Synthesis and biological evaluation of novel 11-azaartemisinin derivatives

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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. Three articles, one of which have been published, are included in this thesis:

CHAPTER 3: Article 1

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ABSTRACT

Malaria is a devastating mosquito-borne disease caused by several species of *Plasmodium* protozoa, of which the most important is *Plasmodium falciparum* (*Pf*). Globally, the disease caused approximately 438 000 deaths in 2014; disease prevalence is highest in the African region. Artemisinin and its derivatives have emerged as the drugs of choice for treatment of malaria where they are used in artemisinin combination therapies (ACTs). However, emergence of resistance to artemisinins poses a global threat to current treatment regimens.

Chapter 3 comprises a review article that examines a relatively new artemisinin derivative, 11-azaartemisinin, and its scientific evolution throughout the past 20 years. Various routes to azaartemisinin derivatives are critically discussed and the biological activities of the azaartemisinin derivatives are examined in order to evaluate if this class of compound is suitable for carrying forward for development into new drugs in the fight against malaria. In general, the azaartemisinins that have been examined display promising antimalarial activities, and would appear to have several advantages over their artemisinin predecessors in being more stable and chemically robust. Decisively, the azaartemisinins cannot provide dihydroartemisinin (DHA) through metabolism or *via* hydrolysis. As the current clinical artemisinins, against which resistance is now emerging, characteristically provide DHA on metabolism or hydrolysis *in vivo*, the newer azaartemisinins will not have this disadvantage, especially as DHA has been fingered as the actual drug that induces resistance among the current clinical artemisinins.

In Chapter 4 the synthesis of *N*-sulfonyl-11-azaartemisinin derivatives are described and the evaluation of the antimalarial activities against intraerythrocytic stages of chloroquine (CQ) sensitive *Pf* NF54 and CQ resistant *Pf* K1 and W2 parasites. The gametocytocidal activities were assessed against *Pf* NF54 blood-stage gametocytes using the luciferase and pLDH assays. Cytotoxicities of the compounds were also evaluated against the human fetal lung fibroblasts WI-38 cell line (HFLF) and were shown to be relatively non-toxic. The *p*-trifluoromethylbenzenesulfonyl-11-azaartemisinin derivative was the most active antimalarial compound with IC_{50} values between 2 – 3 nM, whereas the 2'-thienylsulfonyl derivative demonstrated the best late-stage (IV-V) activity against gametocytes with an IC_{50} value of 8.7 nM. These two compounds are thus potential candidates for further development.

In Chapter 5 the evaluation of nine of the active antimalarial *N*-sulfonylazaartemisinin derivatives against the apicomplexan parasite *Neospora caninum* responsible for bovine abortion in beef and dairy cattle, are described. The antitumor activities were also determined in

order to assess their parasitic versus intracellular activities in general. The 2,5-dichlorothienylsulfonyl-11-azaartemisinin was the most active against neosporosis with an IC_{50} value of 40 nM, whereas the hexadecanesulfonyl derivative demonstrated prominent antitumor activity against breast cancer cells.

Overall, the current study has resulted in the identification of compounds that exhibit varying antimalarial activities, some of which are comparable to the current clinically available artemisinins. These compounds serve as suitable candidates for additional research in order to evaluate their potential as future lead compounds for development into drugs against malaria. Also, several compounds display promising activities against the causative parasite of neosporosis, and likewise require further investigation to evaluate their potential.

Keywords: Plasmodium falciparum, malaria, azaartemisinin, N-sulfonyl-11-azaartemisinin, gametocytocidal, neosporosis

OPSOMMING

Malaria is 'n ernstige muskiet-oordraagbare siekte wat veroorsaak word deur spesies van *Plasmodium* protosoë, waarvan die belangrikste *Plasmodium falciparum* (*Pf*) is. Die siekte veroorsaak wêreldwyd ongeveer 438 000 sterftes per jaar waarvan die voorkoms van die siekte die hoogste is in die Afrika-streek. Artemisinien en sy derivate geniet voorkeur as geneesmiddels vir die behandeling van malaria waar dit gebruik word in kombinasie met artemisinien terapie. Die ontwikkeling van weerstand teen artemisinien hou ongelukkig 'n wêreldwye bedreiging in vir die huidige behandelingsvorm.

Hoofstuk 3 bevat 'n oorsig artikel wat 'n relatiewe nuwe artemisinien derivaat, 11-azaartemisinien, en sy wetenskaplike evolusie van die afgelope 20 jaar, ondersoek. Verskeie sintetiese metodes om derivate van azaartemisinien te bekom, word krities bespreek. Die biologiese aktiwiteite van die azaartemisinien derivate word bestudeer om uiteindelik te bepaal of hierdie klas van geneesmiddels geskik is vir verdere ontwikkeling as nuwe geneesmiddels wat gebruik kan word in die stryd teen malaria. Oor die algemeen vertoon die azaartemisiniene wat ondersoek is belowende anti-malaria aktiwiteit. Dit wil voorkom asof hierdie klas van geneesmiddels menigte voordele inhou in vergelyking met hul artemisinien voorgangers. Die nuwer verbindings is meer stabiel, asook chemies meer robuus. Die azaartemisiniene kan nie dihydroartemisinien (DHA) deur middel van metabolisme of hidrolise voorsien nie. Die huidige kliniese artemisiniene, waar weerstand nou algemeen voorkom, vorm kenmerkende DHA *in vivo* wanneer dié gemetaboliseer word, of hidrolise ondergaan. Die nuwe azaartemisiniene sal dus nie oor hierdie nadelige eienskap beskik nie, veral noudat dit bewys kan word dat DHA die werklike oorsaak is van weerstand onder huidige kliniese artemisiniene.

Hoofstuk 4 beskryf die sintese van *N*-sulfoniel-11-azaartemisinien derivate asook die evaluering van malaria-aktiwiteite teen intra-eritrositiese fases van chlorokien (CQ) sensitiewe *Pf* NF54 en CQ bestande *Pf* K1 en W2 parasiete. Die gametosiet aktiwiteite word ook bepaal teenoor *Pf* NF54 bloed-fase gametosiete met behulp van die lusiferase en pLDH toetse. Sitotoksisiteite van die verbindings is ook geëvalueer teenoor menslike fetale long fibroblaste WI-38 sellyn (HFLF) en het getoon dat die verbindings relatief veilig is. Die *p*-trifluorometielbenseensulfoniel-11-azaartemisinien derivaat was die mees aktiewe malaria verbinding met IC_{50} waardes tussen 2 – 3 nM, terwyl die 2'-tiofeensulfoniel derivaat die beste laat stadium (IV-V) aktiwiteit teen gametosiete gedemonstreer het met 'n IC_{50} waarde van 8,7 nM. Hierdie twee verbindings is potensiële kandidate vir verdere ontwikkeling.

Hoofstuk 5 beskryf die evaluering van nege van die aktiewe malaria *N*-sulfonielazaartemisinien derivate teenoor die apikompleksia parasiet *Neospora caninum*, wat verantwoordelik is vir misgeboortes in vleis- en melkbeeste. Die antikanker aktiwiteite van die verbindings word ook bepaal sodat hul parasitiese aktiwiteite teenoor intrasellulêre aktiwiteite beoordeel kan word. Die 2,5-dichlorotiofeensulfoniel-11-azaartemisinien was die mees aktiewe verbinding teenoor neosporose met 'n IC_{50} waarde van 40 nM, terwyl die heksadekaansulfoniel derivaat prominente anti-kanker aktiwiteit teenoor borskankerselle gedemonstreer het.

