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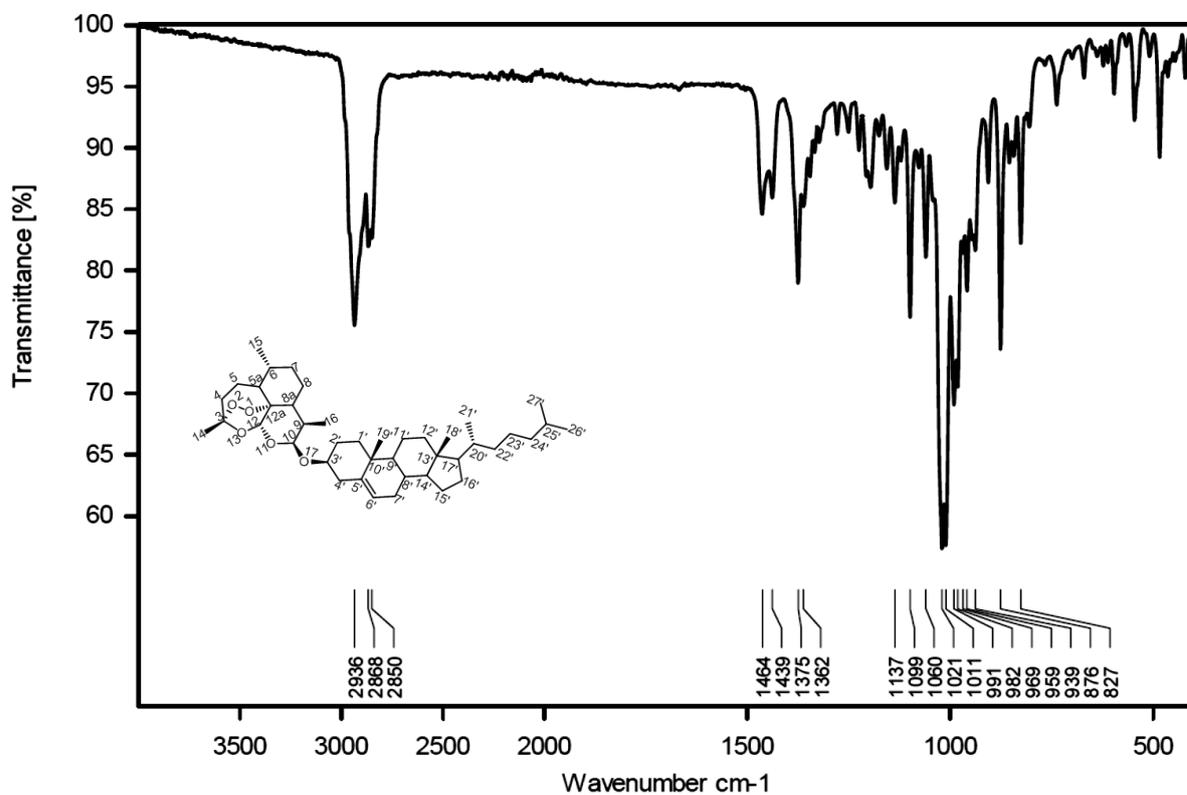
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APPENDIX A: SPECTRA

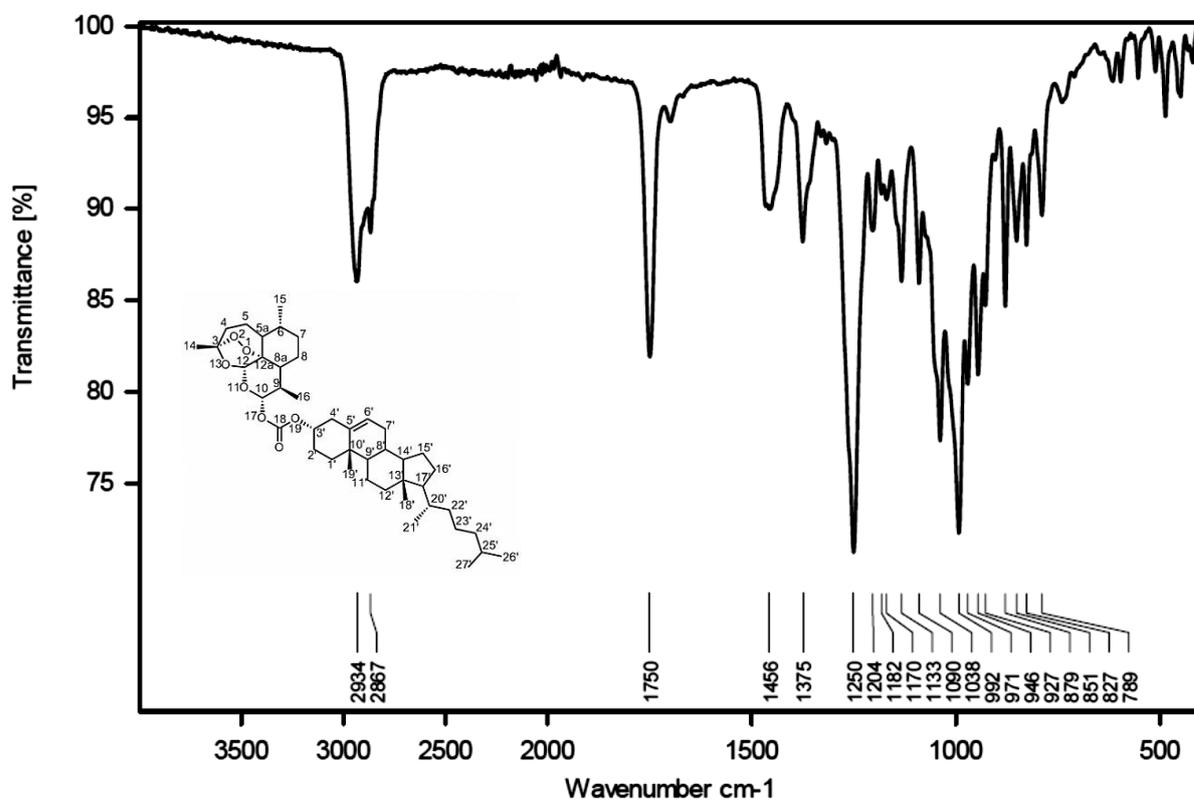
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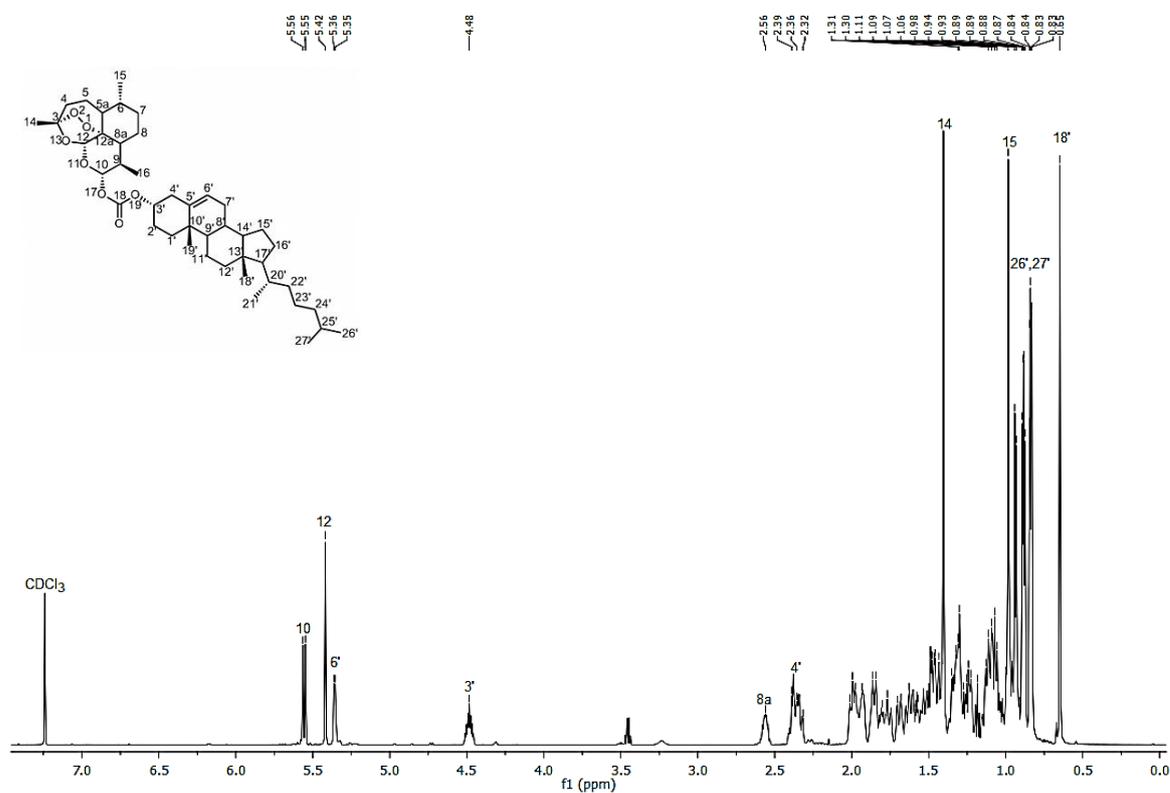


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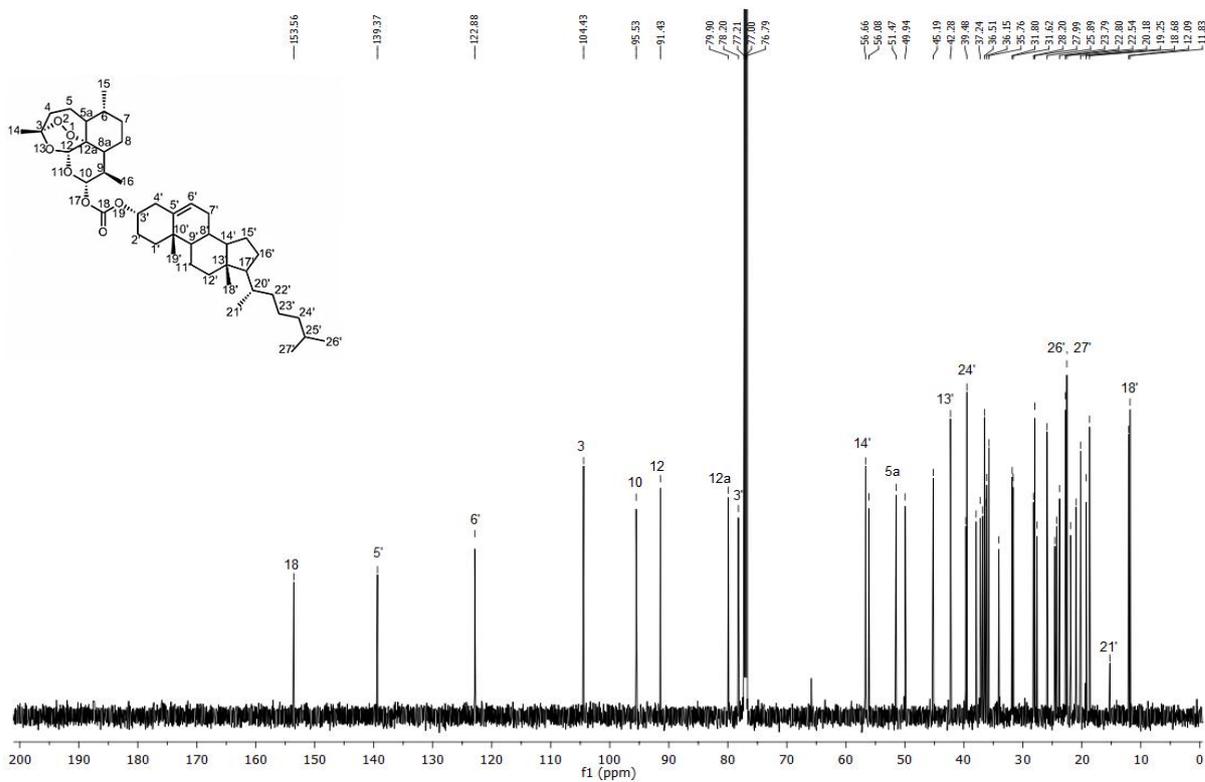
IR



^1H NMR



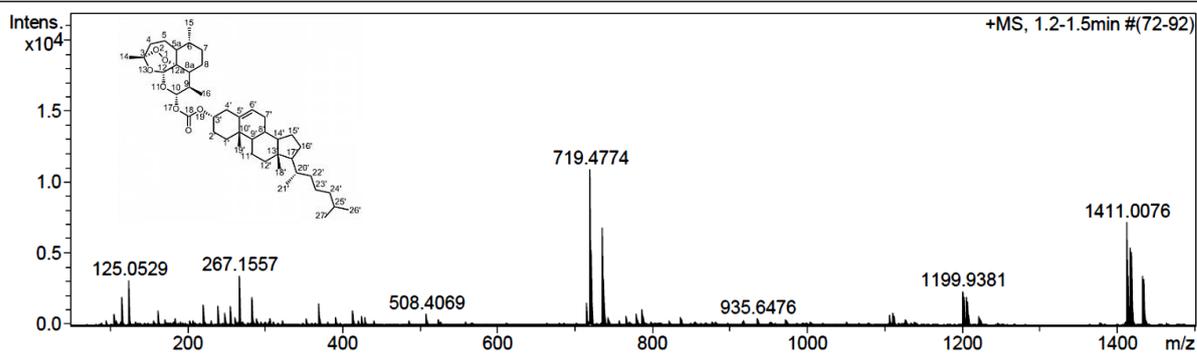
¹³C NMR



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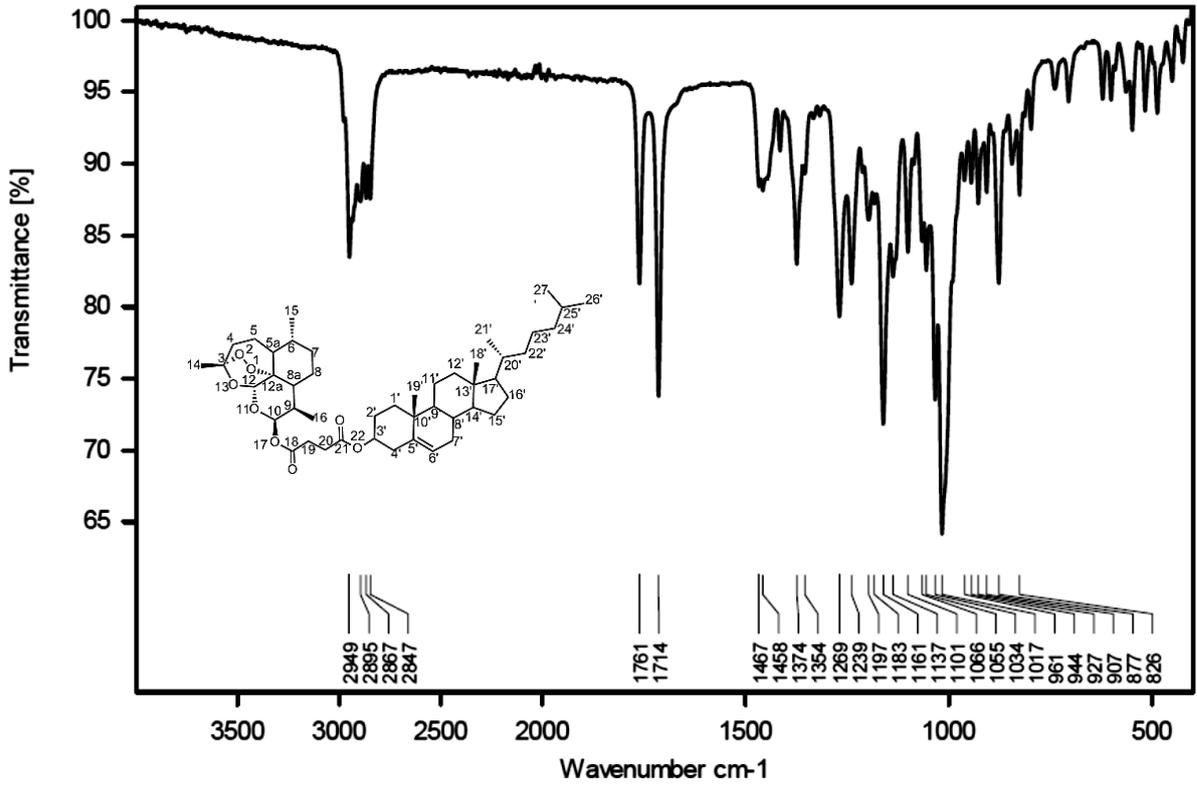
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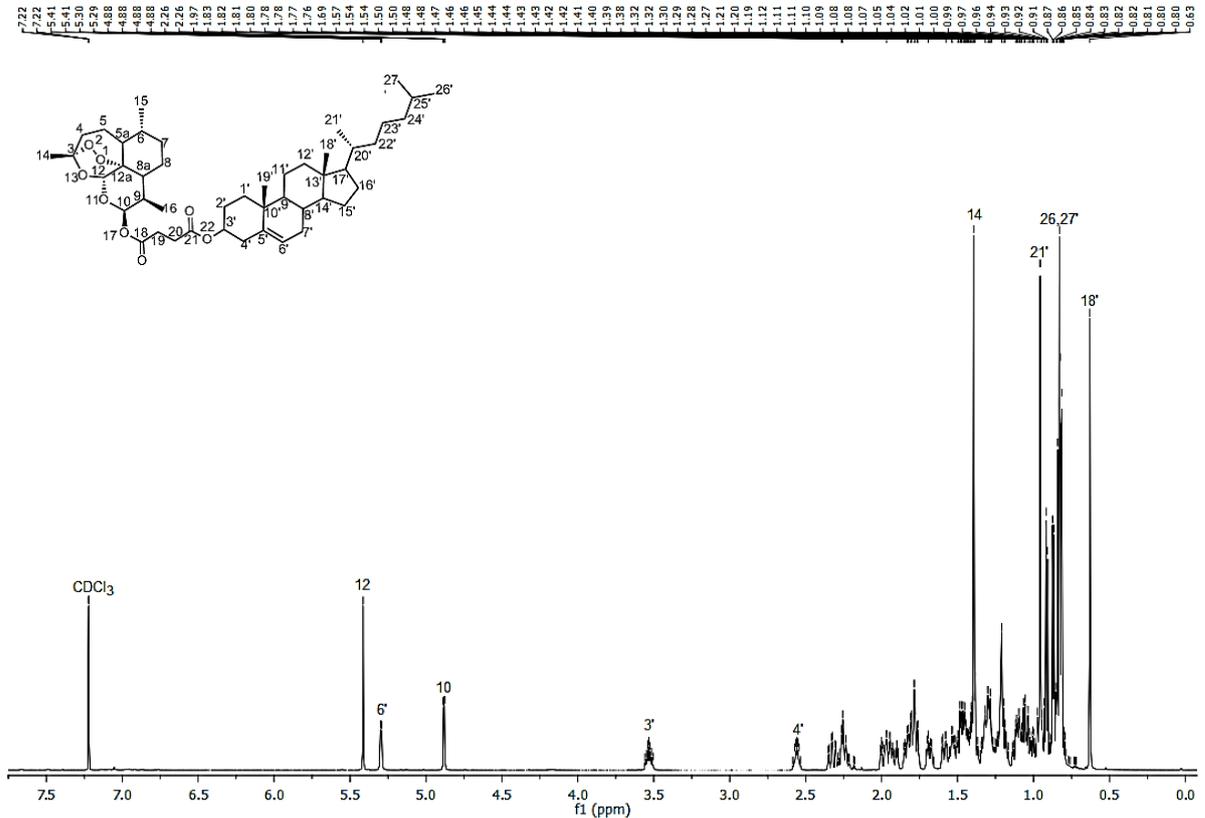