Die huidige studie het gelei tot die identifisering van verbindings wat wissellende malaria aktiwiteite het, waarvan sommige verbindings se aktiwiteite vergelykbaar is met die huidige klinies beskikbare artemisiniene. Hierdie verbindings dien as geskikte kandidate vir verdere navorsing om hul potensiaal as toekomstige leidraadverbindings vir ontwikkeling as geneesmiddels teen malaria te evalueer. Van die verbindings toon belowende aktiwiteite teenoor neosporose en vereis ook verdere ondersoek om hul potensiaal te evalueer.

Sleutelwoorde: *Plasmodium falciparum*, malaria, azaartemisinien, *N*-sulfoniel-11-azaartemisinien, gametosiete, neosporose

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LIST OF ABBREVIATIONS

ACE	-	Associated Chemical Enterprises
ACT	-	Artemisinin combination therapy
ACTs	-	Artemisinin combination therapies
APAD	-	Acetylpyridine adenine dinucleotide
ATP	-	Adenosine triphosphate
ATR	-	Attenuated total reflectance
BHT	-	2,6-Di-*tert*-butyl-4-methylphenol
$CDCl_3$	-	Chloroform-d
^{13}C NMR	-	Carbon NMR
CoMFA	-	Comparative molecular field analysis
CQ	-	Chloroquine
CYP	-	Cytochrome P450
DBU	-	1,8-diazabicyclo(5.4.0)undec-7-ene
DDT	-	Dichlorodiphenyltrichloroethane
DFHS	-	Dihydrofolate synthase
DFO	-	Desferrioxamine
DHA	-	Dihydroartemisinin
DHFR	-	Dihydrofolate reductase
DHPS	-	Dihydropteroate synthase
DIBAL-H	-	Diisobutylaluminium hydride
DIPA	-	*N*,*N*-diisopropylamide
DMAP	-	4-*N*,*N*-dimethyl-aminopyridine
DMF	-	Dimethyl formamide
DMP	-	Dimethyl phthalate
DNA	-	Deoxyribonucleic acid
dTMP	-	Deoxythymidylate
DV	-	Digestive vacuole
$FADH_2$	-	Flavin adenine dinucleotide
FMN	-	Flavin mononucleotide
FPGS	-	Folylpoly-gamma-glutamate synthase
Fre	-	Flavin oxidoreductase
G6PD	-	Glucose-6-phosphate dehydrogenase
GR	-	Glutathione reductase

GSH	-	Glutathione
GTPCH	-	GTP cyclohydrolase I
GPIRM	-	Global plan for insecticide resistance management in malaria vectors
HFLF	-	Human fetal lung fibroblasts
^{1}H NMR	-	Proton NMR
HPPK	-	Hydroxymethyl dihydropterin pyrophosphokinase,
HRMS	-	High resolution mass spectrometry
IC_{50}	-	50% inhibitory concentration
ICH	-	International Conference on Harmonization
IR	-	Infrared
IRS	-	Indoor residual spraying
ITNs	-	Insecticide treated mosquito nets
LDA	-	Lithium diisopropylamide
LMB	-	Leucomethylene blue
MB	-	Methylene blue
MFQ	-	Mefloquine
mp	-	Melting points
MRC	-	Medical Research Council
NADPH	-	Nicotinamide adenine dinucleotide phosphate
NBT	-	Nitro blue tetrazoliumchloride
NMR	-	Nuclear magnetic resonance
NRF	-	National Research Foundation
PES	-	Phenazine ethosulphate
Pf	-	*Plasmodium falciparum*
*Pf*CRT	-	*P*. *falciparum* chloroquine resistance transporter
*Pf*HRP2	-	Plasmodium falciparum histidine-rich protein 2
*Pf*MDR1	-	*P*. *falciparum* multidrug resistance transporter 1
*Pf*MRP	-	*P*. *falciparum* multidrug resistance-associated protein
*Pf*NHE	-	*P*. *falciparum* sodium/proton exchanger
PfPI3K	-	*P*. *falciparum* phosphatidylinositol-3-kinase
Pgh	-	P-glycoprotein homologue
ppm	-	Parts per million
PTPS	-	Pyruvyl tetrahydropterin synthase III
RDTs	-	Rapid detection tests
RF	-	Riboflavin
RFH_2	-	Dihydroriboflavin
RI	-	Resistance index

ROS	-	Reactive oxygen species
SERCA	-	Sarco-endoplasmic reticulum membrane calcium ATPase
SHMT	-	Serine hydroxymethyltransferase
SI	-	Selectivity index
SP	-	Sulfadoxine-pyrimethamine
SRB	-	Sulforhodamine B
TGA	-	Thermogravimetric analysis
THF	-	Tetrahydrofuran
TMS	-	Tetramethylsilane
TrxR	-	Thioredoxin reductase
$TrxS_2$	-	Thioredoxin oxidized
TrxSH	-	Thioredoxin reduced
TS	-	Thymidylate synthase
ULV	-	Ultra-low-volume
WHO	-	World Health Organization

CHAPTER 1
Introduction and objectives

1.1 Introduction

Malaria is a devastating protozoan disease transmitted to humans by the female *Anopheles* mosquito. Five different species of the genus *Plasmodium* including *P*. *falciparum*, *P*. *vivax*, *P*. *ovale*, *P*. *malariae* and *P*. *knowlesi* cause infection in humans (White *et al*., 2014). Infection by *P. falciparum*, if left untreated, leads to cerebral malaria; a major cause for mortality (Opsenica and Šolaja, 2012).

Malaria is endemic in 97 countries inhabited by roughly 3.4 billion people, making it a global health threat. In 2014, 214 million cases were reported and 438 000 people succumbed to the disease (WHO, 2015a). Malaria is most prevalent in the sub-Saharan African region; 88% of cases and 90% of deaths occur within this region. Children under the age of 5 are mostly affected with an infection rate of 70% (WHO, 2015a). In 2014, malaria killed an estimated 306 000 children globally under the age of 5; this translates into a child dying every 2 minutes of malaria (WHO, 2015a).

The World Health Organization (WHO) advocates a multi-faceted strategy to manage malaria; this includes diagnostic testing, preventative therapies, vector control, strong malaria surveillance and treatment with artemisinin combination therapies (ACTs) (WHO, 2015a). Vector control of malaria plays an important role with regard to the physical eradication of the mosquito, as it can decrease the number of people being infected by the disease. Physical eradication measures which include indoor residual spraying and insecticide treated mosquito nets (ITNs) are effective as these methods are shown to offer significant protection in millions of individuals in the African region (WHO, 2015a). Although these vector eradication methods are in place to help prevent the disease, the actual treatment of malaria is still hampered by the ability of the parasite to develop resistance against current clinically available antimalarial drugs (Okombo *et al*., 2012) such as quinine, chloroquine (CQ), mefloquine (MFQ), primaquine and other antimalarial drugs (Opsenica and Šolaja, 2012). Parasite resistance is driven by numerous factors including lack of patient adherence to prescribed drug regimens, inferior treatment practices, the extensive use of monotherapy based on artemisinins and the unfortunate use of sub-standard antimalarial drugs.