Compound 9:

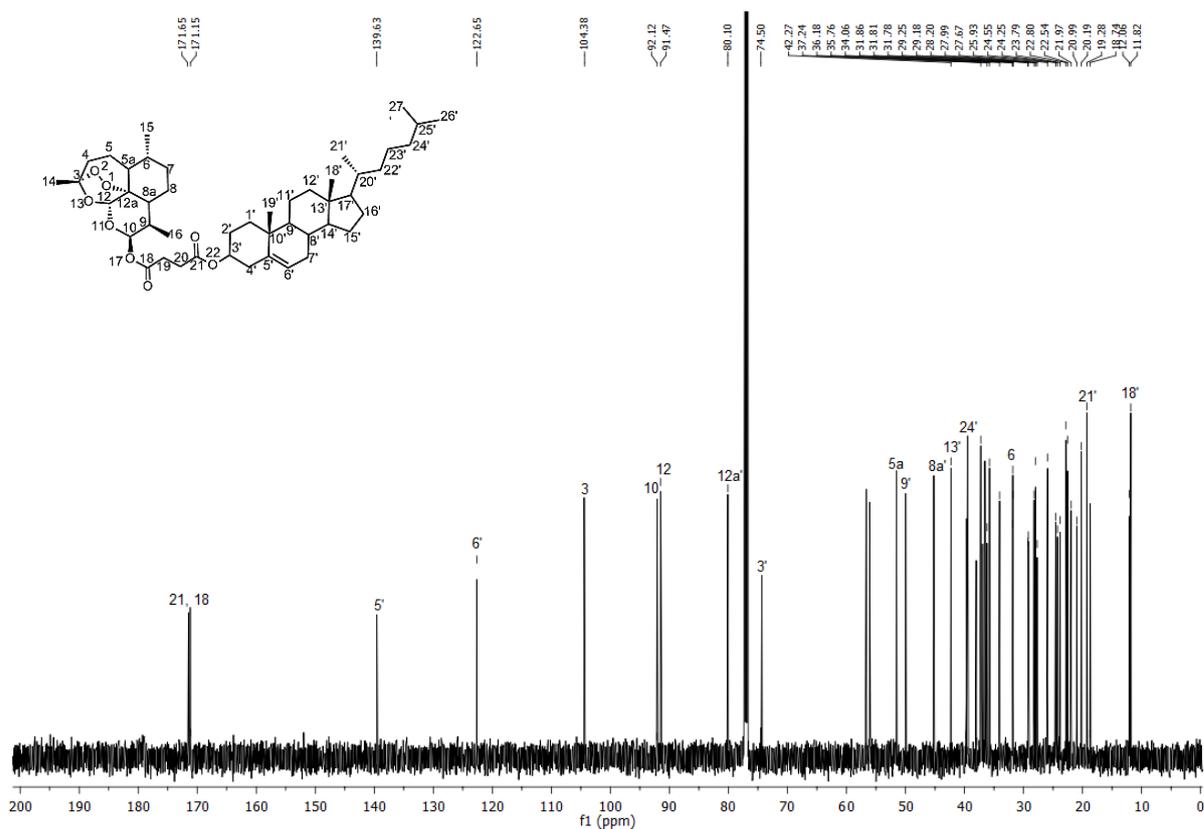
IR



¹H NMR



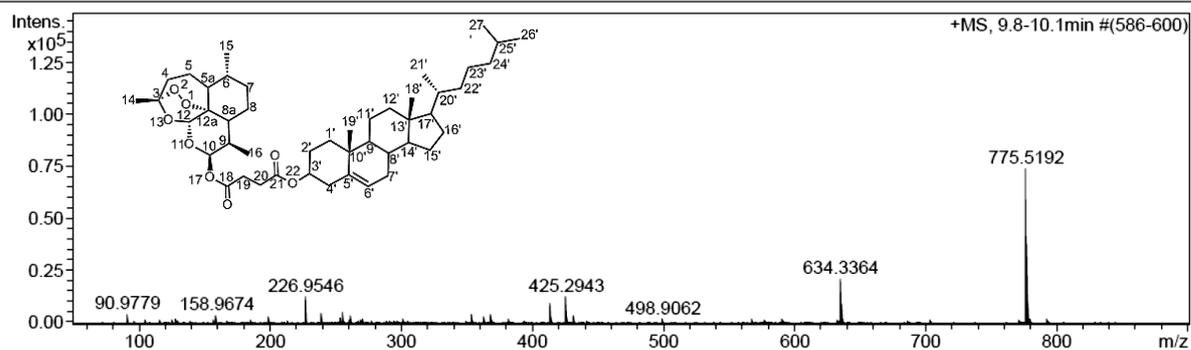
¹³C NMR



HRMS

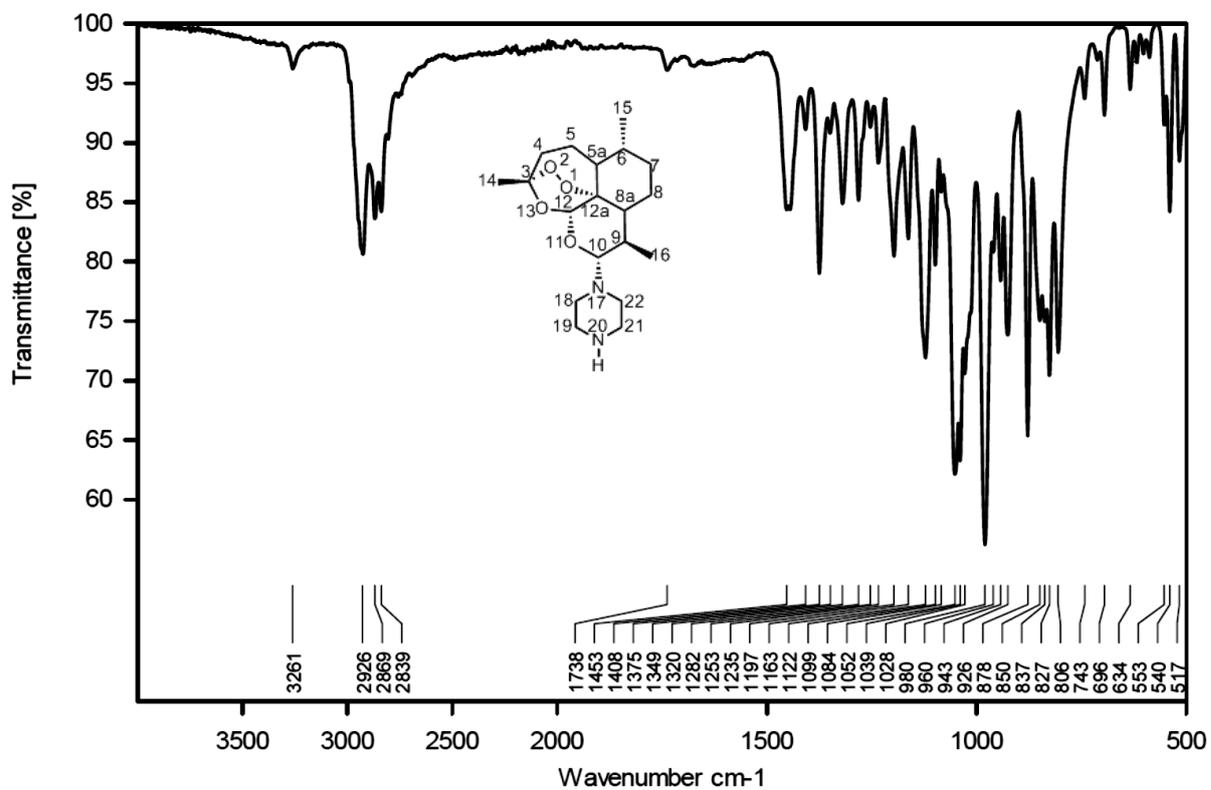
Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	0.4 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	180 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	4.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

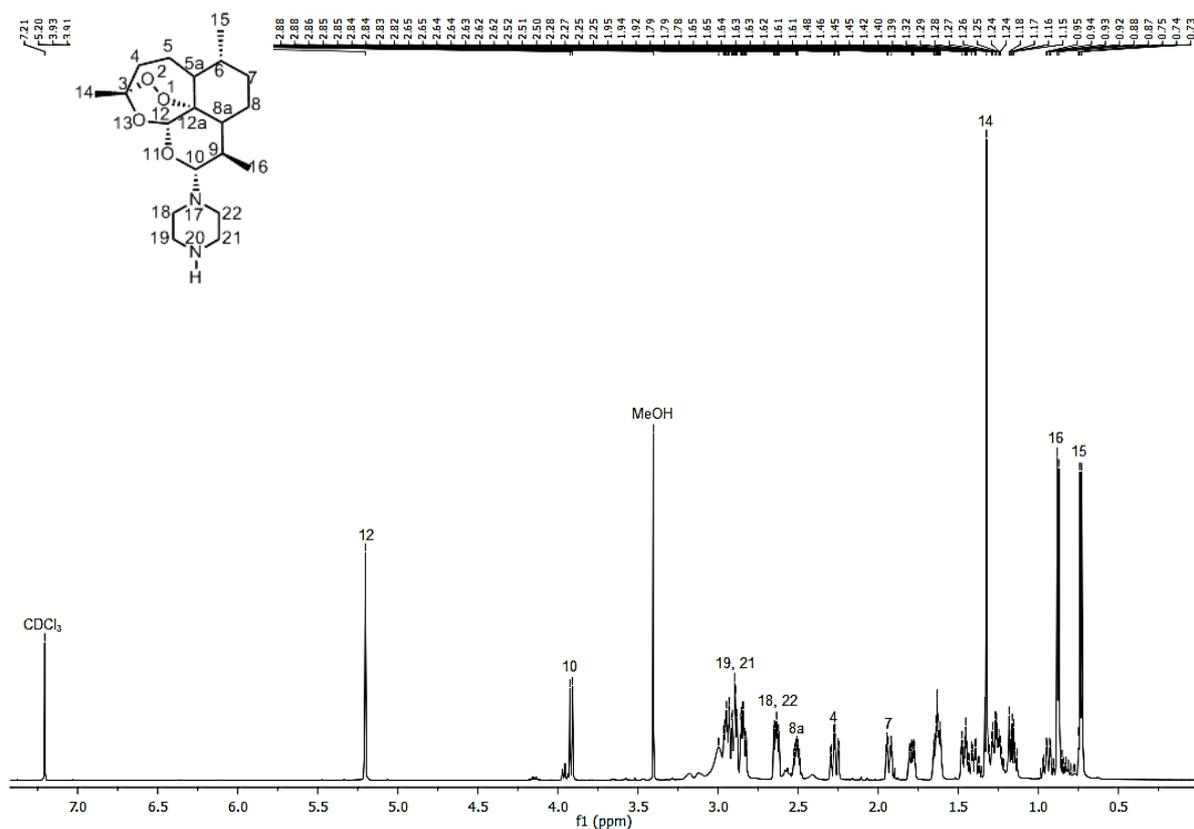


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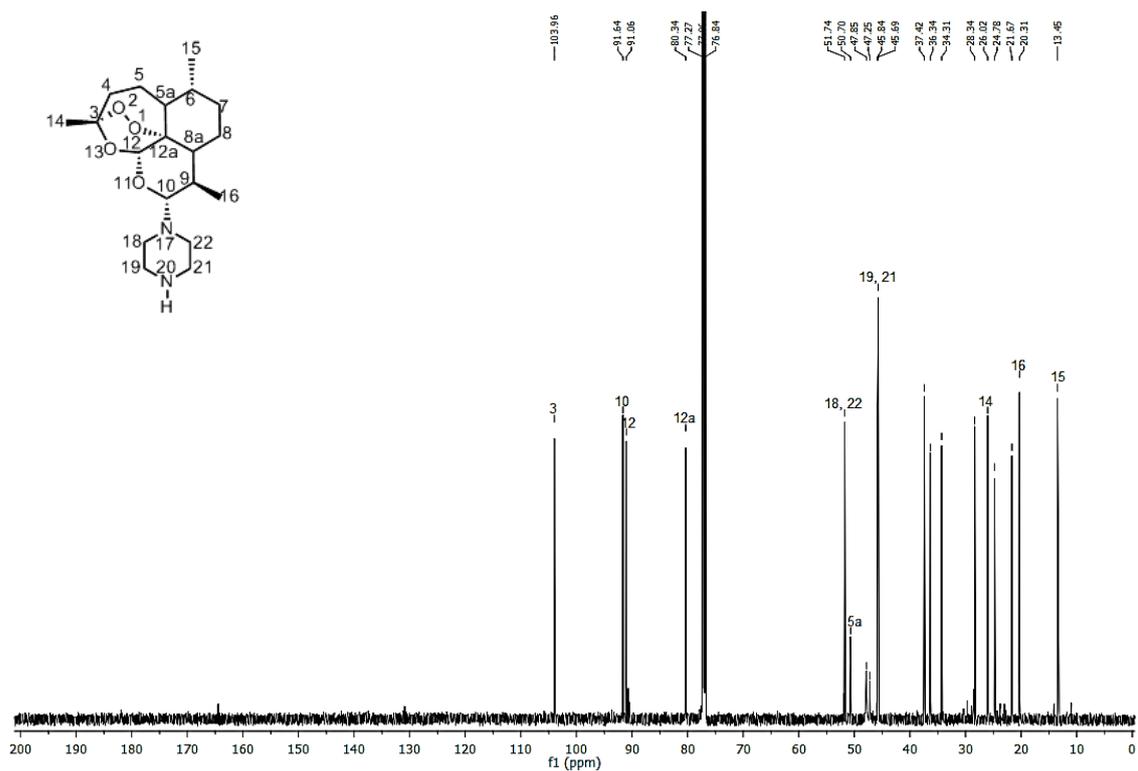
IR



¹H NMR



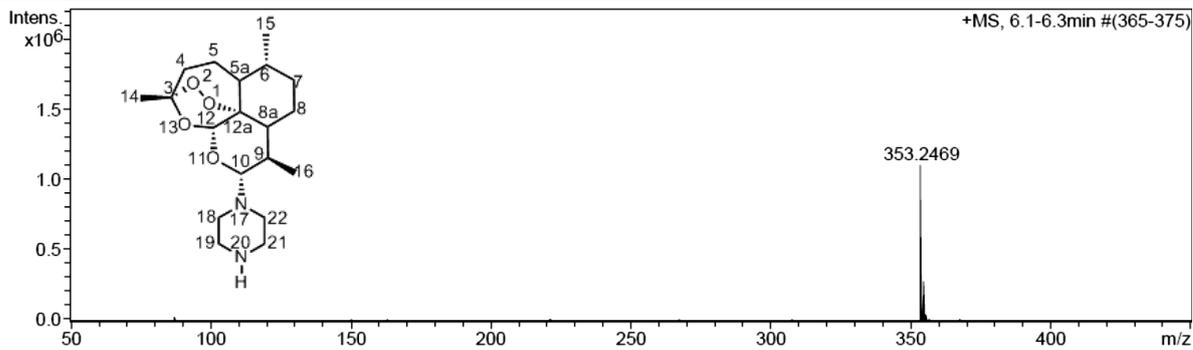
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HRMS