Current WHO recommended chemotherapies for treatment of uncomplicated malaria rely on the use of combinations of drugs known as artemisinin combination therapies (ACTs) that include an artemisinin and a longer half-life partner drug (Kantele and Jokiranta, 2011). Five ACTs, namely dihydroartemisinin-piperaquine, artesunate-amodiaquine, artesunate-mefloquine, artesunate-sulfadoxine-pyrimethamine (SP), and artemether-lumefantrine are currently recommended for use by the WHO. The choice of the ACT is largely dictated by therapeutic efficacy of the combination in the country or area of intended use. Interestingly, the ACT partner drug only targets the asexual life cycle of the parasite which is responsible for the manifestation of the clinical symptoms of the disease. Unfortunately, most drugs in current use are less effective against blood-stages of the parasite that lead to transmission. These stages are referred to as gametocytes that are sexually differentiated stages taken up by the mosquito. Therefore, in order to prevent malaria from being transferred from host to vector it is necessary for patients being treated for malaria to be cleared of gametocytes (Peatey *et al*., 2012). This is difficult in *P*. *falciparum* infections as the gametocytes persist much longer in the blood than the asexual stages that are most susceptible to antimalarial drugs. This applies especially to late blood-stage gametocytes (stages IV – V) that are much less susceptible to antimalarial drugs and metabolic inhibitors (Lang-Unnasch and Murphy, 1998). The one drug currently used that is effective against late-stage gametocytes is primaquine (Moyo *et al*., 2016).

Artemisinin is the highly active antimalarial component of the ancient Chinese traditional plant Qinghao (blue-green herb) or *Artemisia annua.* Also known as sweet wormwood, this plant has been used as a remedy by Chinese herbalists for more than 2 000 years for treatment of fevers and chills (Maude *et al*., 2009). Clinically used derivatives of artemisinin (Fig 1.1) include dihydroartemisinin (DHA), artemether, arteether and artesunate.

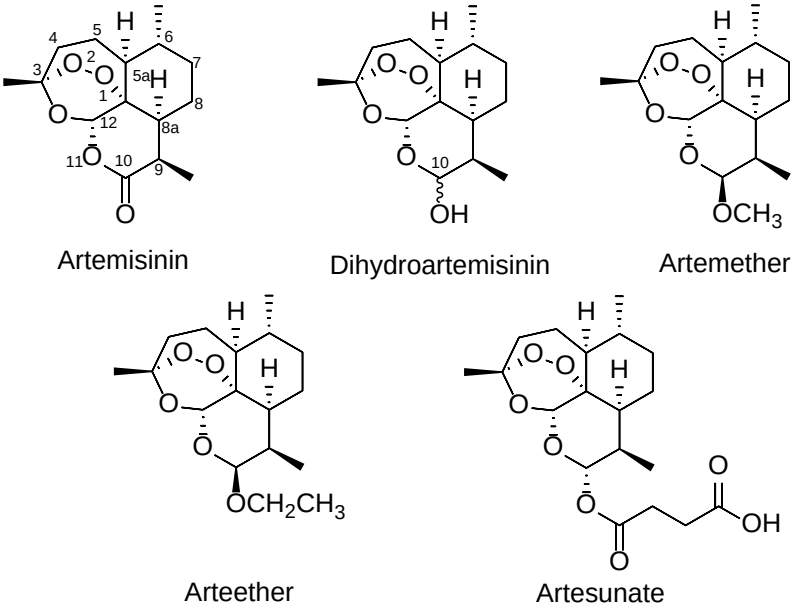


Figure 1.1: Artemisinin and derivatives dihydroartemisinin, artemether, arteether and artesunate.

Neospora caninum, like *P*. *falciparum*, is a protozoan parasite (Dubey *et al*., 1988) causing the economically important disease neosporosis that infects and induces abortion in cattle (Goodswen *et al*., 2013). The parasite life cycle involves both sexual and asexual reproduction. Sexual reproduction usually takes place in a definite host (canids such as dogs, coyotes, grey wolves and dingoes) (McAllister *et al*., 1998, Gondim *et al*., 2004, King *et al*., 2010, Dubey *et al*., 2011), while asexual reproduction only takes place in cattle. Unlike *P*. *falciparum*, no vector is involved in transmission. Dogs become infected by consuming contaminated meat containing oocysts that pass through the animal and are expelled within the faeces. Thereafter the oocysts in the faeces can persist in the environment. Cattle become infected by consuming pasture or water contaminated with the faeces containing the oocysts. Once ingested, the oocysts transform into tachyzoites, that transfer from an infected dam (mother) to foetus *via* the placenta (Goodswen *et al*., 2013). However, how dogs become infected with *N*. *caninum* in the first place is not properly understood, in spite of considerable research (Dubey *et al*., 2007). Interestingly, of the microbes that are known to infect cattle, *N*. *caninum* is one of the most efficiently transmitted across the placenta (Dubey *et al*., 2006).

The prevalence of neosporosis substantially differs between countries, regions within countries and between beef and dairy cattle. Remarkably, the prevalence of *Neospora* associated abortion appears to depend upon the particular region or country, and may display endemic, epidemic or sporadic patterns (Goodswen *et al*., 2013). Sporadic abortions

within a herd rarely take place, whereas endemic abortion is characterized by chronic long-term infection of a herd. In such a case, the parasite can be found in family lines as a consequence of recurrent transplacental transmission (Hall *et al*., 2005). Primary infection of previously uninfected dams that are exposed to a single source of infection are thought to be the cause of epidemic abortion patterns (McAllister *et al*., 2000). The epidemic pattern can result in an abortion "storm"; pregnant cows abort within a 12-week period, which can have a devastating economic impact on the region or country where it takes place.

Although a lot of time and effort have been spent on the development of a vaccine, there has been little success so far. Use of chemotherapeutic agents as treatment against neosporosis has not been considered as an economical viable option until recently. This was due to the potentially long withdrawal period during which milk and meat from drug-treated cattle remains unacceptable (Dubey *et al*., 2007). Therefore, there are no safe and effective treatment regimens currently available for neosporosis (Hemphill and Müller, 2015). Recent studies indicated that several compounds derived from screening against *Plasmodium* present potentially interesting effects (Müller and Hemphill, 2011). Their application as agents against neosporosis would establish a good example of drug repurposing (Sateriale *et al*., 2014).

1.2 Rationale

Chemoprophylaxis and chemotherapy are the primary means of combating malaria infections in a human host as there are no vaccines available yet. Since the introduction of synthetic and semisynthetic antimalarials, only a small number of compounds was found to be suitable for clinical use and this limited arsenal is further compromised by the parasites' ability to develop resistance. Although the WHO has strategies in place to suppress development of resistance, artemisinin-resistant *P. falciparum* has been reported in five countries in Southeast Asia namely Cambodia, Laos, Myanmar, Thailand and Vietnam (O'Brien *et al*., 2011, Ashley *et al*., 2014). Although the resistance is known to be associated with delayed clearance of the early asexual blood-stages of the parasite from the blood, patients do respond to combination treatment so long as the partner drug retains activity (WHO, 2015a). Therefore, use of artemisinins in monotherapy for the treatment of uncomplicated malaria is prohibited, as poor adherence to the essential 7-day course of treatment results only in the partial clearance of malaria parasites; this will enable resistant parasites to survive, thus contributing to the spread of artemisinin resistance (WHO, 2015a). Also, artemisinin monotherapy causes high rates of parasite recrudescence (Cheng *et al*.,

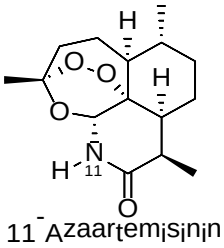
2012) and this not uncommonly occurs even when an ACT is used. Some studies (Teuscher *et al*., 2010, Cheng *et al*., 2012) even suggest that parasite recrudescence can be linked to artemisinin-induced dormancy where the parasite responds to stress caused by artemisinin in such a way that parasite development at the ring-stage is temporarily halted for a period of time. After artemisinin drug concentrations decrease, a small portion of dormant ring-stage parasites recover and resume growth which in turn causes the recrudescence that ultimately leads to treatment failure. It stands to reason that the efficacy of an ACT is achieved by the companion drug having an impact on the dormant parasites and in the case of a long-acting companion drug, by direct suppression of growth of the recovering parasites (Cheng *et al*., 2012).

Resistance and parasite recrudescence are not the only problems the artemisinins face as there are a host of other challenges that threaten their use as viable drugs against malaria. Indeed, the majority of artemisinins that have been synthesized are mostly esters, ethers or urethane derivatives of the hydroxyl group of DHA (Dayan *et al*., 1999) with the clinically most useful artemisinins, *viz*. artemether and artesunate, being metabolized *in vivo* to DHA (Krishna *et al*., 2004). DHA is an unstable drug with a very short plasma half-life and is erratically absorbed. Of concern is the fact that DHA has proved to be neurotoxic in cell and animal assays (Smith *et al*., 2001, Gordi and Lepist, 2004, Toovey, 2006, Efferth and Kaina, 2010) which raise questions about its safety in humans. The longer half-life partner drug in ACT therapy compensates for the short half-life of the artemisinins. However, significantly prolonged *in vivo* parasite clearance times have been observed in Southeast Asia for ACTs containing artesunate and artemether (Dondorp *et al*., 2009, Phyo *et al*., 2012); in other words, the parasite is evolving increased tolerance towards the combination partner in the ACT.

Although ACTs are still effective for now, one cannot help but notice the barrier that keeps us from reaching full-blown malaria drug resistance has grown remarkably thin. Considering the above mentioned problems and challenges the artemisinins face, a new artemisinin derivative that is thermally more stable than the current derivatives, does not provide DHA *in vivo*, is non-cytotoxic, and has a longer half-life, is urgently required.

One such compound that might meet such demands is 11-azaartemisinin (Fig 1.2). This can be easily obtained from artemisinin by a well-established method (Haynes *et al*., 2007a). 11-Azaartemisinin represents a rather different structural type in that O-11 is substituted by a nitrogen atom whereas all current clinical derivatives rely on modification and substitution at the C-10 position on the artemisinin moiety. The substitution of O-11 by nitrogen results in a significantly more stable compound under acidic conditions (Avery *et al*., 1995) that also

shows an increase in bioavailability when compared to other artemisinins. Also, the compound and its derivatives can't undergo decomposition to DHA as the azaartemisinins are at a higher oxidation level than DHA (Haynes *et al*., 1999). Whether or not azaartemisinin itself has an increased half-life remains to be established. Azaartemisinin has an enhanced thermal stability when compared to other artemisinins. By using thermogravimetric analysis (TGA), Haynes and co-workers were able to compare the thermal stabilities of current clinically used artemisinins with their novel series of *N*-sulfonyl- and *N*-carbonyl-11-azaartemisinins (Haynes *et al*., 2007a). Not only did these compounds, especially the *N*-sulfonyl-11-azaartemisinins, possess greatly enhanced thermal stabilities but also some of these derivatives were potent against CQ-sensitive and -resistant strains of *P*. *falciparum*.



11-Azaartemisinin

Figure 1.2: 11-Azaartemisinin contains the lactam moiety instead of the lactone of artemisinin, making the compound chemically more robust.

As most of the current clinically used drugs for malaria only target the asexual blood-stages of the parasite, it is of value to investigate the gametocytocidal activity of the azaartemisinins. Some evidence does suggest that artemisinins can reduce gametocyte carriage (Price *et al*., 1996, Sutherland *et al*., 2005) but transmission still occurs after the use of ACTs (Bousema *et al*., 2006) which may partially reflect the rapid clearance of artemisinin-based compounds (Baker, 2010). Whereas artemisinin itself is capable of killing the early-stage gametocytes it does not appear to affect the mature gametocytes (stage IV/V) crucial for transmission (Kumar and Zheng, 1990, Pukrittayakamee *et al*., 2004, Sutherland *et al*., 2005, Shekalaghe *et al*., 2007, Czesny *et al*., 2009). Currently, of the artemisinins it is only artesunate and artemether that show moderate activity towards late-stage gametocytes but these drugs are threatened by emerging resistance and concerns regarding toxicity. Procuring a compound that can target late-stage gametocytes would be of great importance and this is therefore an objective of the research.

Given that artemisinins display activity against tumour cell lines, it is of interest to establish if 11-azaartemisinins are also active. There appears to be a correlation between the antimalarial and antitumour activities of artemisinins (Jones *et al*., 2009, Lombard *et al*.,

2012), although IC_{50} values against tumour cell lines are generally in the low micromolar range, that is, they are several orders of magnitude less active against tumour cells than against the malaria parasite. Thus, artemisinin (Lu, 2003), artemether (Singh and Panwar, 2006) and artesunate (Singh and Verma, 2002, Berger *et al*., 2005, Zhang *et al*., 2008) display IC_{50} values against different tumour cell lines in the low micromolar range. Nevertheless, the encouraging nature of the results have resulted in substantial follow up studies involving evaluation of the effects of artemisinins against cancer tissue xenografts in mice, and, in several cases, of the conduct of Phase II trials in humans with artesunate coupled with other treatment modalities. Extensive mechanistic studies aimed at determining how artemisinins exert their cytotoxicity towards cancer cells have also been carried out, and quite a lot is understood now as to how artemisinins exert their antitumour effects. Therefore, it is planned to evaluate the activities of the azaartemisinins against tumour cell lines as one of the objectives of the current research.

As *P*. *falciparum* and *N*. *caninum* are both protozoan apicomplexan parasites, there is the possibility that *N*. *caninum* can also be treated with artemisinin and its derivatives. There is currently no vaccine available for *N*. *caninum* and the disease has a staggering worldwide economic impact of over US$1.3 billion (Reichel *et al*., 2014). A couple of studies already suggest that artemisinin is indeed effective against the disease (Kim *et al*., 2002, Mazuz *et al*., 2012). Another study involving an examination of the use of artemisone and other aminoartemisinin derivatives against *N*. *caninum* (Müller *et al*., 2015) reported good activity and very low toxicity towards human foreskin fibroblasts infected with *N. caninum*. The efficacy of 11-azaartemisinins on the other hand has not been evaluated against *N*. *caninum*.

1.3 Aim and objectives

1.3.1 Aim of this study

In light of the above considerations, the aim of this study was to prepare new *N*-sulfonyl-11-azaartemisinin derivatives, to evaluate their antimalarial activity against both CQ-sensitive and -resistant strains of *P. falciparum*, to assess their gametocytocidal activity against early- and late-stage gametocytes and to determine their toxicity against mammalian cells with the ultimate goal to finding new artemisinin derivatives for the treatment of malaria. At the same time, the efficacy of the *N*-sulfonyl-11-azaartemisinin derivatives would be evaluated against *N*. *caninum*, with the eventual goal of evolving new treatments for neosporosis.