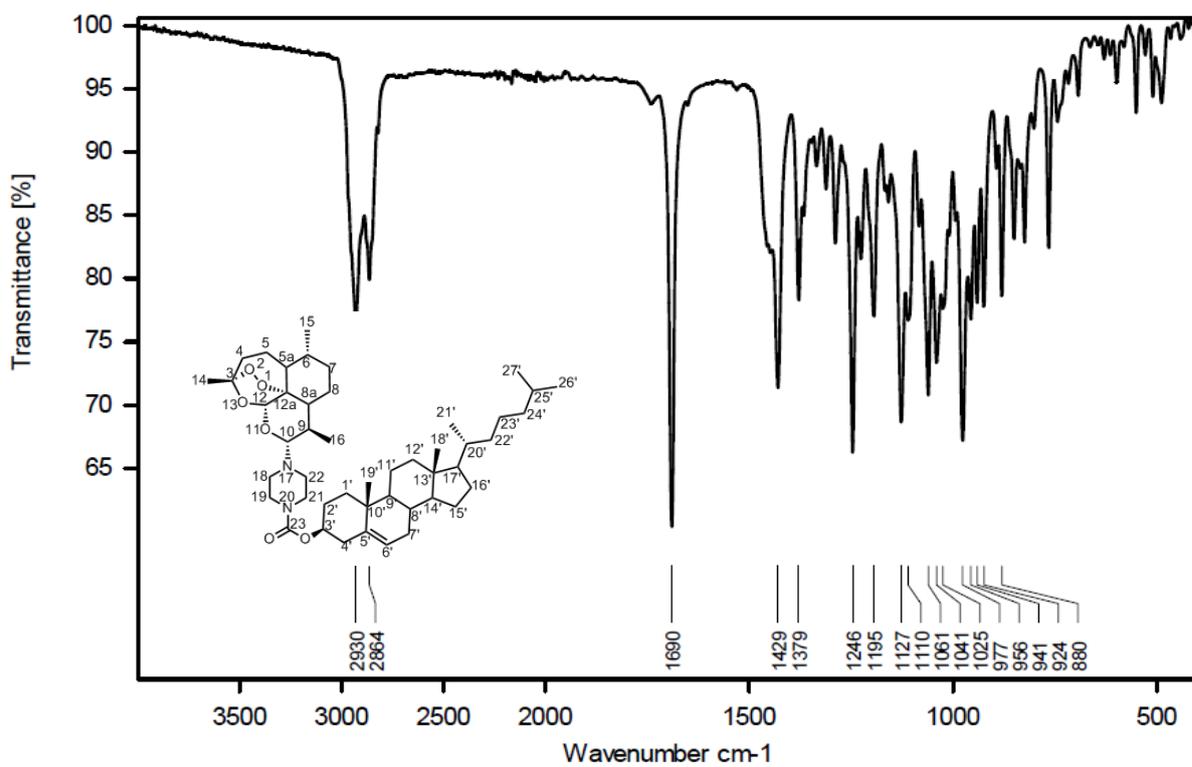
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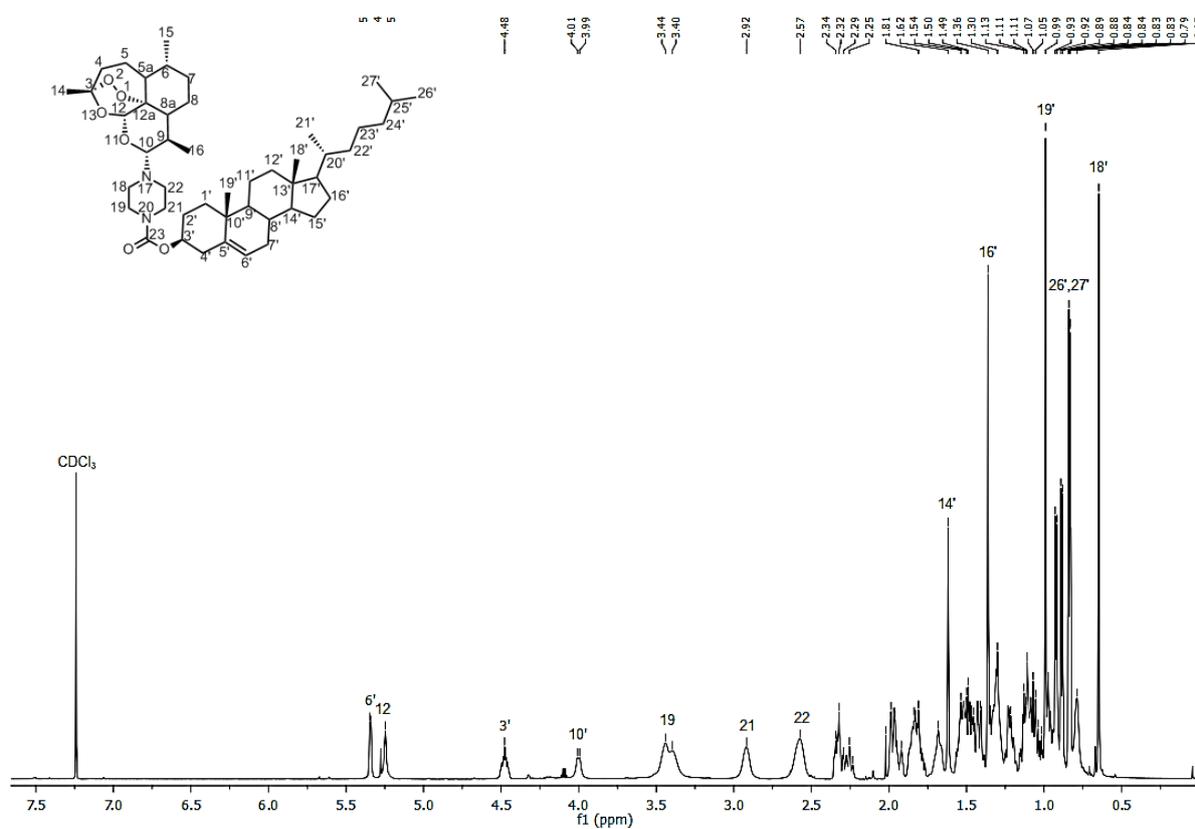


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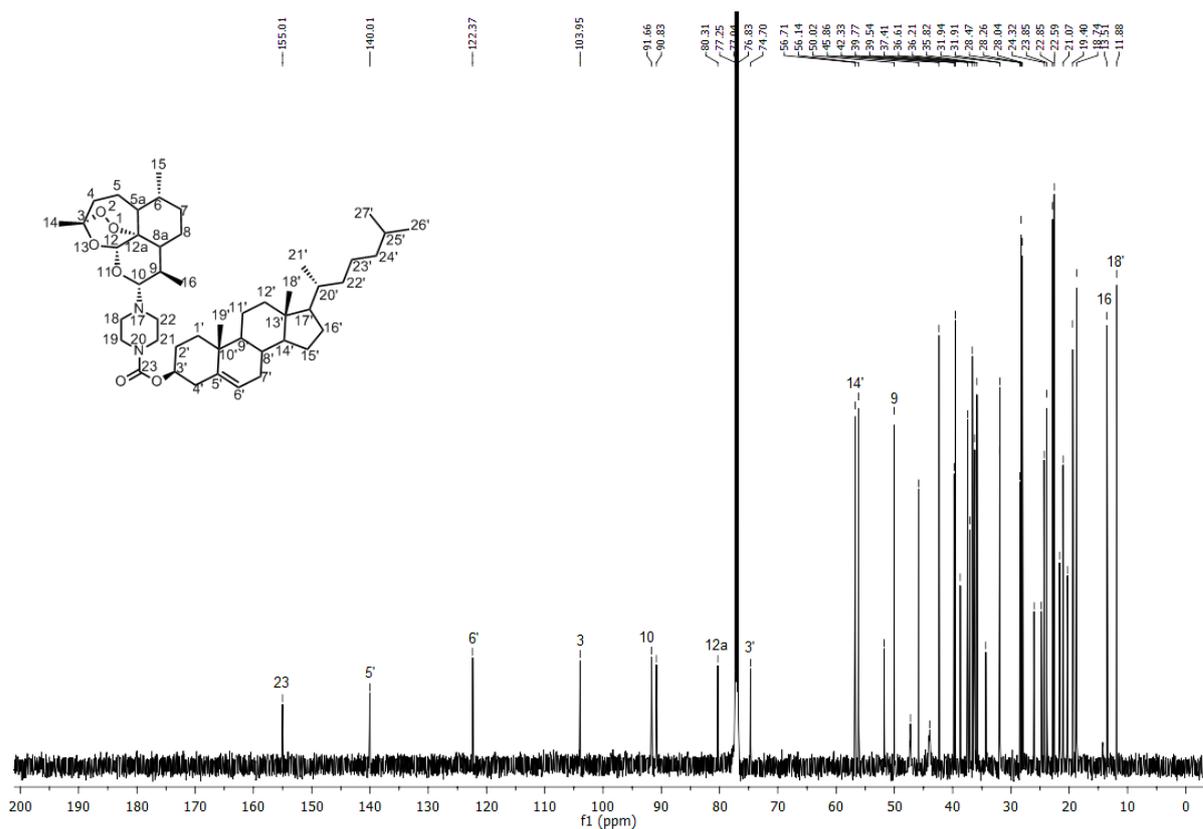
IR



^1H NMR



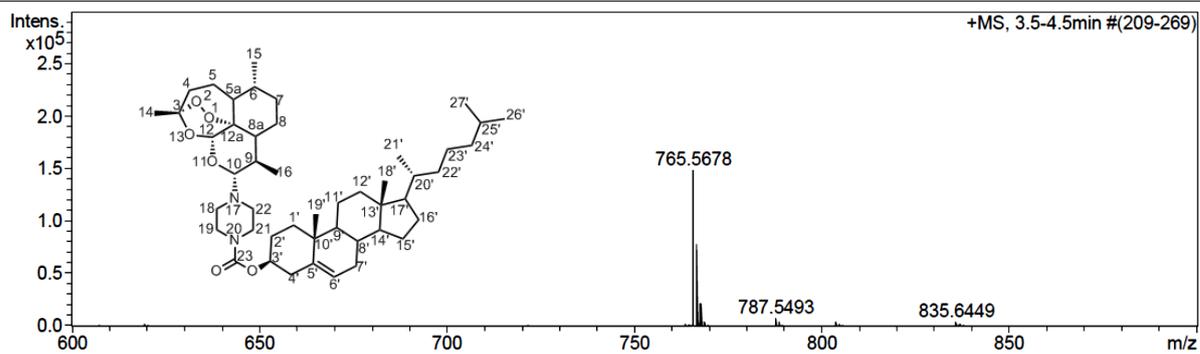
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HRMS

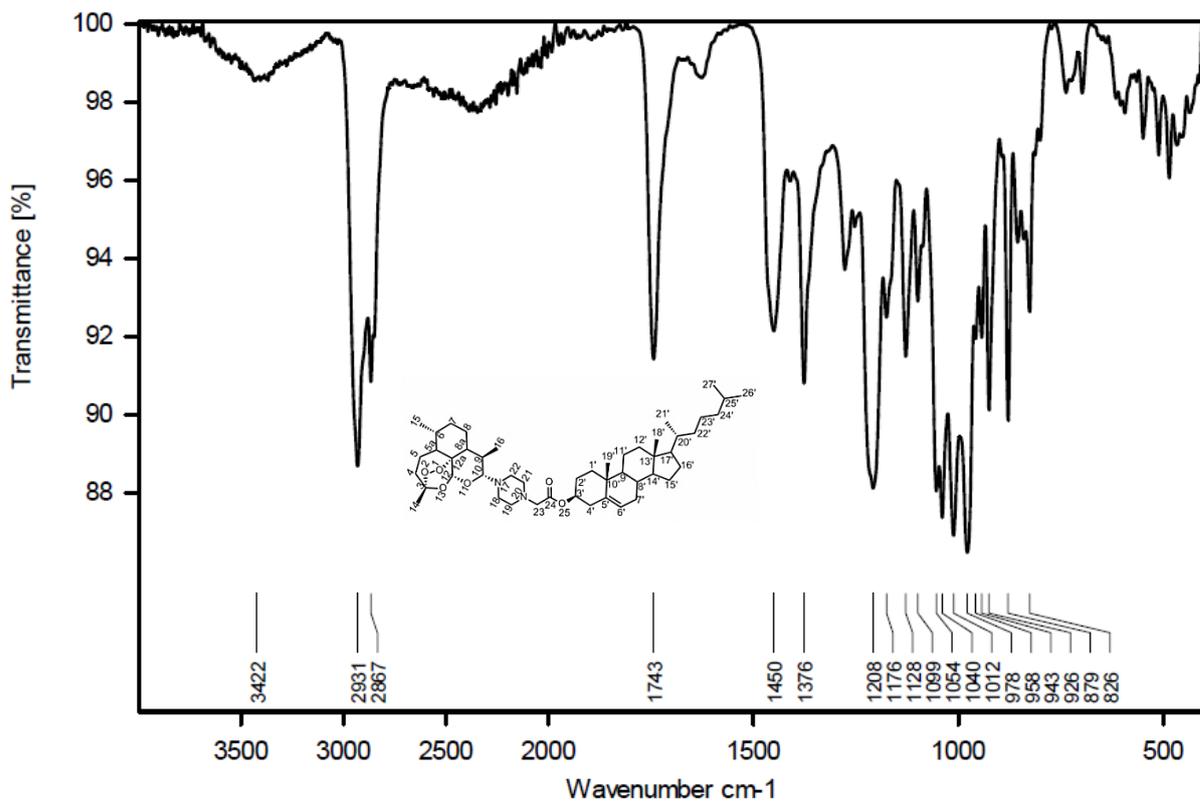
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Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

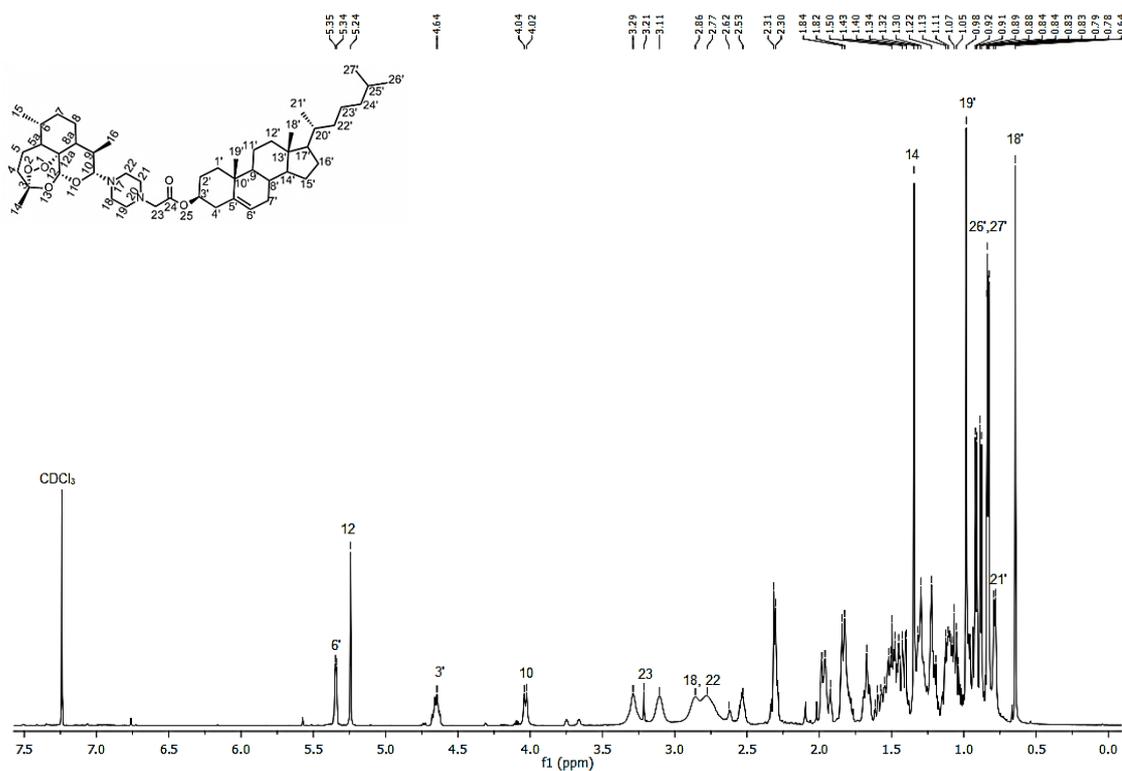


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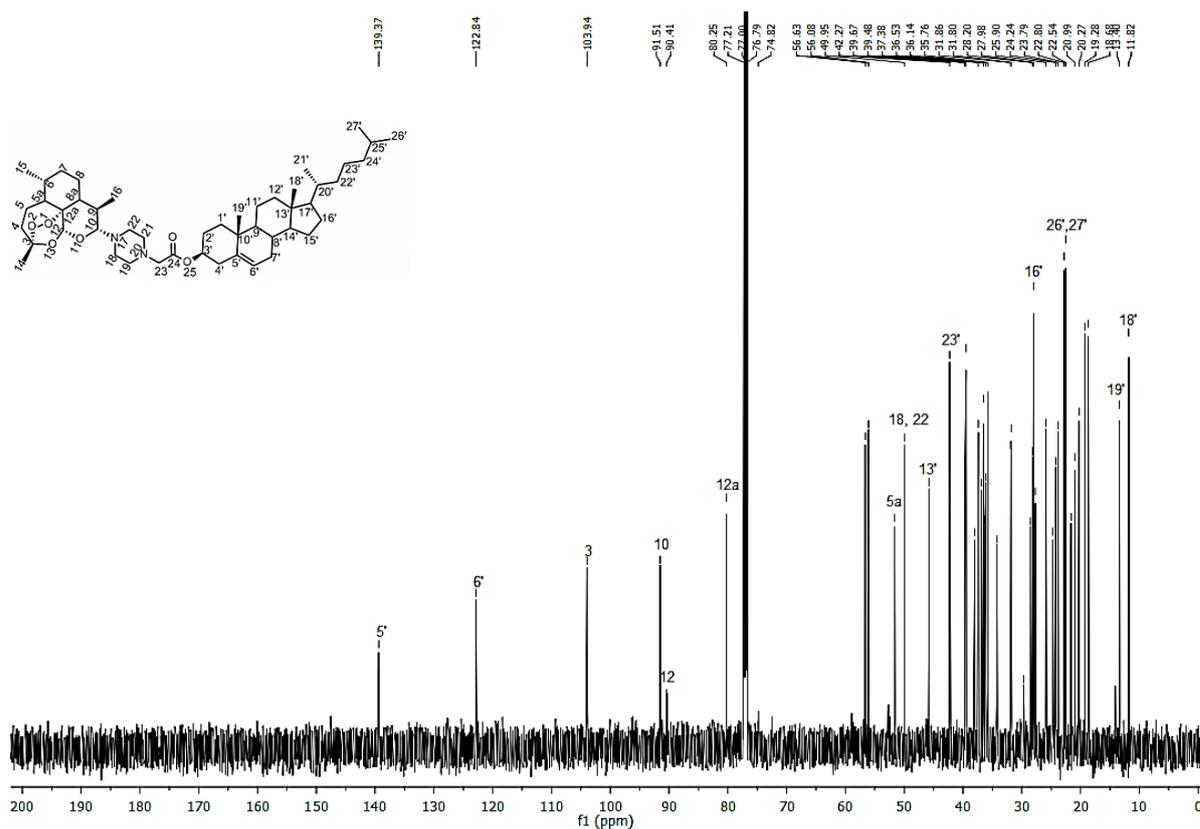
IR



¹H NMR



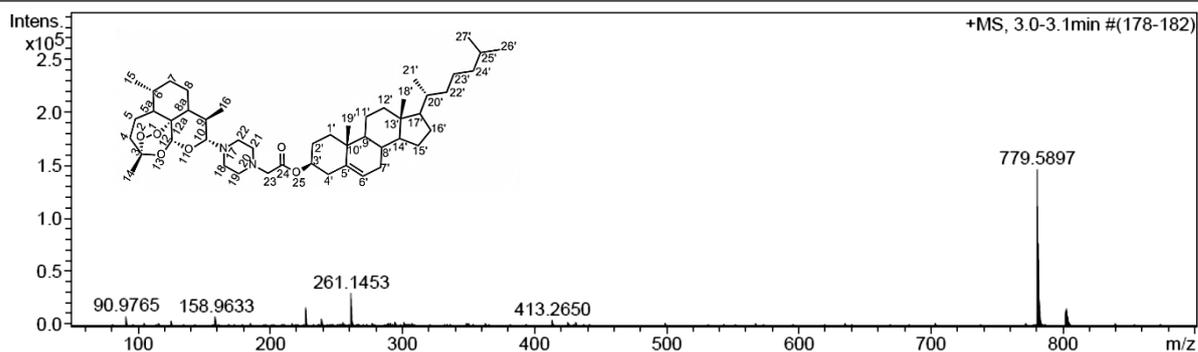
¹³C NMR



HRMS

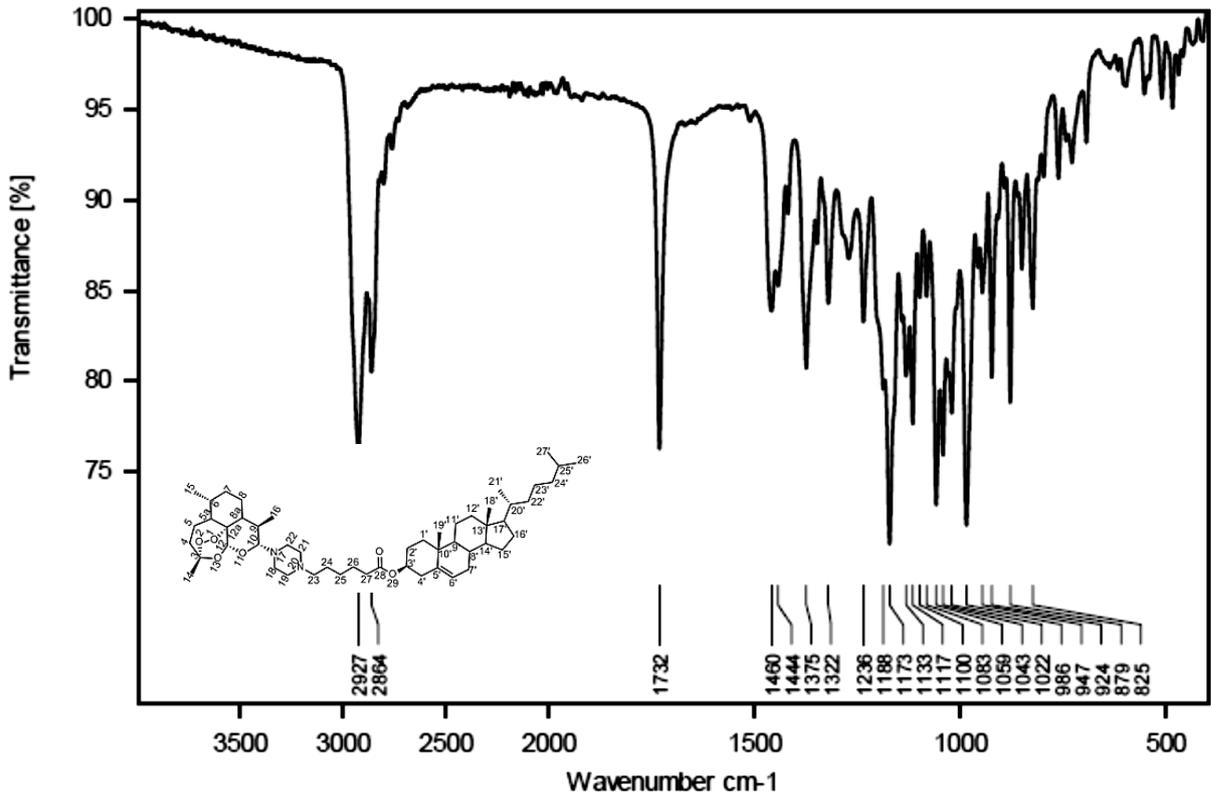
Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	0.4 Bar
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Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

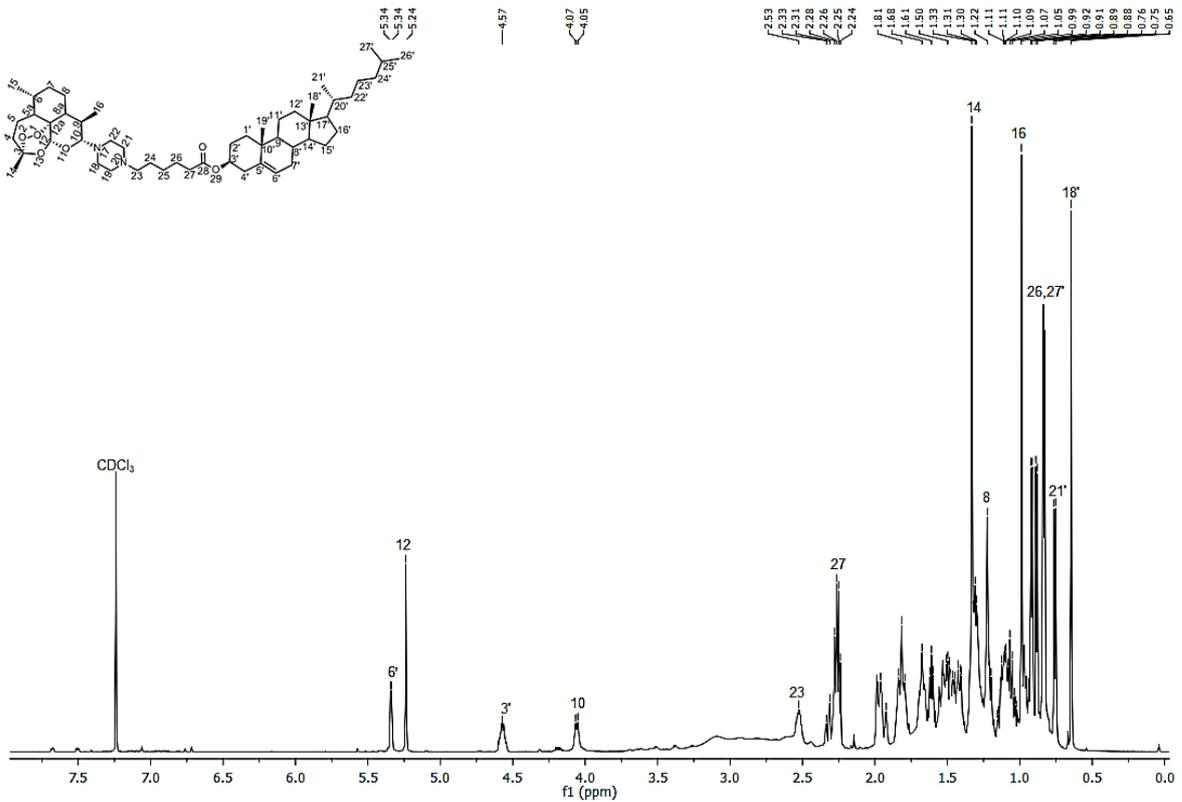


Compound 15:

IR

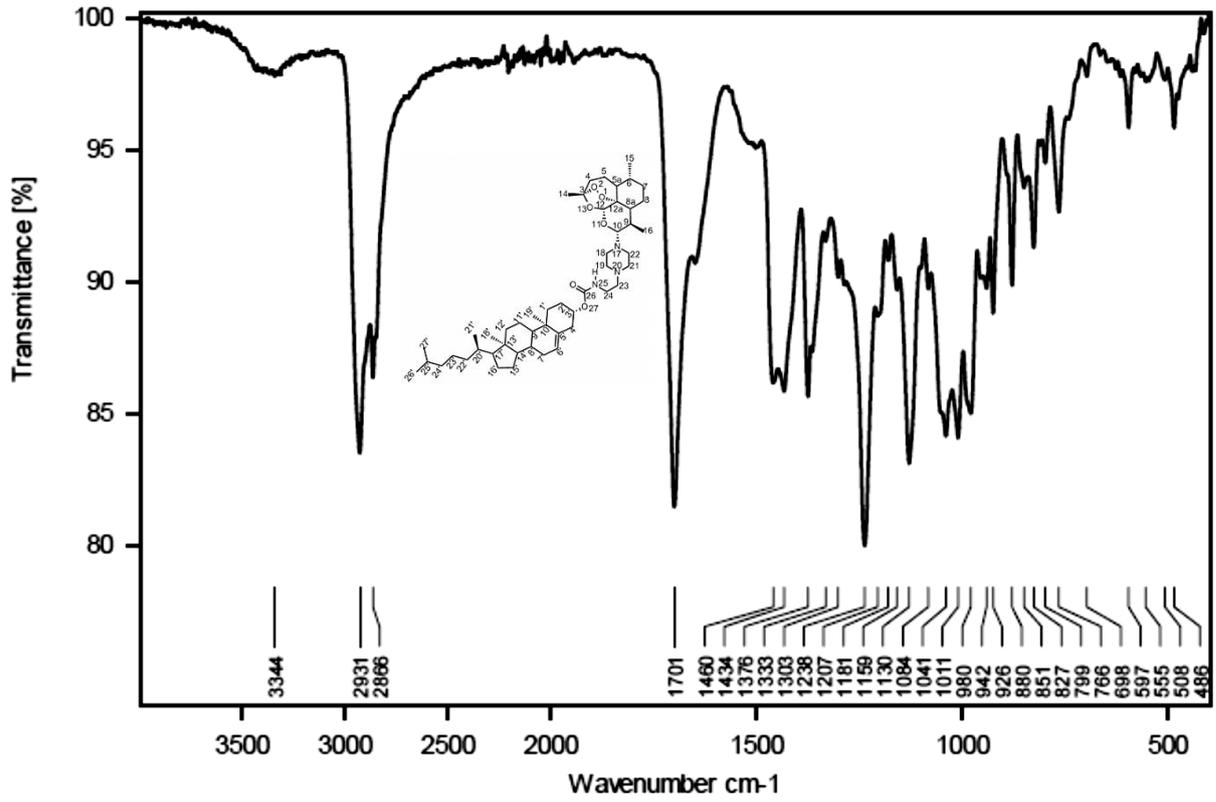


¹H NMR

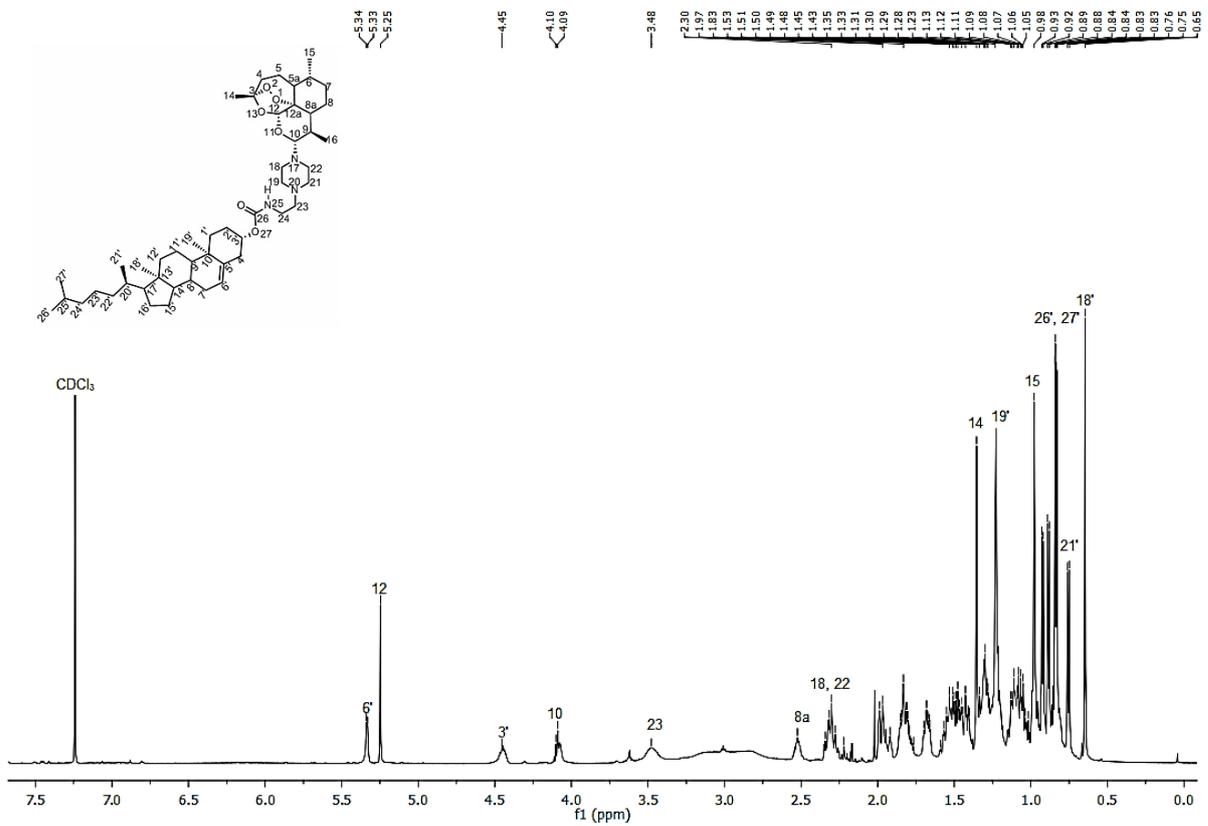


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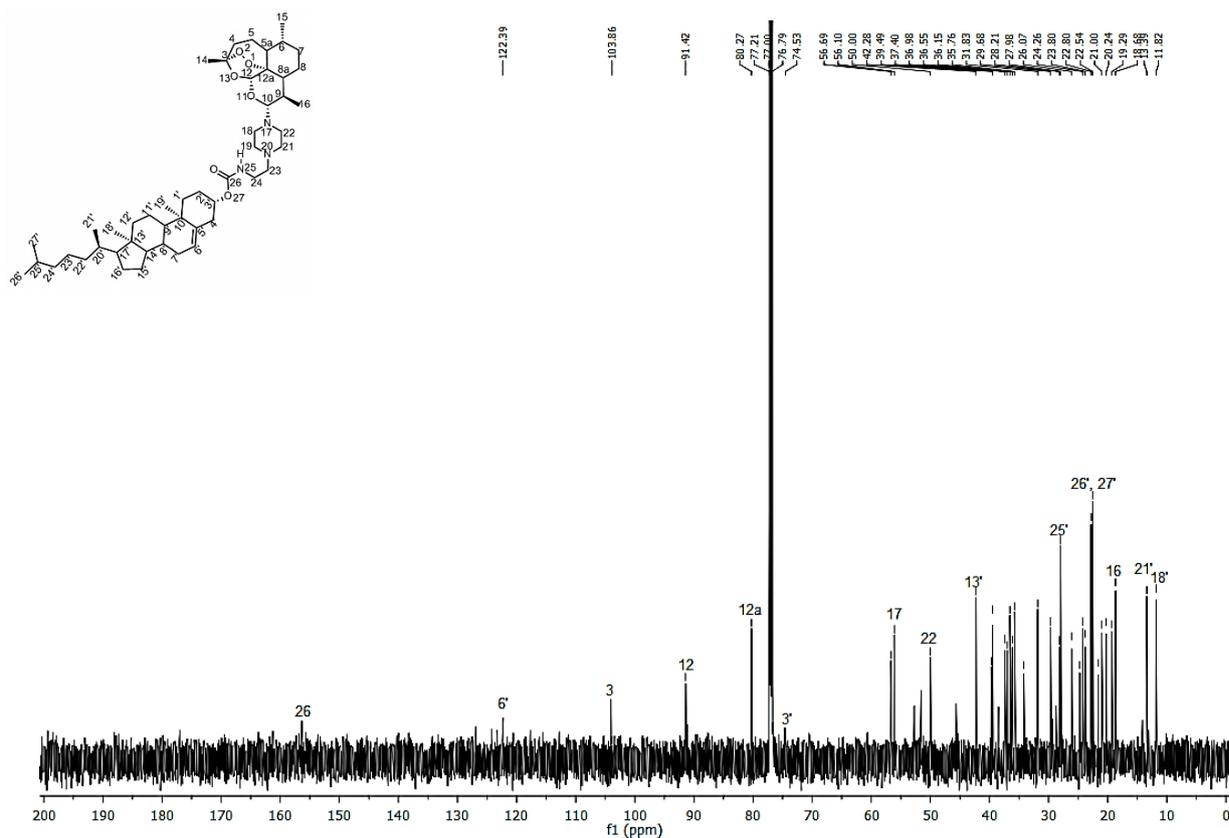
IR