1.3.2 Specific objectives of this study

The initial objectives of the study are as follows:

- To synthesize new *N*-sulfonyl-11-azaartemisinins and to carry out their structural characterization by means of nuclear magnetic resonance (NMR) and infrared (IR) spectroscopy, and high resolution mass spectrometry (HRMS) (Chapter 4).
- To determine the *in vitro* activities of the new azaartemisinins against *P. falciparum* and cytotoxicities against mammalian cells (Chapter 4).

The next objectives of the study are as follows:

- To evaluate the gametocytocidal activities of the new compounds with emphasis on discovering compounds displaying potent activities against late-stage gametocytes (Chapter 4).
- To determine the antitumour activities of the new derivatives and to establish if the derivatives are selectively cytotoxic towards tumour cells but not against non-proliferating mammalian cells (Chapter 5).
- To evaluate the potential of these compounds for use as drugs against diseases caused by other apicomplexan parasites like *Neospora caninum* by determining efficacies against this parasite (Chapter 5).

REFERENCES

Ashley, E. A., Dhorda, M., Fairhurst, R. M., Amaratunga, C., Lim, P., Suon, S., Sreng, S., Anderson, J. M., Mao, S. & Sam, B. (2014) Spread of artemisinin resistance in *Plasmodium falciparum* malaria. *New England Journal of Medicine,* 371**,** 411-423.

Avery, M. A., Bonk, J. D., Chong, W. K. M., Mehrotra, S., Miller, R., Milhous, W., Goins, D. K., Venkatesan, S. & Wyandt, C. (1995) Structure-activity relationships of the antimalarial agent artemisinin. 2. Effect of heteroatom substitution at O-11: synthesis and bioassay of N-Alkyl-11-aza-9-desmethylartemisinins. *Journal of Medicinal Chemistry,* 38**,** 5038-5044.

Baker, D. A. (2010) Malaria gametocytogenesis. *Molecular and Biochemical Parasitology,* 172**,** 57-65.

Berger, T. G., Dieckmann, D., Efferth, T., Schultz, E. S., Funk, J.-O., Baur, A. & Schuler, G. (2005) Artesunate in the treatment of metastatic uveal melanoma-first experiences. *Oncology Reports,* 14**,** 1599-1603.

Bousema, J. T., Schneider, P., Gouagna, L. C., Drakeley, C. J., Tostmann, A., Houben, R., Githure, J. I., Ord, R., Sutherland, C. J. & Omar, S. A. (2006) Moderate effect of artemisinin-based combination therapy on transmission of *Plasmodium falciparum*. *Journal of Infectious Diseases,* 193**,** 1151-1159.

Cheng, Q., Kyle, D. E. & Gatton, M. L. (2012) Artemisinin resistance in *Plasmodium falciparum*: A process linked to dormancy? *International Journal for Parasitology: Drugs and Drug Resistance,* 2**,** 249-255.

Czesny, B., Goshu, S., Cook, J. L. & Williamson, K. C. (2009) The proteasome inhibitor epoxomicin has potent *Plasmodium falciparum* gametocytocidal activity. *Antimicrobial Agents and Chemotherapy,* 53**,** 4080-4085.

Dayan, F. E., Hernändez, A., Allen, S. N., Moraes, R. M., Vroman, J. A., Avery, M. A. & Duke, S. O. (1999) Comparative phytotoxicity of artemisinin and several sesquiterpene analogues. *Phytochemistry,* 50**,** 607-614.

Dondorp, A. M., Nosten, F. O., Yi, P., Das, D., Phyo, A. P., Tarning, J., Lwin, K. M., Ariey, F., Hanpithakpong, W. & Lee, S. J. (2009) Artemisinin resistance in *Plasmodium falciparum* malaria. *New England Journal of Medicine,* 361**,** 455-467.

Dubey, J. P., Carpenter, J. L., Speer, C. A., Topper, M. J. & Uggla, A. (1988) Newly recognized fatal protozoan disease of dogs. *Journal of the American Veterinary Medical Association,* 192**,** 1269-1285.

Dubey, J. P., Buxton, D. & Wouda, W. (2006) Pathogenesis of bovine neosporosis. *Journal of Comparative Pathology,* 134**,** 267-289.

Dubey, J. P., Schares, G. & Ortega-Mora, L. M. (2007) Epidemiology and control of neosporosis and *Neospora caninum*. *Clinical Microbiology Reviews,* 20**,** 323-367.

Dubey, J. P., Jenkins, M. C., Rajendran, C., Miska, K., Ferreira, L. R., Martins, J., Kwok, O. C. H. & Choudhary, S. (2011) Gray wolf (*Canis lupus*) is a natural definitive host for *Neospora caninum*. *Veterinary Parasitology,* 181**,** 382-387.

Efferth, T. & Kaina, B. (2010) Toxicity of the antimalarial artemisinin and its derivatives. *Critical Reviews in Toxicology,* 40**,** 405-421.

Gondim, L. F. P., McAllister, M. M., Pitt, W. C. & Zemlicka, D. E. (2004) Coyotes (*Canis latrans*) are definitive hosts of *Neospora caninum*. *International Journal for Parasitology,* 34**,** 159-161.

Goodswen, S. J., Kennedy, P. J. & Ellis, J. T. (2013) A review of the infection, genetics, and evolution of *Neospora caninum*: from the past to the present. *Infection, Genetics and Evolution,* 13**,** 133-150.

Gordi, T. & Lepist, E.-I. (2004) Artemisinin derivatives: toxic for laboratory animals, safe for humans? *Toxicology Letters,* 147**,** 99-107.

Hall, C. A., Reichel, M. P. & Ellis, J. T. (2005) *Neospora* abortions in dairy cattle: diagnosis, mode of transmission and control. *Veterinary Parasitology,* 128**,** 231-241.

Haynes, R. K., Pai, H. H.-O. & Voerste, A. (1999) Ring opening of artemisinin (qinghaosu) and dihydroartemisinin and interception of the open hydroperoxides with formation of *N*-oxides - a chemical model for antimalarial mode of action. *Tetrahedron Letters,* 40**,** 4715-4718.

Haynes, R. K., Wong, H. N., Lee, K. W., Lung, C. M., Shek, L. Y., Williams, I. D., Croft, S. L., Vivas, L., Rattray, L. & Stewart, L. (2007a) Preparation of *N*-Sulfonyl- and *N*-Carbonyl-11-azaartemisinins with greatly enhanced thermal stabilities: *in vitro* antimalarial activities. *ChemMedChem,* 2**,** 1464-1479.

Hemphill, A. & Müller, J. (2015) Vaccines and drugs against *Neospora caninum*, an important apicomplexan causing abortion in cattle and other farm animals. *Reports in Parasitology,* 4**,** 31-41.

Jones, M., Mercer, A. E., Stocks, P. A., La Pensèe, L. J. I., Cosstick, R., Park, B. K., Kennedy, M. E., Piantanida, I., Ward, S. A. & Davies, J. (2009) Antitumour and antimalarial activity of artemisinin-acridine hybrids. *Bioorganic & Medicinal Chemistry Letters,* 19**,** 2033-2037.