¹H NMR



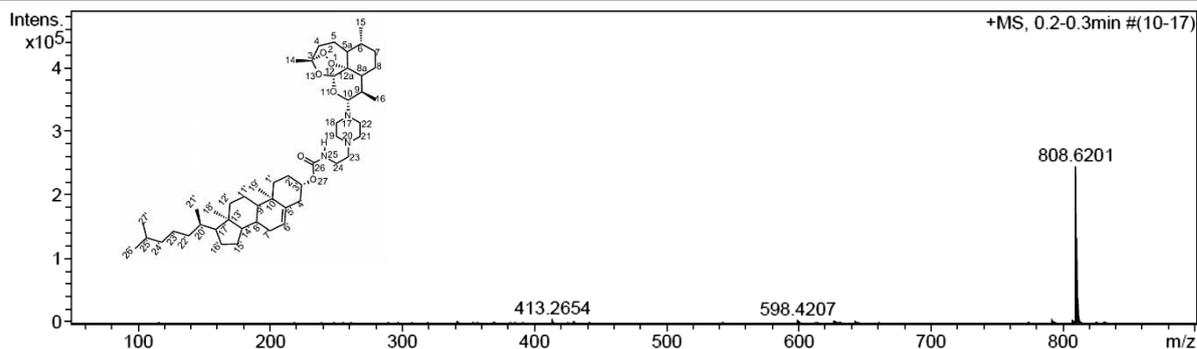
¹³C NMR



HRMS

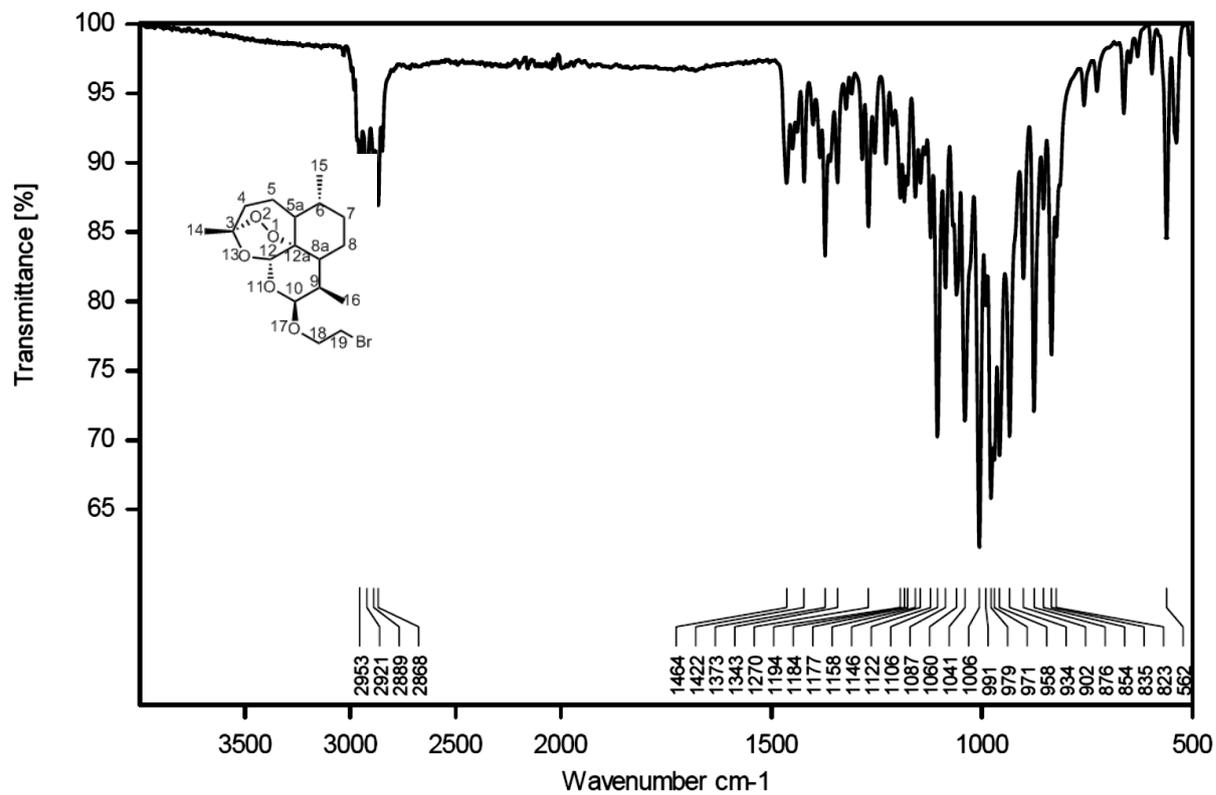
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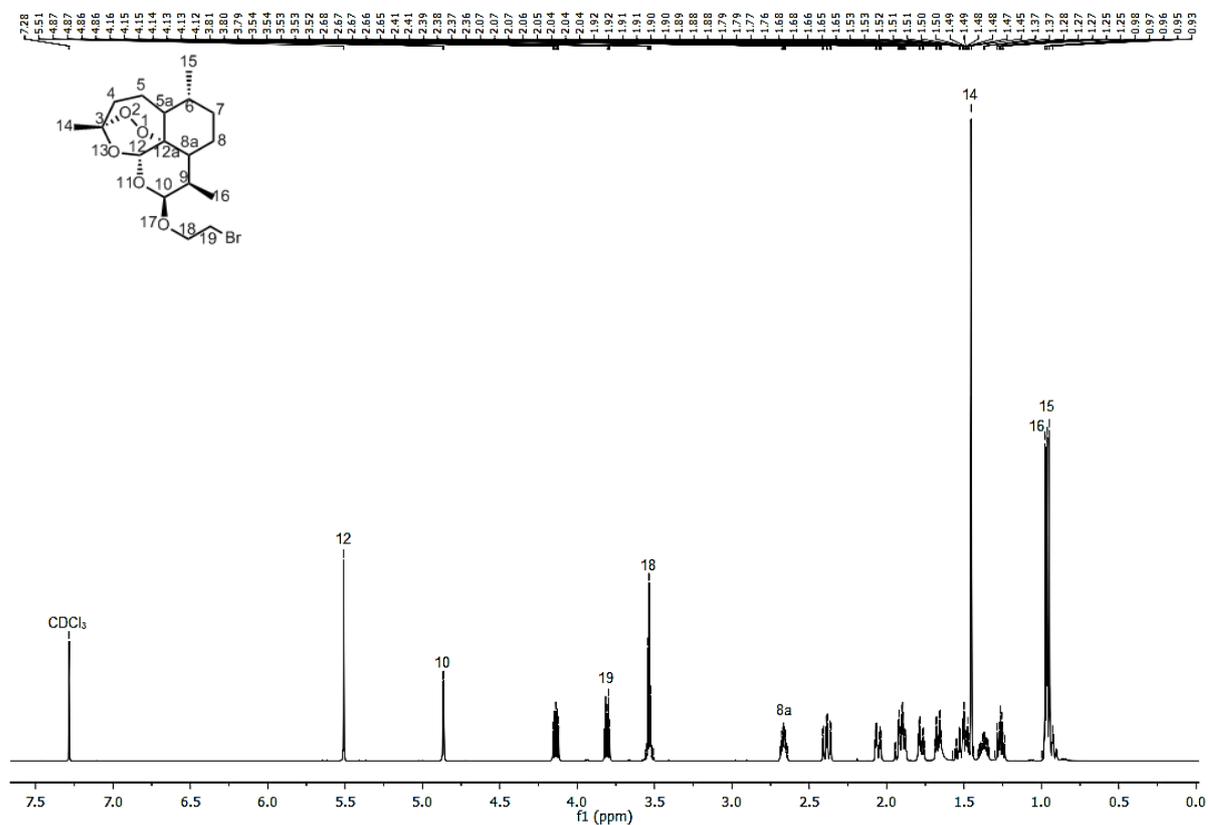


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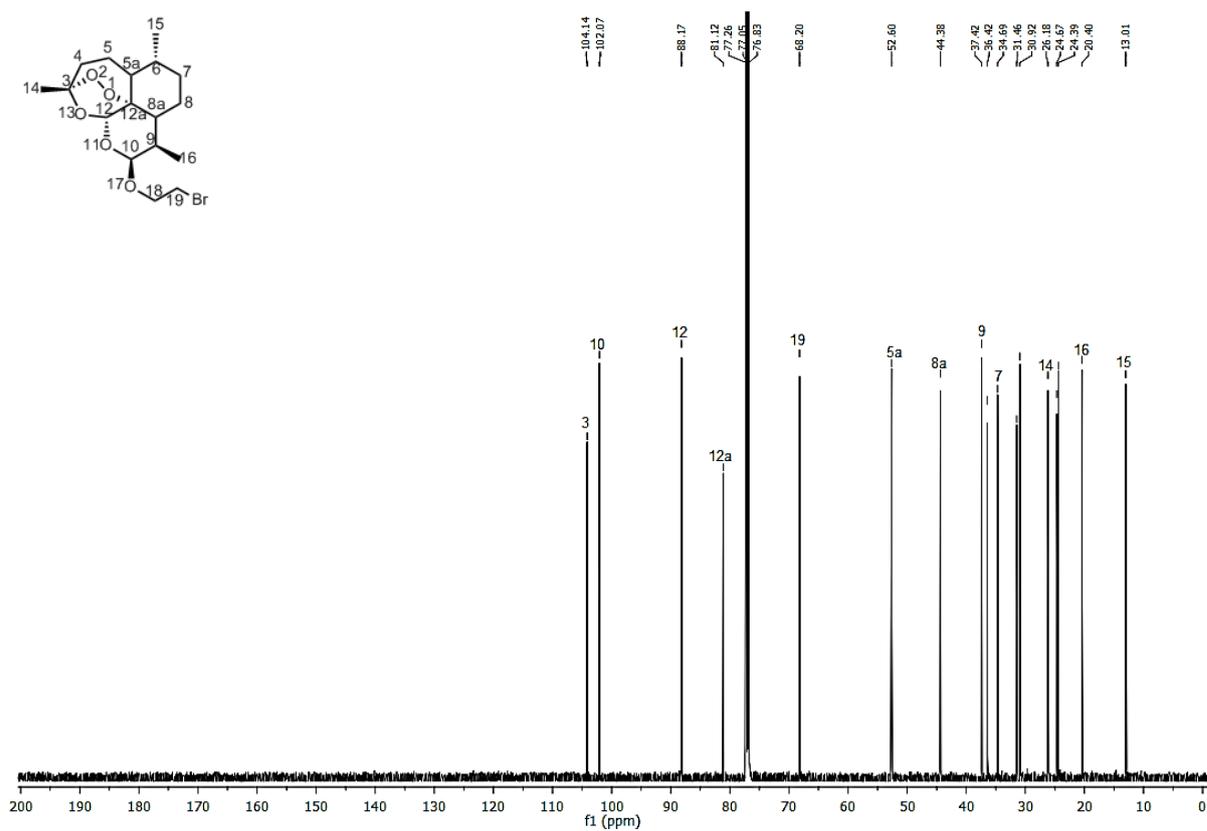
IR



¹H NMR

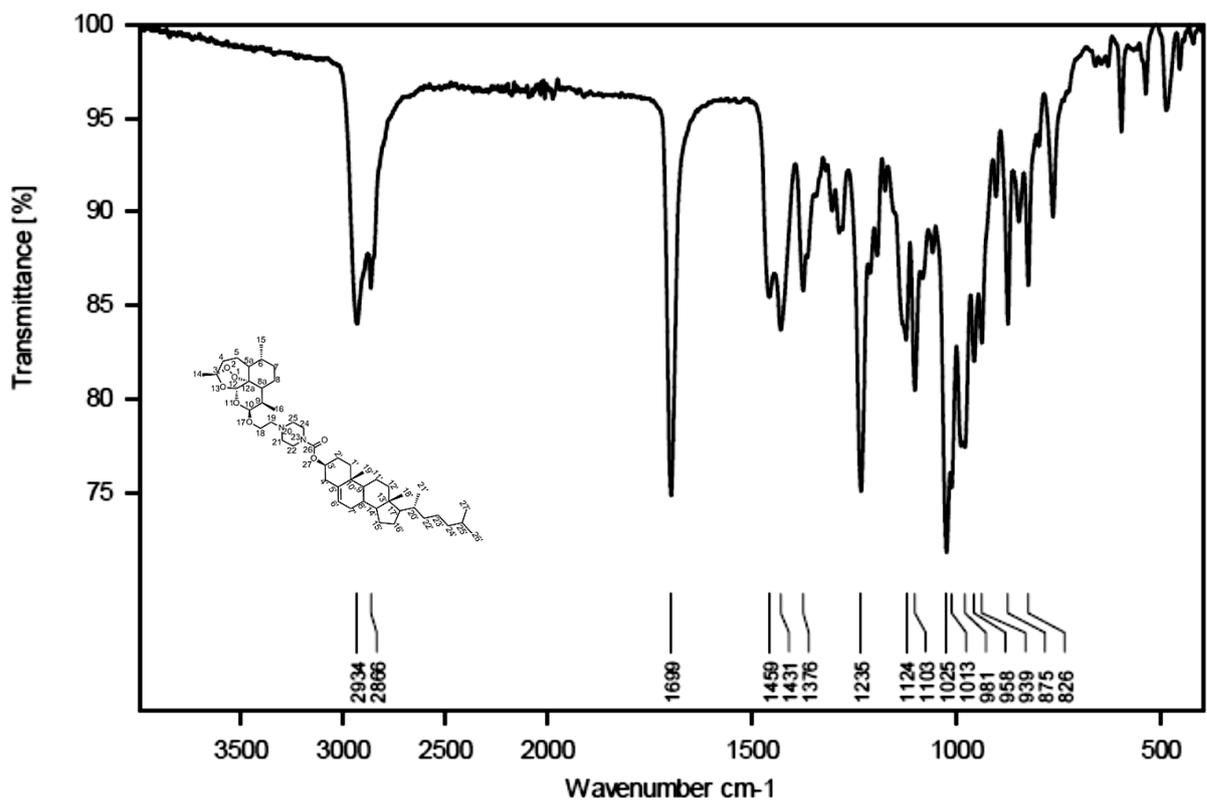


¹³C NMR

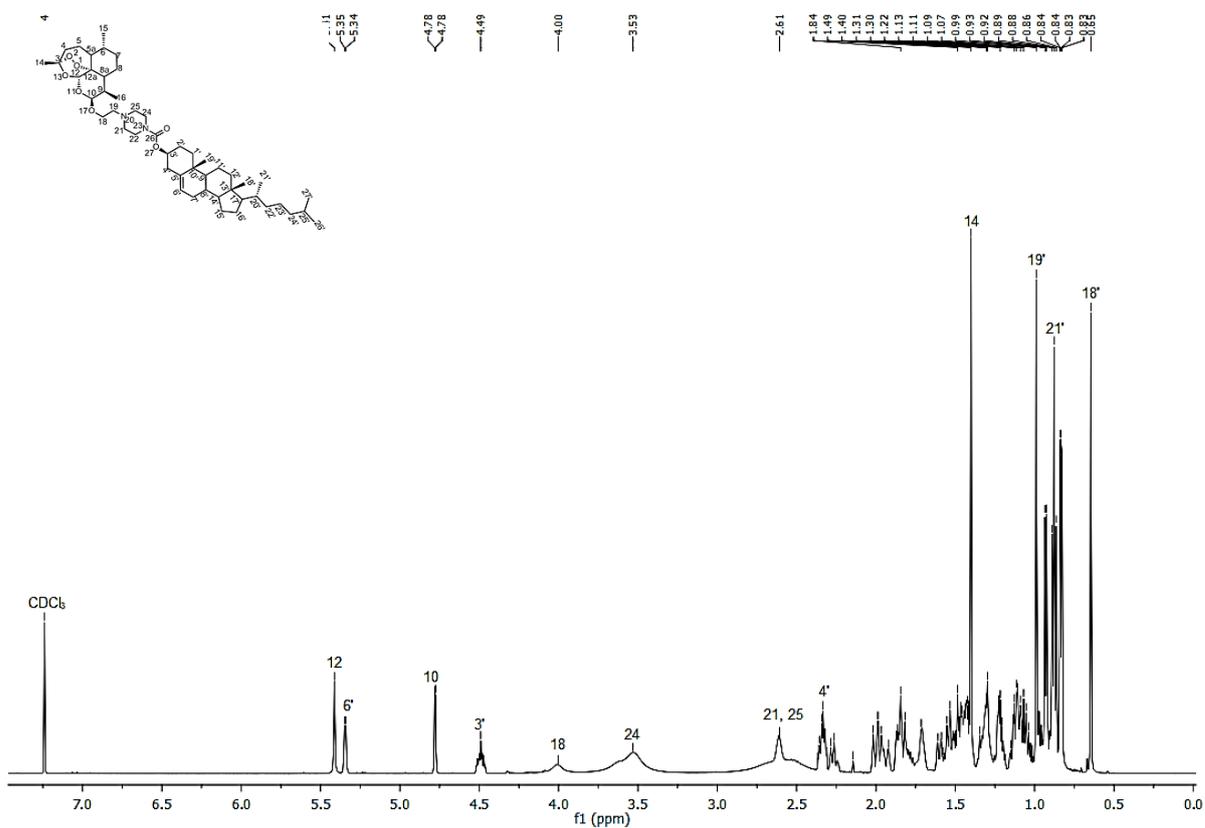


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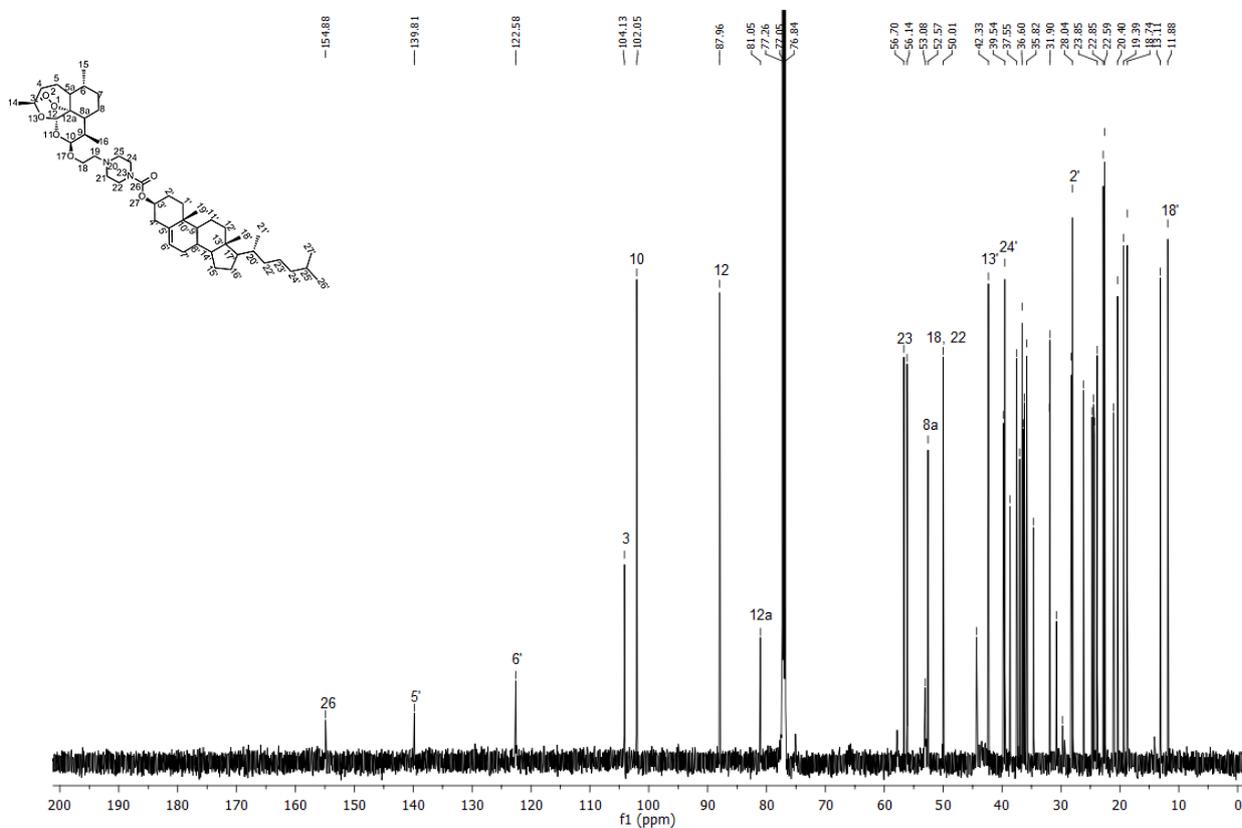
IR



¹H NMR



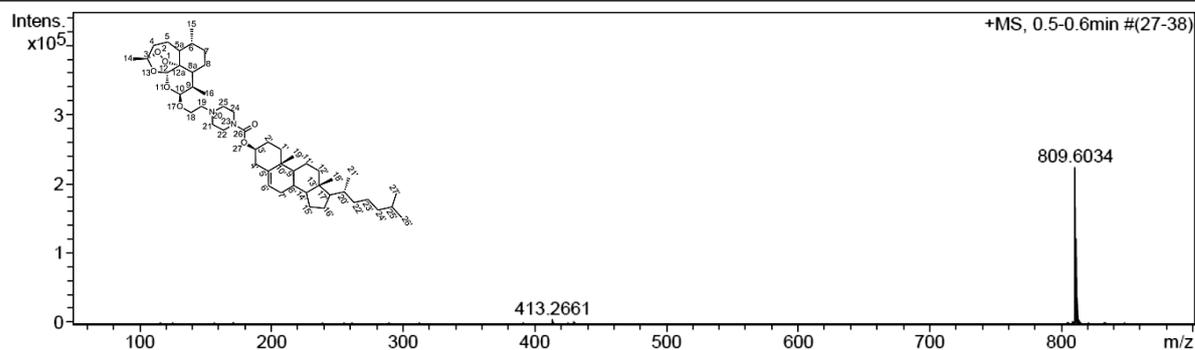
¹³C NMR



HRMS

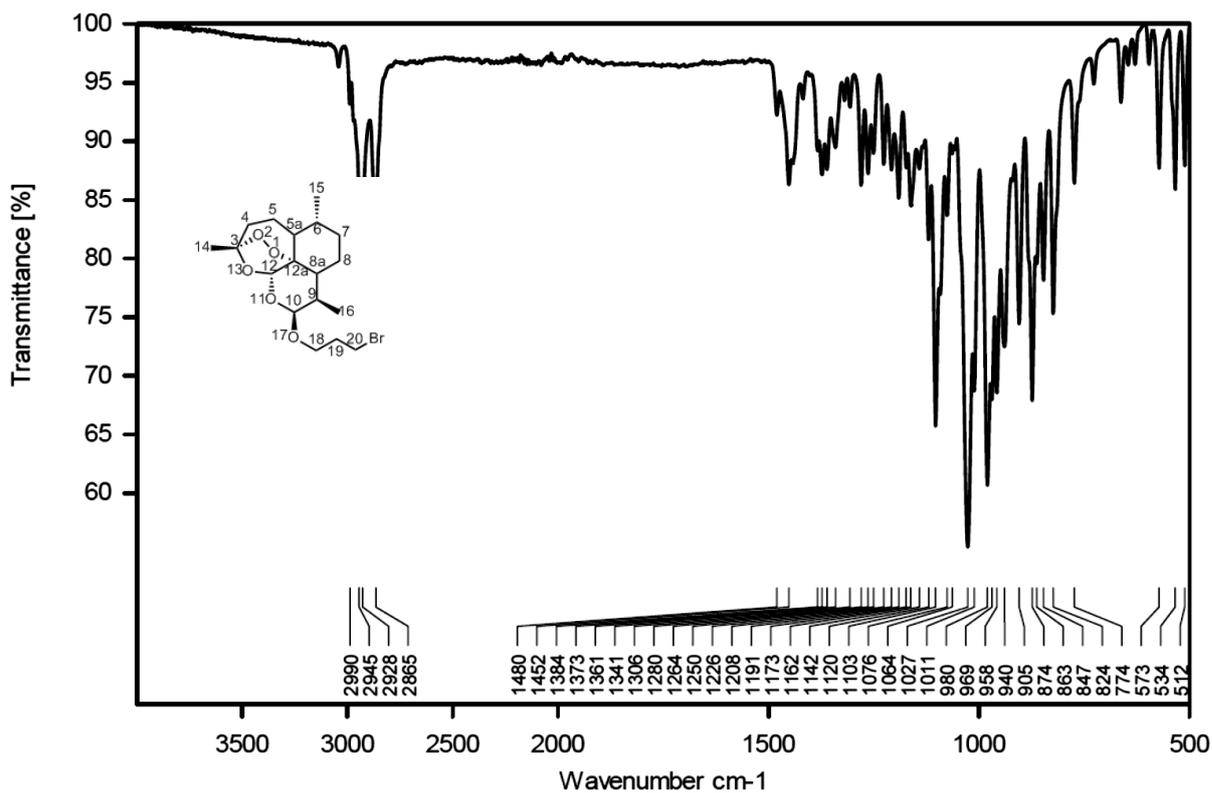
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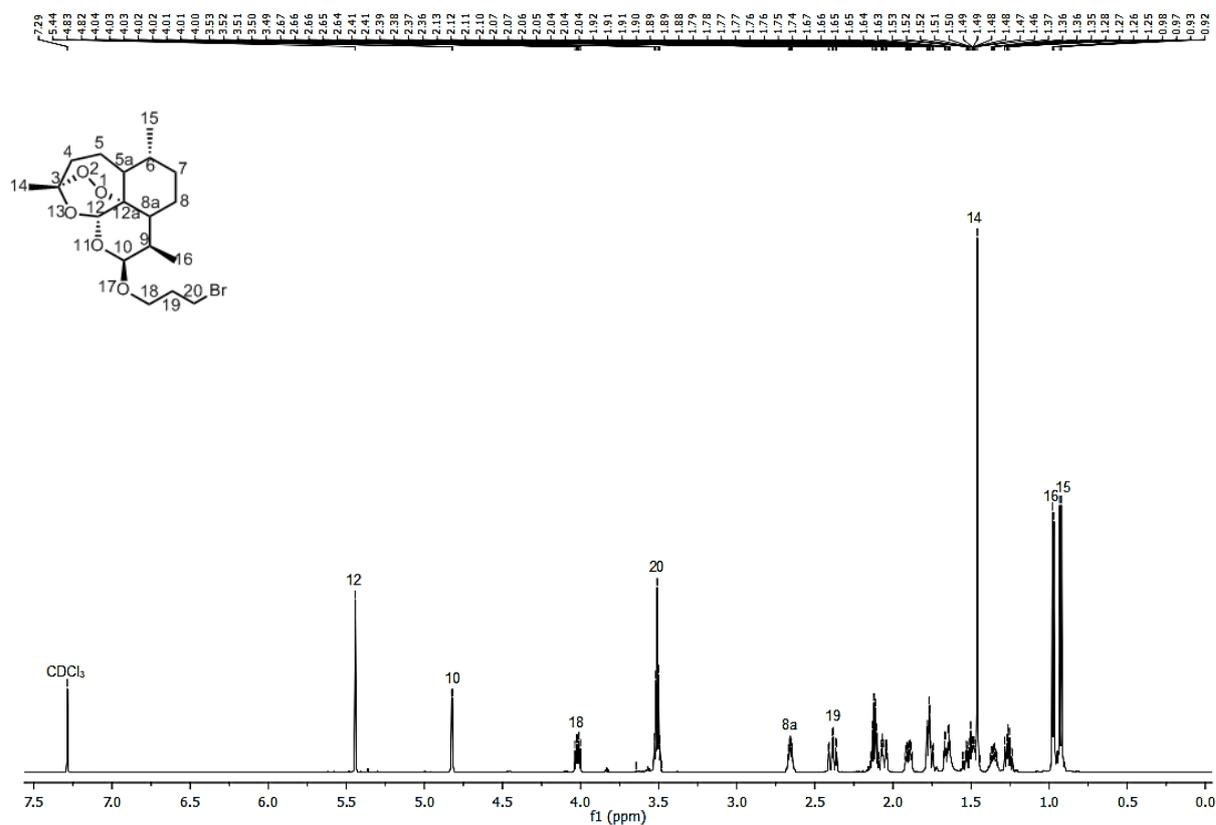


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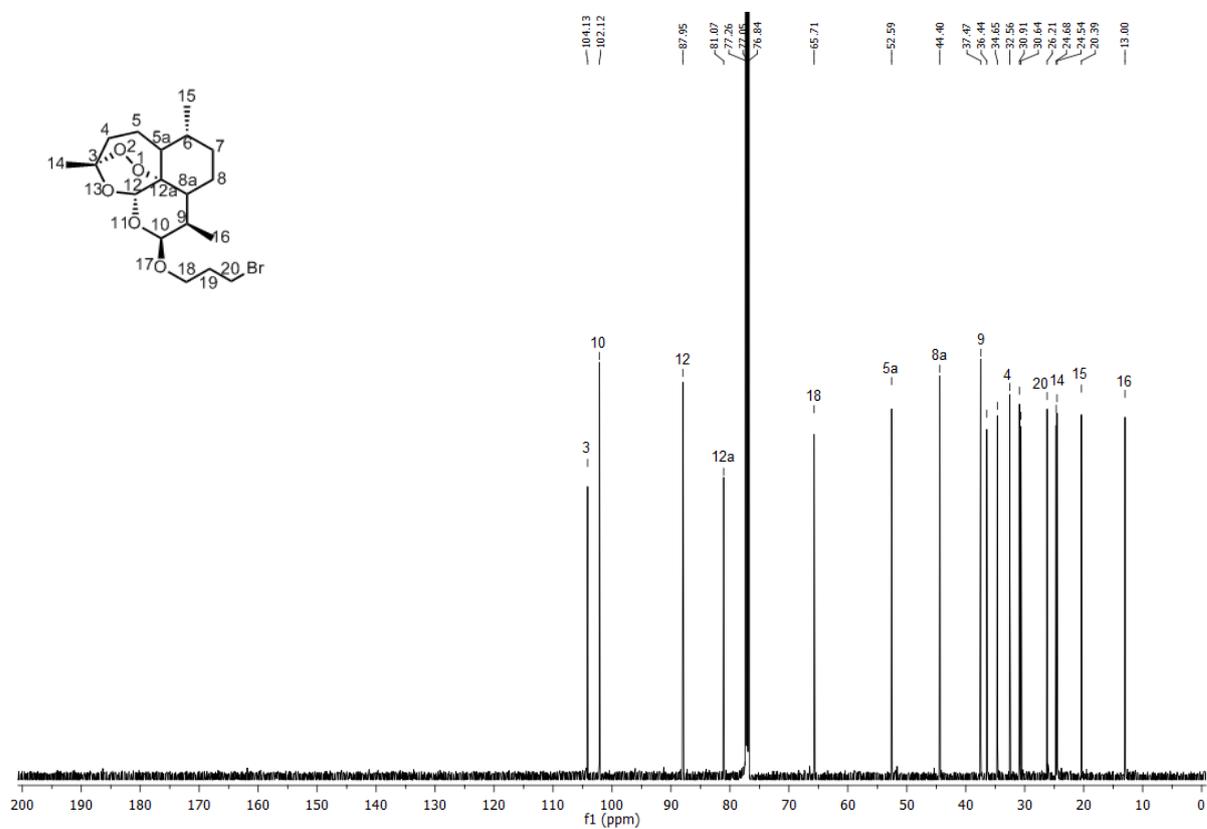
IR



¹H NMR

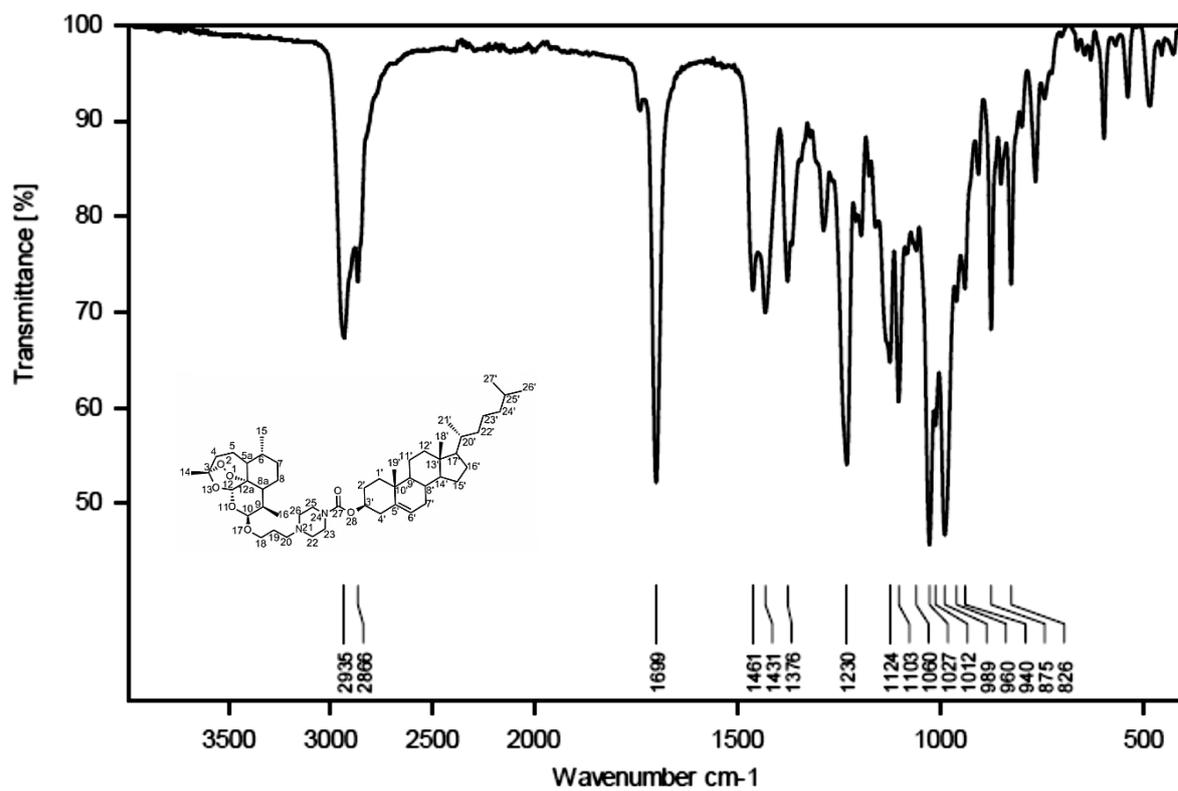


¹³C NMR

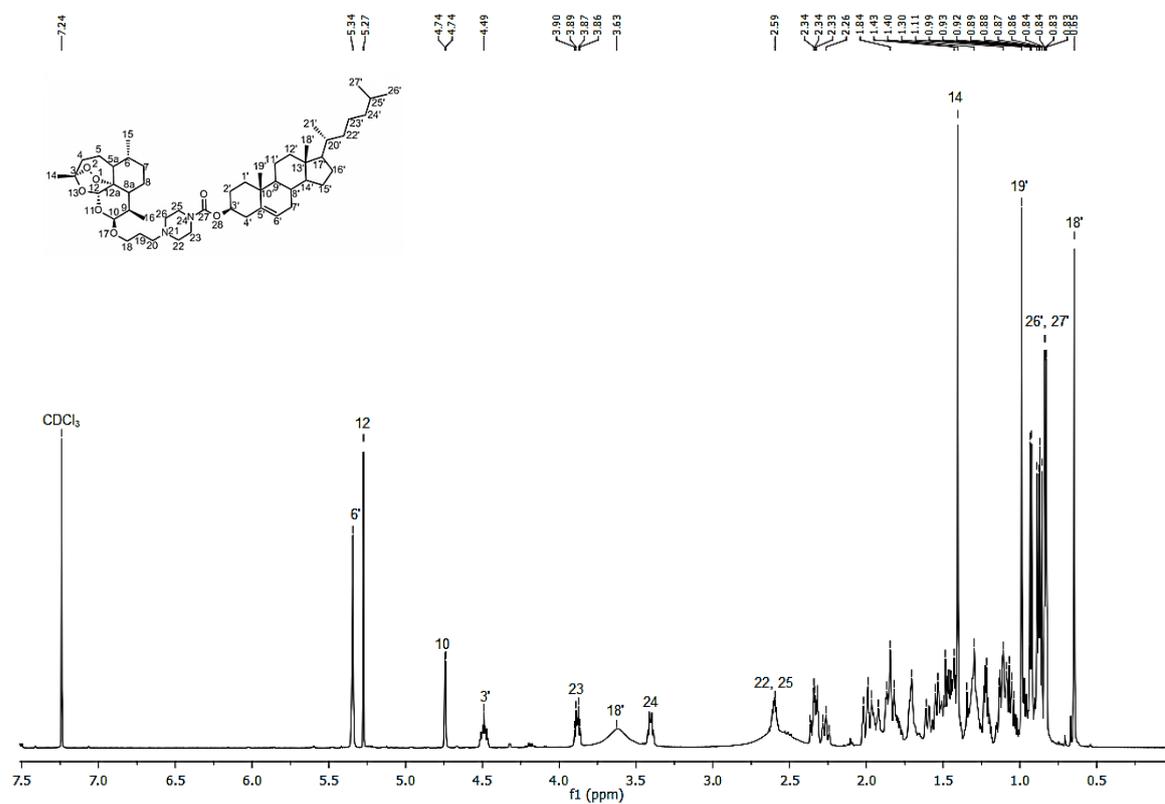


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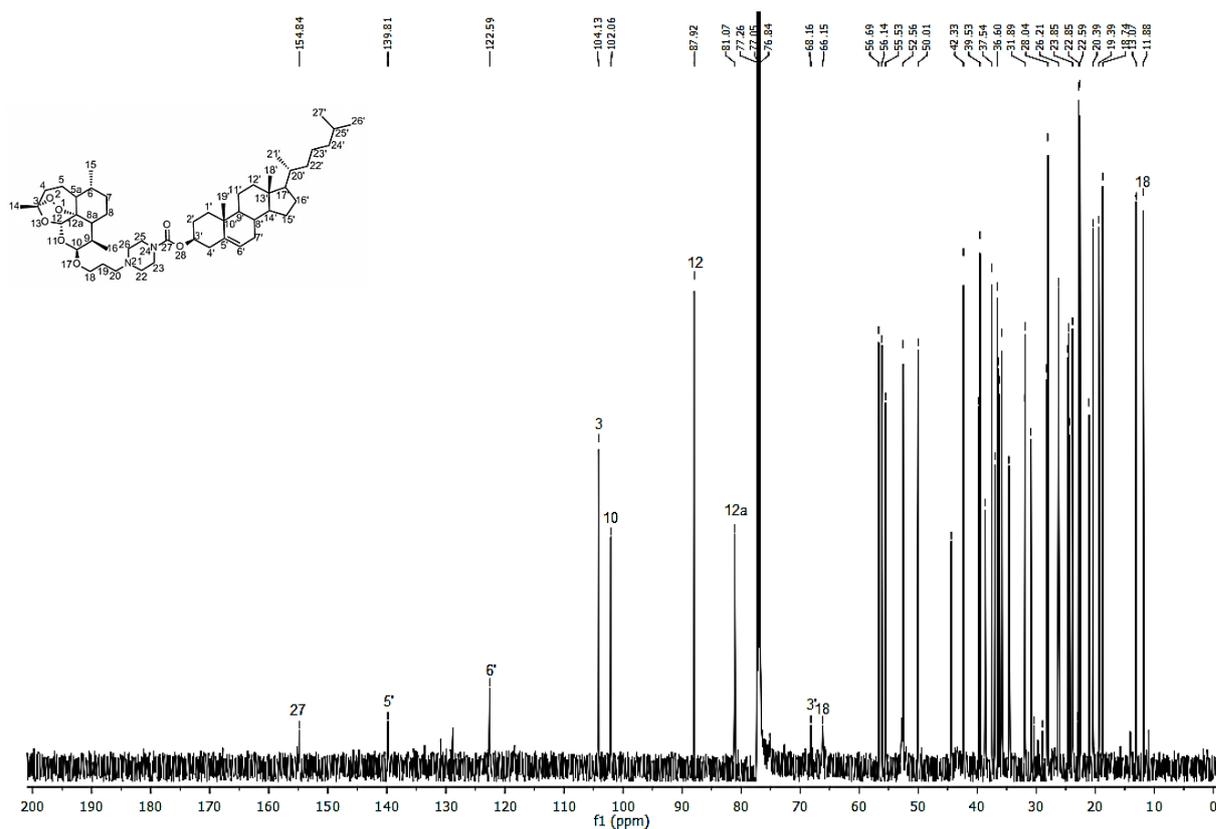
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¹H NMR