Kantele, A. & Jokiranta, T. S. (2011) Review of cases with the emerging fifth human malaria parasite, *Plasmodium knowlesi*. *Clinical Infectious Diseases,* 52**,** 1356-1362.

Kim, J.-T., Park, J.-Y., Seo, H.-S., Oh, H.-G., Noh, J.-W., Kim, J.-H., Kim, D.-Y. & Youn, H.-J. (2002) *In vitro* antiprotozoal effects of artemisinin on *Neospora caninum*. *Veterinary Parasitology,* 103**,** 53-63.

King, J. S., Slapeta, J., Jenkins, D. J., Al-Qassab, S. E., Ellis, J. T. & Windsor, P. A. (2010) Australian dingoes are definitive hosts of *Neospora caninum*. *International Journal for Parasitology,* 40**,** 945-950.

Krishna, S., Uhlemann, A.-C. & Haynes, R. K. (2004) Artemisinins: mechanisms of action and potential for resistance. *Drug Resistance Updates,* 7**,** 233-244.

Kumar, N. & Zheng, H. (1990) Stage-specific gametocytocidal effect in vitro of the antimalaria drug qinghaosu on *Plasmodium falciparum*. *Parasitology Research,* 76**,** 214-218.

Lang-Unnasch, N. & Murphy, A. D. (1998) Metabolic changes of the malaria parasite during the transition from the human to the mosquito host. *Annual Reviews in Microbiology,* 52**,** 561-590.

Lombard, M. C., N'da, D. D., Breytenbach, J. C., Kolesnikova, N. I., Van Ba, C. T., Wein, S., Norman, J., Denti, P., Vial, H. & Wiesner, L. (2012) Antimalarial and anticancer activities of artemisinin-quinoline hybrid-dimers and pharmacokinetic properties in mice. *European Journal of Pharmaceutical Sciences,* 47**,** 834-841.

Lu, L. (2003) Study on effect of *Cordyceps sinensis* and artemisinin in preventing recurrence of lupus nephritis. *Alternative Medicine Review,* 8**,** 209-210.

Maude, R. J., Pontavornpinyo, W., Saralamba, S., Aguas, R., Yeung, S., Dondorp, A. M., Day, N. P. J., White, N. J. & White, L. J. (2009) The last man standing is the most resistant: eliminating artemisinin-resistant malaria in Cambodia. *Malaria Journal,* 8**,** 31.

Mazuz, M. L., Haynes, R., Shkap, V., Fish, L., Wollkomirsky, R., Leibovich, B., Molad, T., Savitsky, I. & Golenser, J. (2012) *Neospora caninum*: *in vivo* and *in vitro* treatment with artemisone. *Veterinary Parasitology,* 187**,** 99-104.

McAllister, M. M., Dubey, J. P., Lindsay, D. S., Jolley, W. R., Wills, R. A. & Mcguire, A. M. (1998) Rapid communication: Dogs are definitive hosts of *Neospora caninum*. *International Journal for Parasitology,* 28**,** 1473-1479.

McAllister, M. M., Björkman, C., Anderson-Sprecher, R. & Rogers, D. G. (2000) Evidence of point-source exposure to *Neospora caninum* and protective immunity in a herd of beef cows. *Journal of the American Veterinary Medical Association,* 217**,** 881-887.

Moyo, P., Botha, M. E., Nondaba, S., Niemand, J., Maharaj, V. J., Eloff, J. N., Louw, A. I. & Birkholtz, L. (2016) *In vitro* inhibition of *Plasmodium falciparum* early and late-stage gametocyte viability by extracts from eight traditionally used South African plant species. *Journal of Ethnopharmacology,* 185**,** 235-242.

Müller, J., Balmer, V., Winzer, P., Rahman, M., Manser, V., Haynes, R. K. & Hemphill, A. (2015) *In vitro* effects of new artemisinin derivatives in *Neospora caninum*-infected human fibroblasts. *International Journal of Antimicrobial Agents,* 46**,** 88-93.

Müller, J. & Hemphill, A. (2011) Drug target identification in intracellular and extracellular protozoan parasites. *Current Topics in Medicinal Chemistry,* 11:(16)**,** 2029-2038.

O'Brien, C., Henrich, P. P., Passi, N. & Fidock, D. A. (2011) Recent clinical and molecular insights into emerging artemisinin resistance in *Plasmodium falciparum*. *Current Opinion in Infectious Diseases,* 24**,** 570-585.

Okombo, J., Mwai, L. & Nzila, A. (2012) Tackling the Problem of Antimalarial Resistance. IN CHIBALE, K., DAVIES-COLEMAN, M. & MASIMIREMBWA, C. (Eds.) *Drug Discovery in Africa: Impacts of Genomics, Natural Products, Traditional Medicines, Insights into Medicinal Chemistry, and Technology Platforms in Pursuit of New Drugs.* London, Springer. p. 301-324.

Opsenica, D. M. & Šolaja, B. A. (2012) Artemisinins and synthetic peroxides as highly efficient antimalarials. *Macedonian Journal of Chemistry and Chemical Engineering,* 31**,** 137-182.

Peatey, C. L., Leroy, D., Gardiner, D. L. & Trenholme, K. R. (2012) Antimalarial drugs: how effective are they against *Plasmodium falciparum* gametocytes? *Malaria Journal,* 11**,** 1-4.

Phyo, A. P., Nkhoma, S., Stepniewska, K., Ashley, E. A., Nair, S., McGready, R., Ler Moo, C., Al-Saai, S., Dondorp, A. M. & Lwin, K. M. (2012) Emergence of artemisinin-resistant malaria on the western border of Thailand: a longitudinal study. *The Lancet,* 379**,** 1960-1966.

Price, R. N., Nosten, F., Luxemburger, C., Ter Kuile, F. O., Paiphun, L., Chongsuphajaisiddhi, T. & White, N. J. (1996) Effects of artemisinin derivatives on malaria transmissibility. *The Lancet,* 347**,** 1654-1658.

Pukrittayakamee, S., Chotivanich, K., Chantra, A., Clemens, R., Looareesuwan, S. & White, N. J. (2004) Activities of artesunate and primaquine against asexual-and sexual-stage parasites in falciparum malaria. *Antimicrobial Agents and Chemotherapy,* 48**,** 1329-1334.

Reichel, M. P., McAllister, M. M., Pomroy, W. E., Campero, C., Ortega-Mora, L. M. & Ellis, J. T. (2014) Control options for *Neospora caninum* - is there anything new or are we going backwards? *Parasitology,* 141**,** 1455-1470.

Sateriale, A., Bessoff, K., Sarkar, I. N. & Huston, C. D. (2014) Drug repurposing: mining protozoan proteomes for targets of known bioactive compounds. *Journal of the American Medical Informatics Association,* 21**,** 238-244.

Shekalaghe, S., Drakeley, C., Gosling, R., Ndaro, A., Van Meegeren, M., Enevold, A., Alifrangis, M., Mosha, F., Sauerwein, R. & Bousema, T. (2007) Primaquine clears submicroscopic *Plasmodium falciparum* gametocytes that persist after treatment with sulfadoxine-pyrimethamine and artesunate. *PloS One,* 2**,** e1023.

Singh, N. P. & Panwar, V. K. (2006) Case report of a pituitary macro-adenoma treated with artemether. *Integrative Cancer Therapies,* 5**,** 391-394.

Singh, N. P. & Verma, K. B. (2002) Case report of a laryngeal squamous cell carcinoma treated with artesunate. *Archive of Oncology,* 10**,** 279-280.

Smith, S. L., Sadler, C. J., Dodd, C. C., Edwards, G., Ward, S. A., Park, B. K. & Mclean, W. G. (2001) The role of glutathione in the neurotoxicity of artemisinin derivatives *in vitro*. *Biochemical Pharmacology,* 61**,** 409-416.

Sutherland, C. J., Ord, R., Dunyo, S., Jawara, M., Drakeley, C. J., Alexander, N., Coleman, R., Pinder, M., Walraven, G. & Targett, G. A. T. (2005) Reduction of malaria transmission to *Anopheles* mosquitoes with a six-dose regimen of co-artemether. *PLoS Medicine,* 2**,** e92.

Teuscher, F., Gatton, M. L., Chen, N., Peters, J., Kyle, D. E. & Cheng, Q. (2010) Artemisinin-induced dormancy in *Plasmodium falciparum*: duration, recovery rates, and implications in treatment failure. *Journal of Infectious Diseases,* 202**,** 1362-1368.

Toovey, S. (2006) Are currently deployed artemisinins neurotoxic? *Toxicology Letters,* 166**,** 95-104.

White, N. J., Pukrittayakamee, S., Hien, T. T., Faiz, M. A., Mokuolu, O. A. & Dondorp, A. M. (2014) Malaria. *The Lancet,* 383**,** 723-735.

Who (2015a) Global Malaria Programme: World Malaria Report. Geneva, World Health Organization. 8-17p.

Zhang, Z. Y., Yu, S. Q., Miao, L. Y., Huang, X. Y., Zhang, X. P., Zhu, Y. P., Xia, X. H. & Li, D. Q. (2008) Artesunate combined with vinorelbine plus cisplatin in treatment of advanced non-small cell lung cancer: a randomized controlled trial. *Zhong Xi Yi Jie He Xue Bao = Journal of Chinese Integrative Medicine,* 6**,** 134-138.

CHAPTER 2
Literature overview

2.1 Introduction

Malaria is an ancient disease with descriptions more or less corresponding to the disease dating back to 2700 BC in ancient Chinese texts (Cox, 2010). For the next 2500 years it was largely believed that the fevers and spleen enlargement associated with malaria was caused by “bad air” arising from swamps; this lead to the use of the Italian word “*mal’aria”* during the Renaissance for the disease meaning "bad air". However, in 1880 Charles Louis Alphonse Laveran discovered that the cause of malaria was protozoan, when he observed parasites in a blood smear of a patient that died from malaria in a military hospital in Algeria (Bruce-Chwatt, 1981). In 1897, Ronald Ross made the discovery that mosquitoes are the vectors for malaria (Ross, 1897). These important discoveries laid the foundation for modern malaria research.

Thanks to the ground-breaking discoveries made by Laveran and Ross and the ensuing research, we know now that malaria is caused by a protozoan parasite of the genus *Plasmodium* transmitted by infected female *Anopheles* mosquitos when these take a blood meal from a human. There are five known species of *Plasmodium* capable of infecting humans namely *P*. *falciparum*, *P*. *vivax*, *P*. *ovale*, *P*. *malariae* and *P*. *knowlesi*. Most deaths are caused by *P*. *falciparum* while the other species largely result in milder cases of malaria. *P*. *knowlesi*, on the other hand rarely causes infection in humans. Malaria can give rise to flu-like symptoms including headaches, fever, vomiting and fatigue with symptoms usually beginning to show after 10 to 15 days post-infection.

In this chapter, the epidemiology of malaria, including some notable statistics from the World Health Organization (WHO) are discussed. An overview of the malaria parasite life cycle is examined as well as the clinical features of this deadly disease, including pathogenesis and complications. Different methods of diagnoses of malaria will also be discussed. The effect of malaria control and prevention and the effect it has on the burden of malaria are explored. Finally, the different chemotherapeutic agents used to treat malaria as well as the mechanisms used by the parasite to develop drug resistance are reviewed.

2.2 Epidemiology

Density, feeding habits and efficiency of transmission associated with the mosquito vector are key factors that influence the transmission of malaria (White *et al*., 2014). There are approximately 400 species of *Anopheles* mosquitoes, but only 30 of these are of major importance for transmitting malaria (Sinka *et al*., 2012, WHO, 2015a). The most successful vectors are relatively robust to environmental change, occur in high densities in tropical climates, breed readily, and preferentially take blood meals from humans. Transmission of malaria cannot occur at temperatures above 35 °C or below 25 °C. Humidity also plays a role in transmission as relative humidity values of 75% usually ensure optimal survival of adult vectors, where values below 35% shorten the life span to a level that is incompatible with malaria transmission. Water is crucial for breeding and the optimal amount of water needed differs greatly between species (Wernsdorfer, 2012). Another factor in the epidemiology of malaria is the behaviour of humans. There are a variety of human factors that play a role in not only the transmission of malaria, but also prevention. Such factors can include access to health care, socio-economic status, gender, migration and land (Protopopoff *et al*., 2009).

According to the WHO, an estimated 3.3 billion people in 97 countries were at risk of being infected with malaria in 2014 (Fig. 2.1) .In 2014, 214 million cases of malaria and approximately 438,000 deaths were reported (WHO, 2015a).

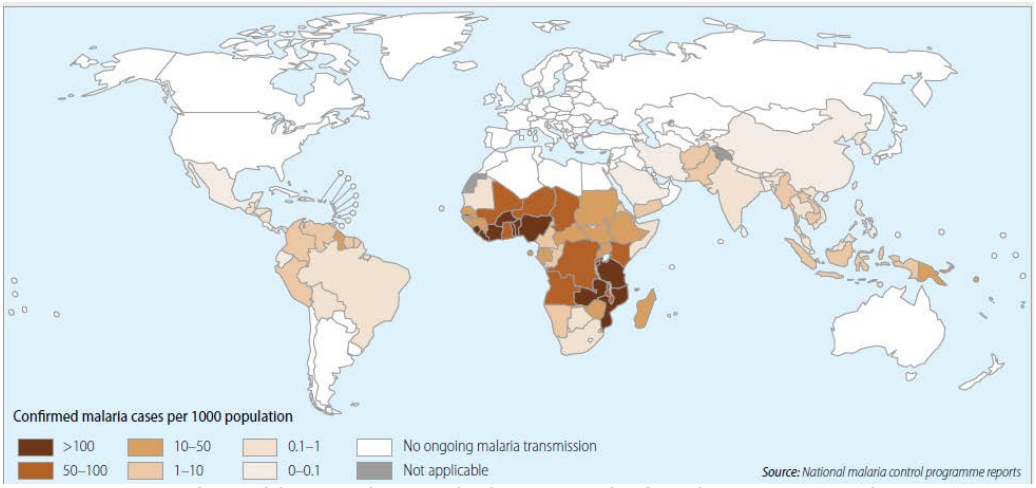


Figure 2.1: Countries with ongoing malaria transmission (WHO, 2015a).

P. *falciparum* and *P*. *vivax* malaria have roughly equal prevalences in Asia and in South and Central America because transmission is low and mainly seasonal (Gething *et al*., 2011a, Gething *et al*., 2011b). In these areas, most humans characteristically receive one or fewer infectious bites per year. In sub-Saharan Africa however, where *P*. *falciparum* predominates,

the malaria burden is much greater. Transmission intensities are much higher (Gething *et al*., 2011a, Gething *et al*., 2011b), and it is in this region that an estimated 90% of all malaria deaths occur, with children under the age of 5 accounting for 71% of all deaths (WHO, 2015a).