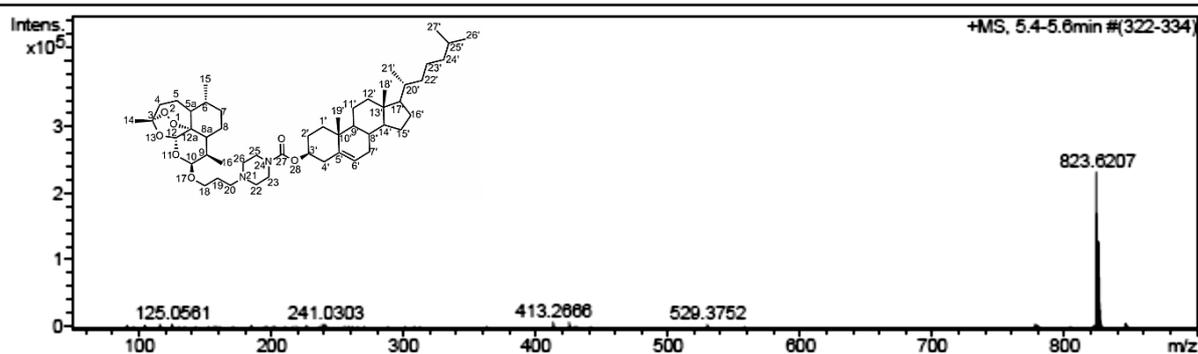
¹³C NMR



HRMS

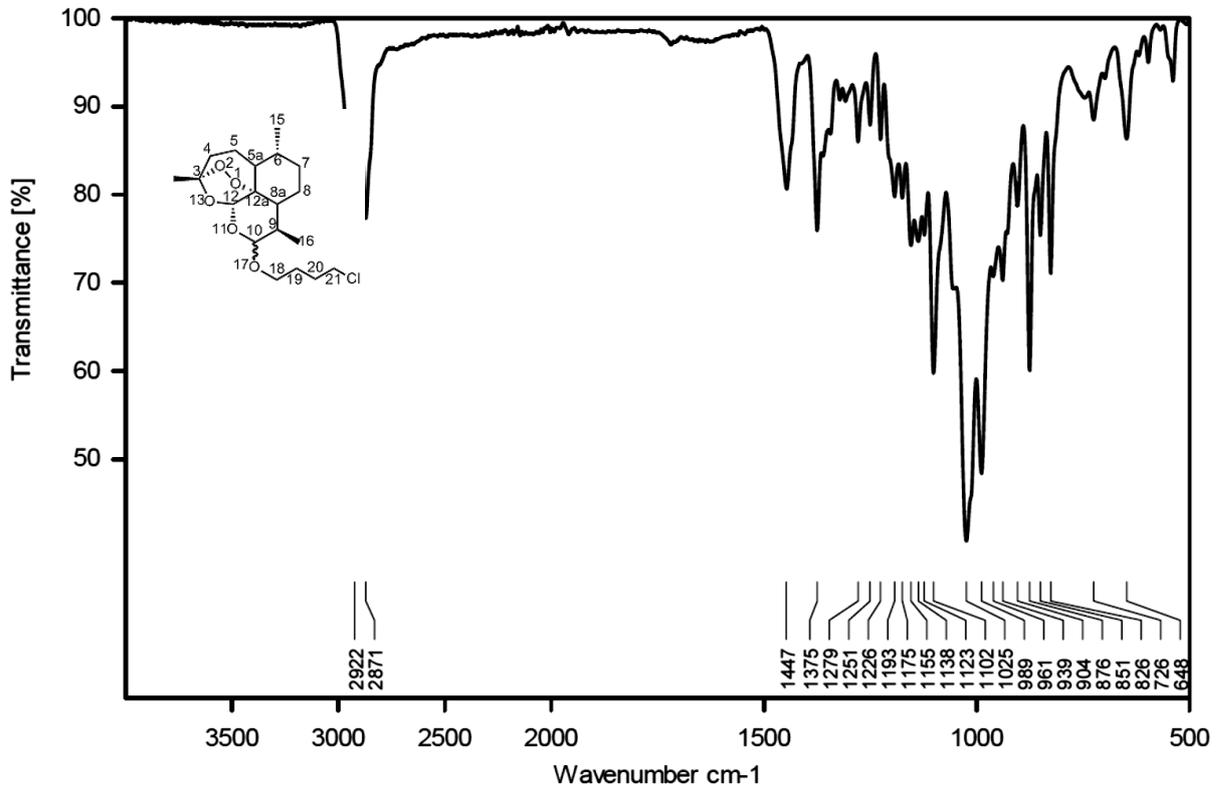
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Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

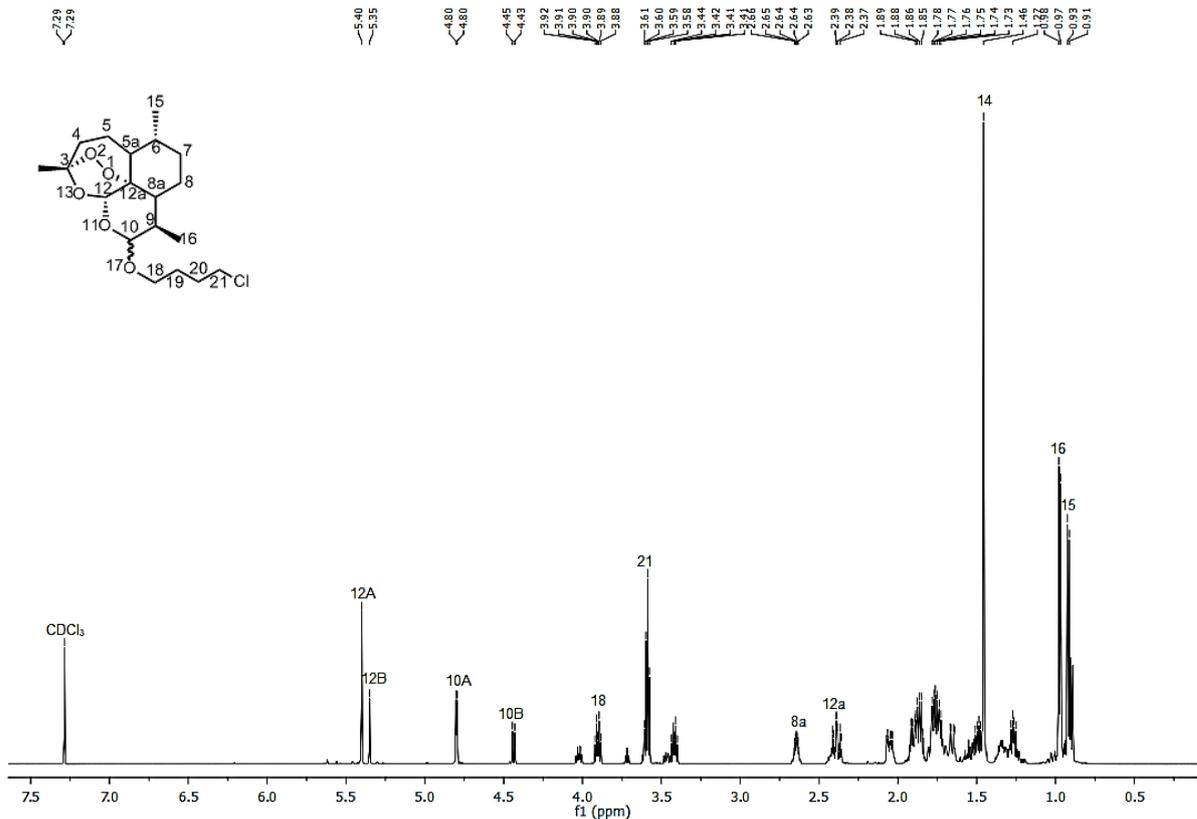


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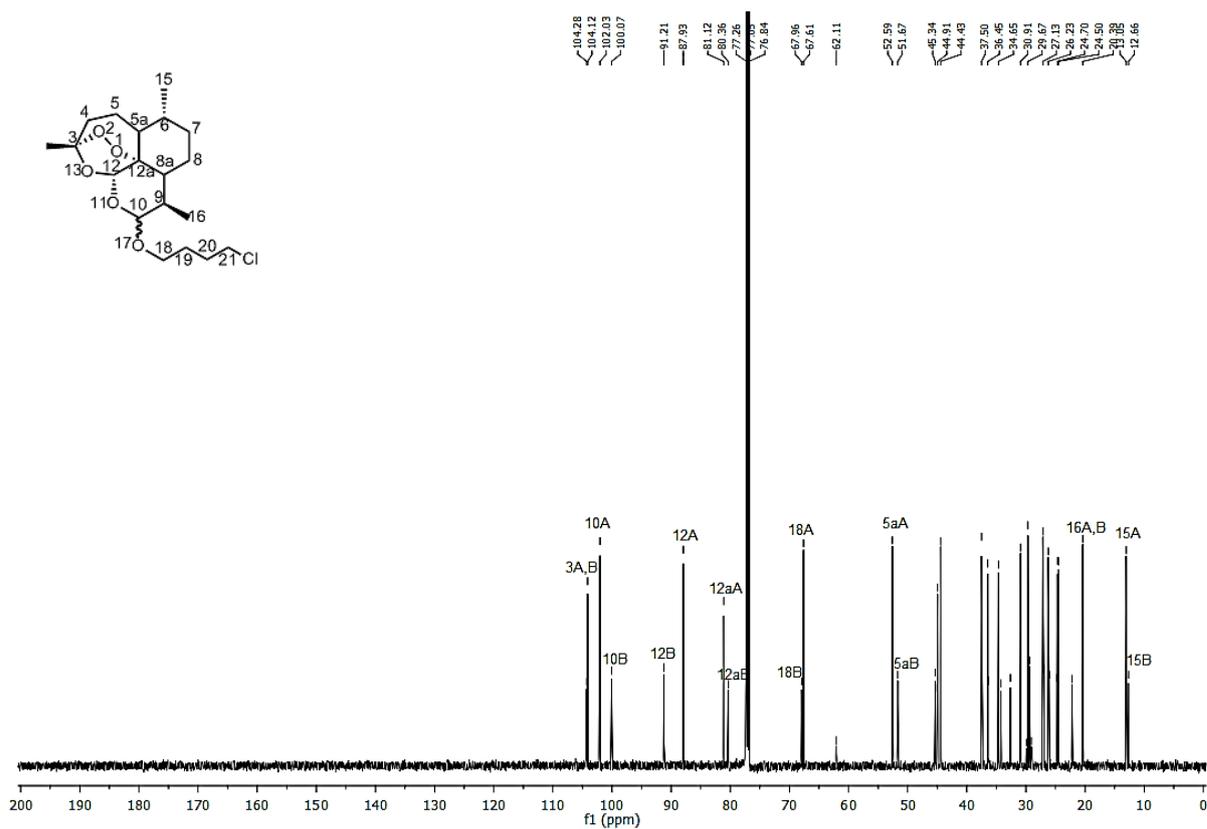
IR



H NMR

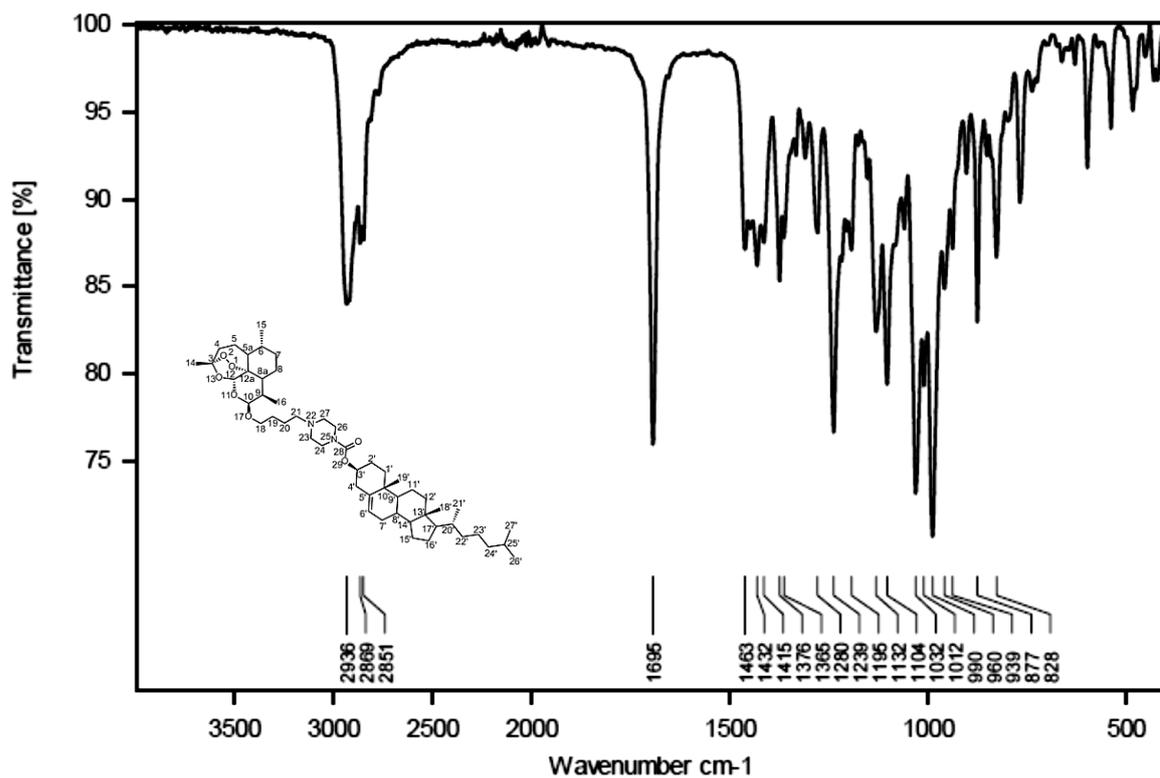


¹³C NMR

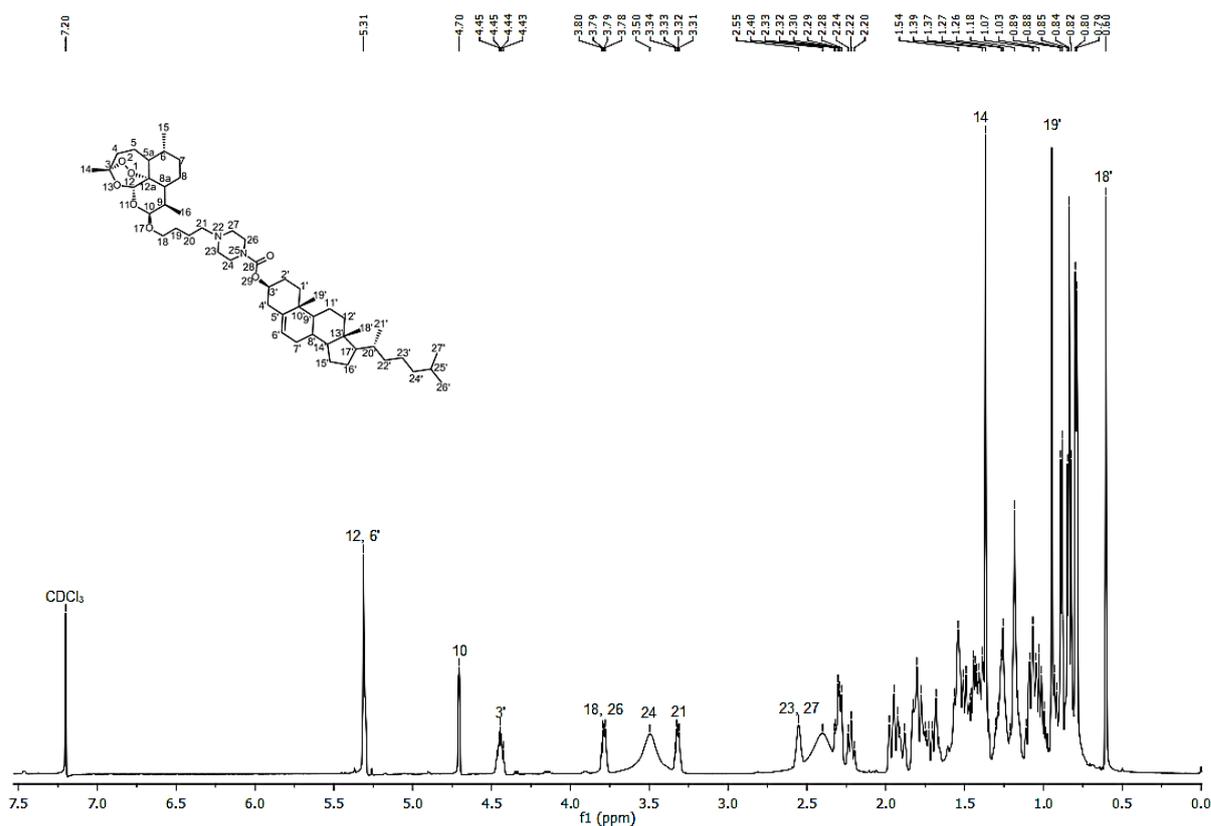


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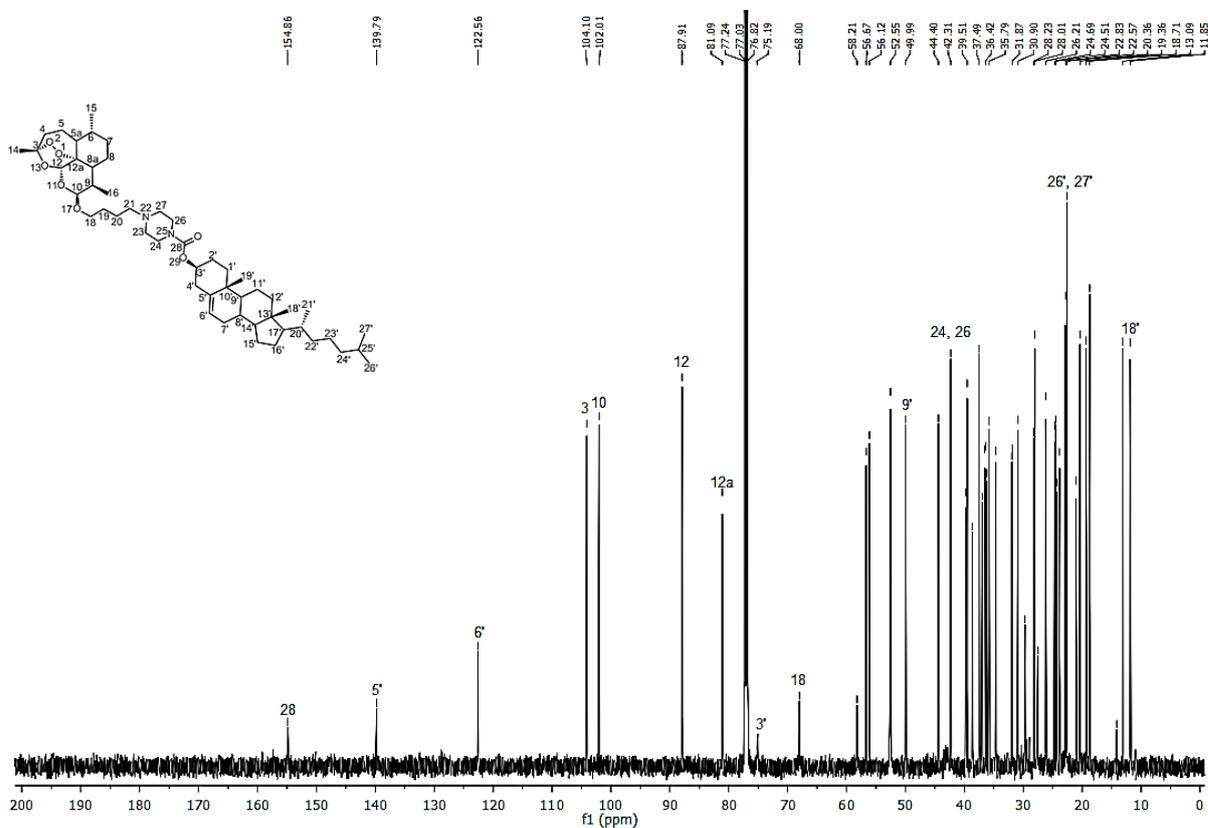
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¹H NMR



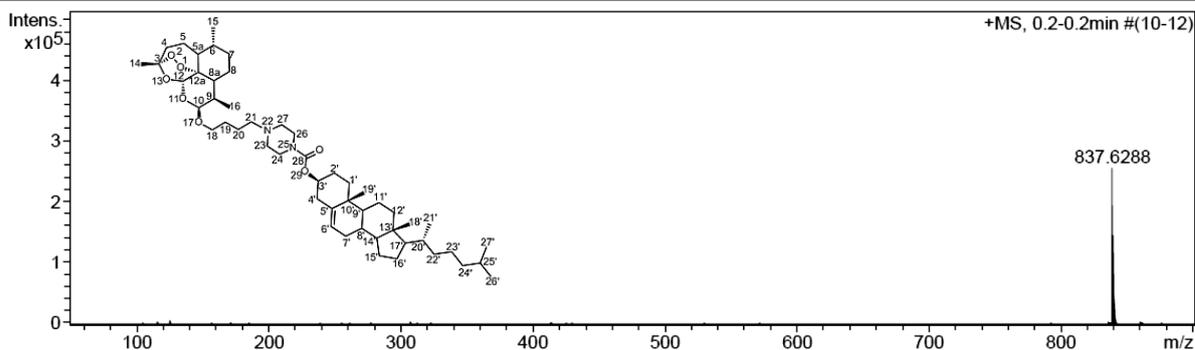
¹³C NMR



HRMS

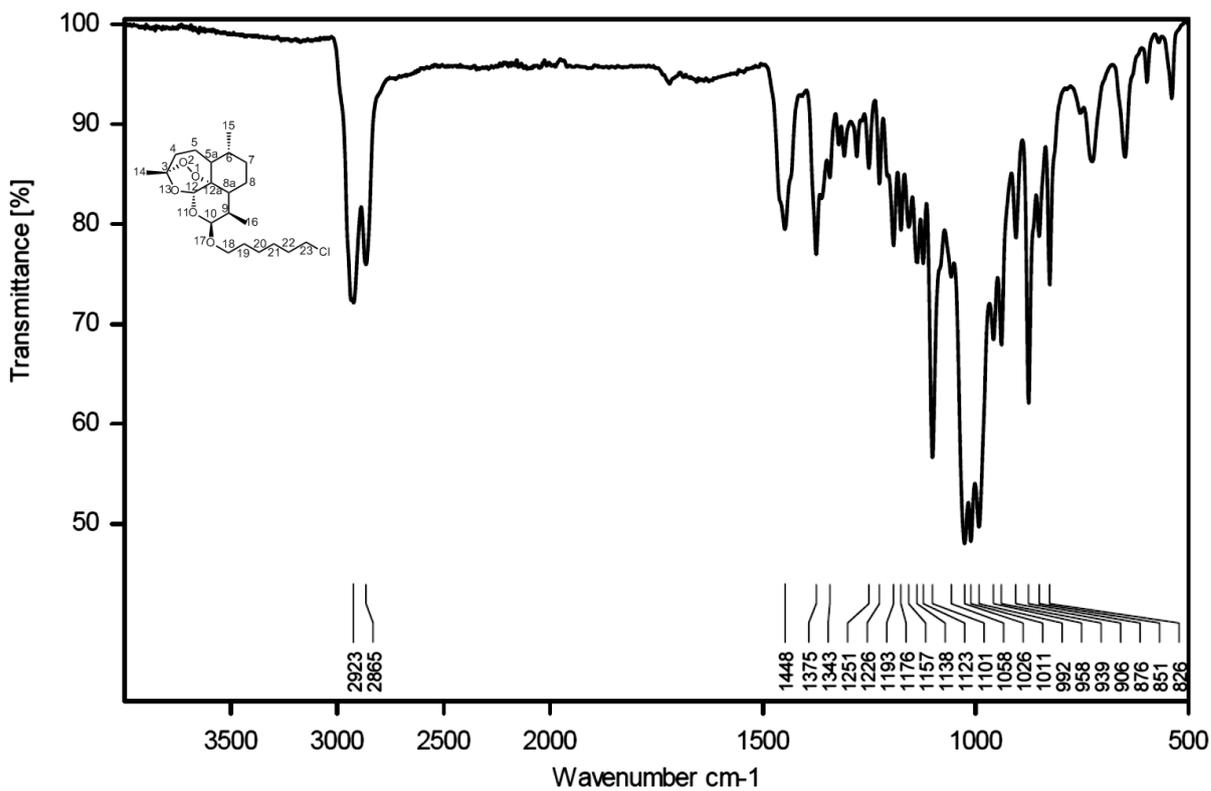
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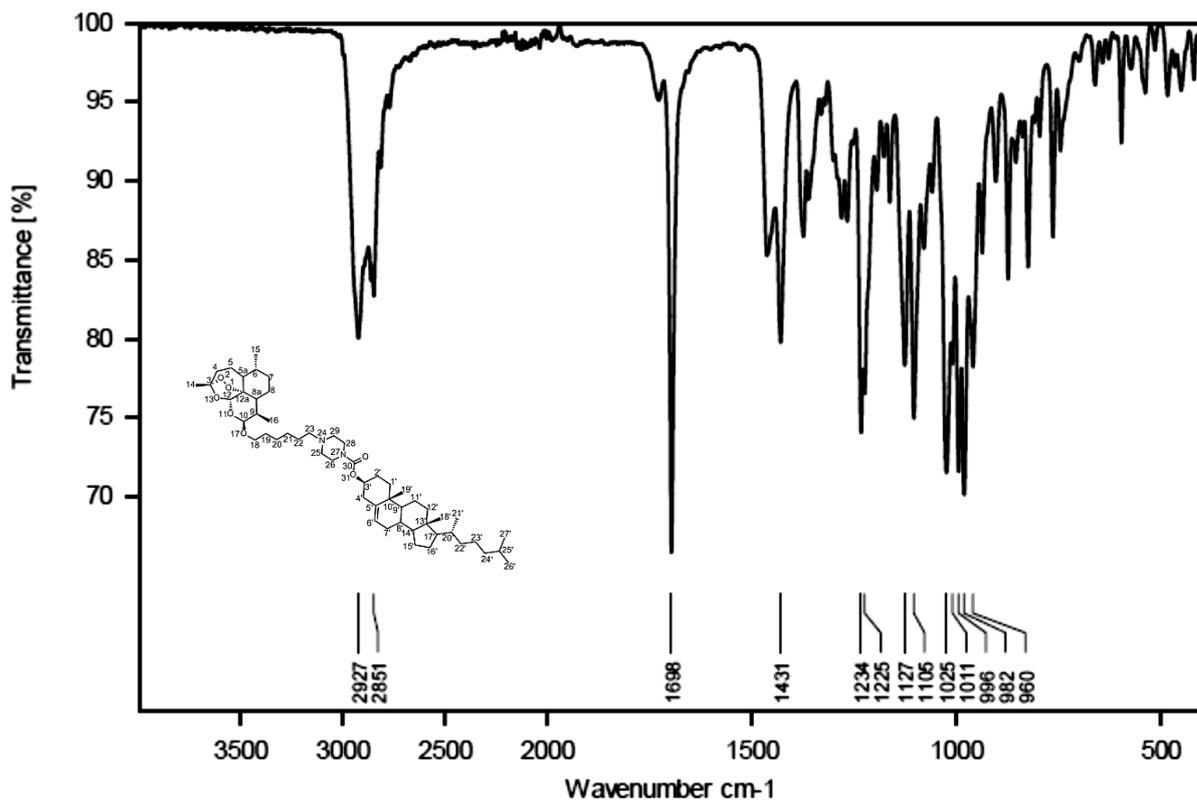
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IR

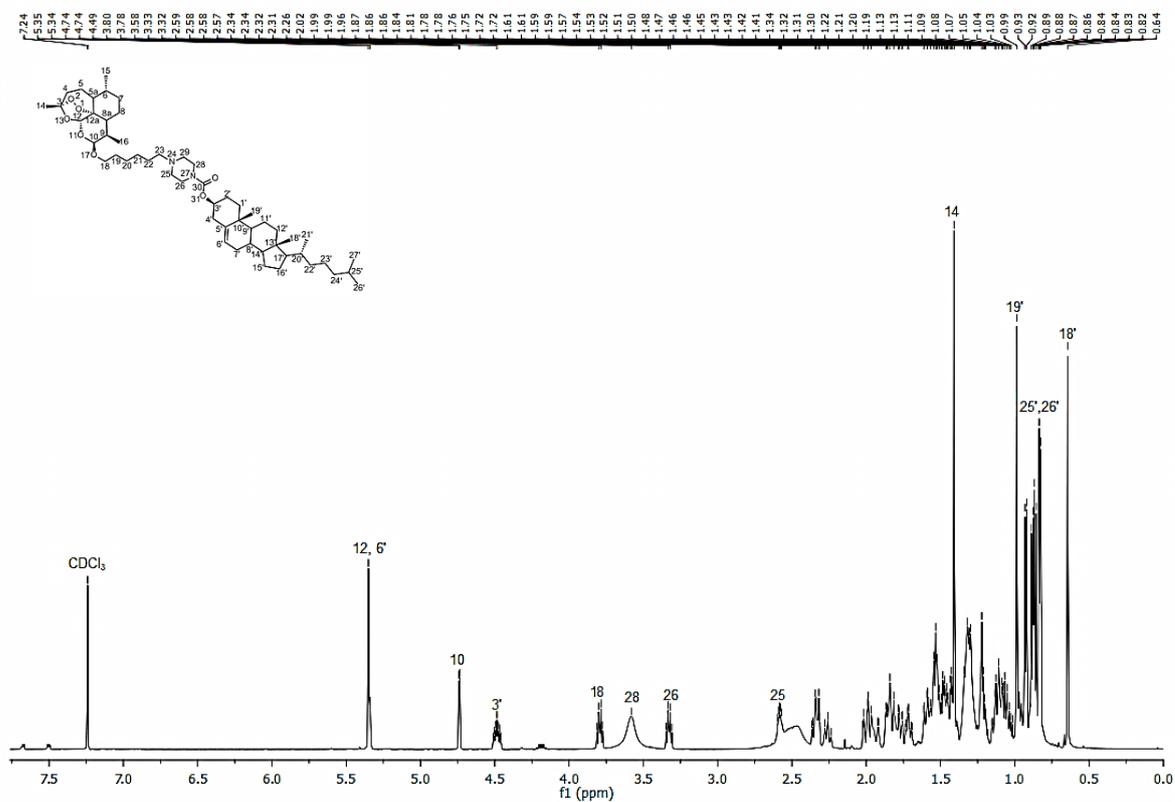


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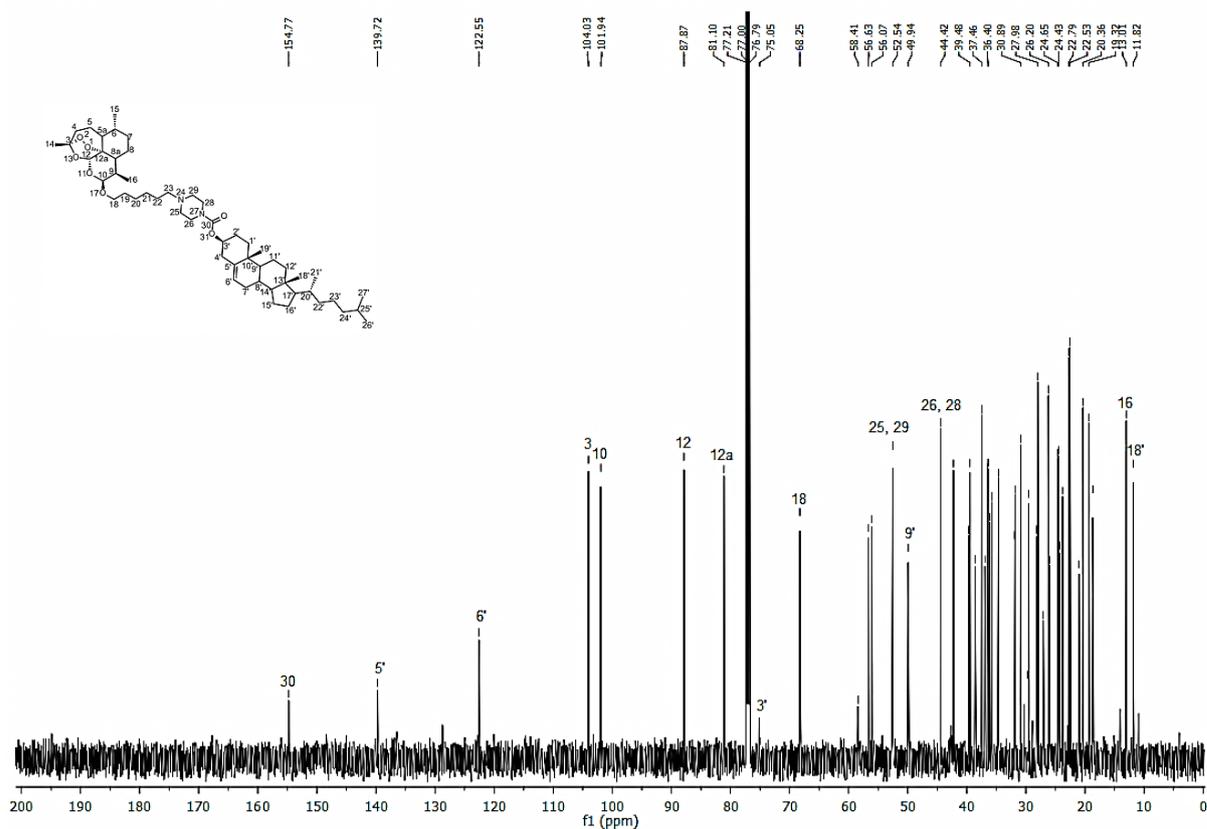
IR



^1H NMR



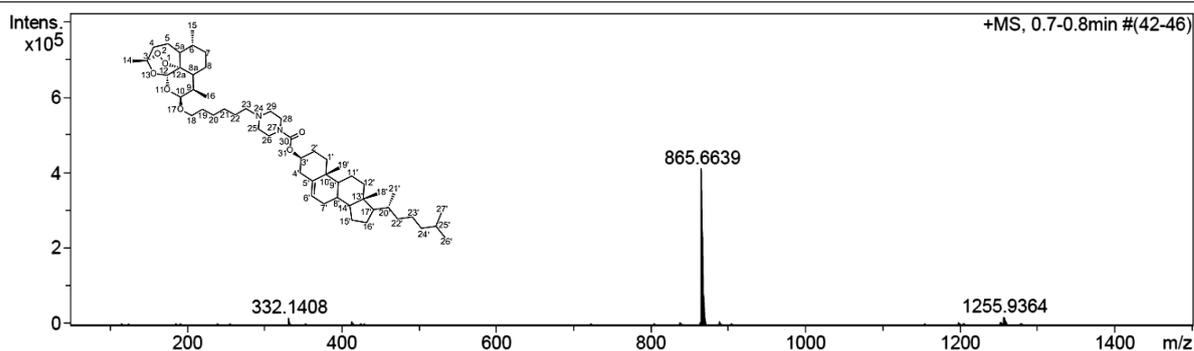
¹³C NMR



HRMS

Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	0.4 Bar
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Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	4.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



APPENDIX B: GUIDE FOR AUTHORS



EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY

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AUTHOR INFORMATION PACK

TABLE OF CONTENTS

• Description	p.1
• Audience	p.1
• Impact Factor	p.1
• Abstracting and Indexing	p.2
• Editorial Board	p.2
• Guide for Authors	p.3



ISSN: 0223-5234

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