2.3 Malaria life cycle and pathogenesis

The life cycle of the malaria parasite starts when a human host is bitten by an infected female *Anopheles* mosquito. The life of the parasite is therefore spent in two hosts, namely the female *Anopheline* mosquito and the human. The life cycle can be divided into three phases where the first two phases, known as the exoerythrocytic and erythrocytic phases, occur inside the human host while the third and final phase, also known as the sexual stage, occur inside the mosquito (Fig. 2.2).

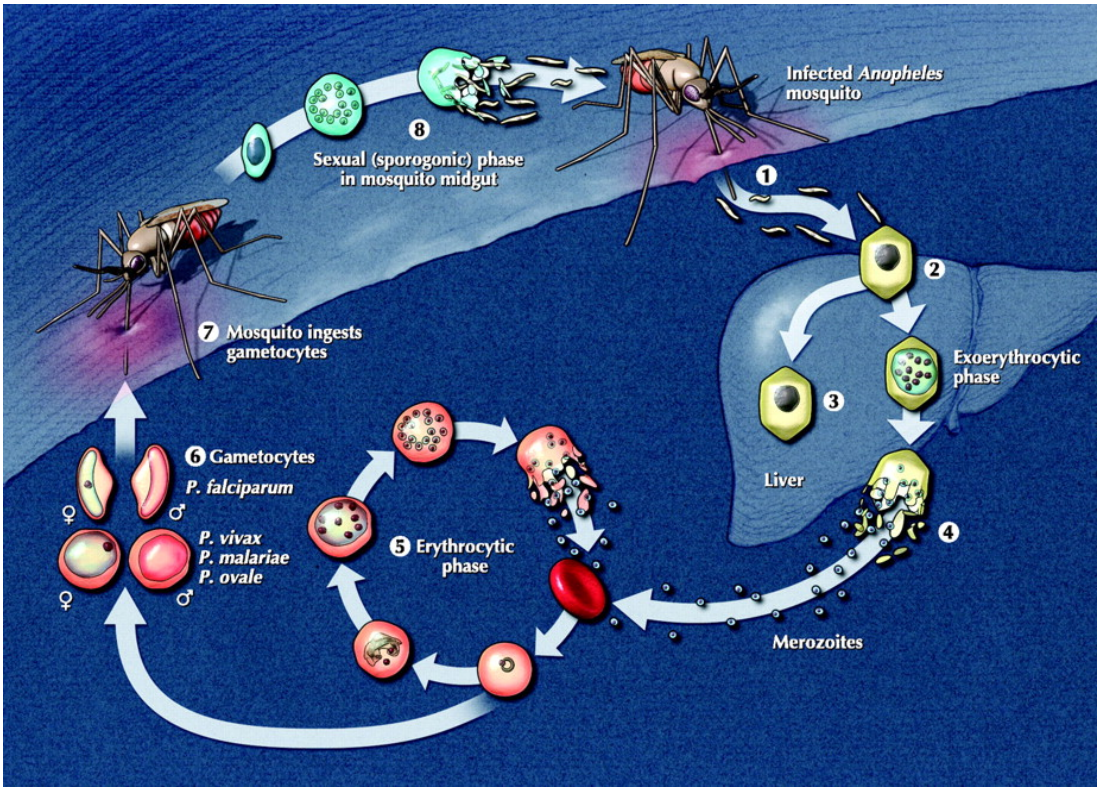


Figure 2.2: The various stages involved in the life cycle of the malaria parasite (Suh *et al*., 2004).

Infection of the human host starts as the female *Anopheline* takes a blood meal and sporozoites are inoculated into the bloodstream. This is known as the start of the exoerythrocytic phase and is completed when the first generations of merozoites are released from the hepatocytes into the bloodstream (Ménard *et al*., 2008). Hereafter, the

sporozoites have up to 3 hours of motility which allow them to reach the liver (Amino *et al*., 2006, Ménard *et al*., 2008). Some of the sporozoites are blocked by the human immune system *via* antibodies and only a certain portion of the skin sporozoites invade the blood capillaries to make their way to the target liver cells (Plebanski and Hill, 2000). After reaching the liver and invading the hepatocytes, the sporozoites undergo mitotic replication called schizogony, lasting between two to ten days, as they develop into liver-stage trophozoites (Doolan *et al*., 2009). The nucleus of the trophozoite divides several times producing thousands of exoerythrocytic merozoites that causes the hepatic schizont to rupture. The release of the merozoites into the bloodstream, marks the end of the exoerythrocytic phase (Ashley *et al*., 2006, White, 2008a, Doolan *et al*., 2009). A successful sporozoite can produce between 10,000 to 30,000 daughter merozoites in 5 – 8 days within a hepatocyte (White *et al*., 2014). Unlike the case with *P. falciparum*, a certain portion of the sporozoites of both *P*. *vivax* and *P*. *ovale* form dormant hypnozoites which can remain in the liver for years before developing into schizonts. Such development ultimately leads to relapse of malaria caused by *P. vivax* and *P. ovale* (Fujioka and Aikawa, 2002, Dembele *et al*., 2011).

The erythrocytic phase starts as the exoerythrocytic merozoites enter the bloodstream, now known as blood-stage merozoites. The merozoite with its very small size (~1 – 2 µm) (Fig. 2.3), is elegantly adapted for entering the erythrocytes (Bannister *et al*., 1986, Cowman *et al*., 2012).

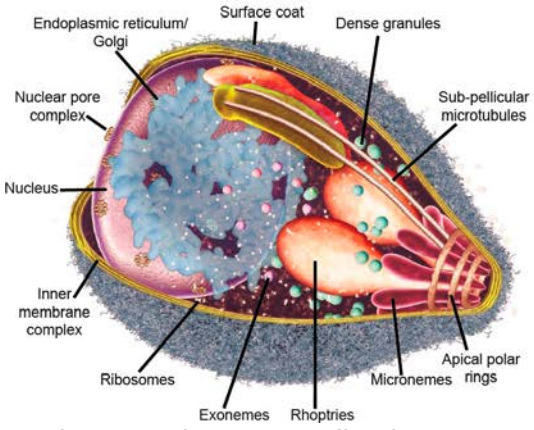


Figure 2.3: The merozoite comprises organelles incorporated within the cell exoskeleton (Morrissette and Sibley, 2002, Cowman *et al*., 2012). This includes an apical complex of secretory organelles (rhoptries, dense granules and micronemes), nucleus, mitochondrion and apicoplast (McFadden, 1996, Roos *et al*., 1999, Cowman *et al*., 2012). Underlying the plasma membrane is the inner membrane complex lined by two to three subpellicular microtubules (Bannister *et al*., 2000, Cowman *et al*., 2012).

As the schizont ruptures, the mature merozoites are ejected; these then again enter the erythrocytes (Fig. 2.4a) (Cowman *et al*., 2012). Initially, distortion of the erythrocyte surface allows for an interaction between the merozoite and erythrocyte (Fig. 2.4b), where a reorientation places the merozoite apex adjacent to the erythrocyte membrane. The parasite enters the erythrocyte after major buckling of the erythrocyte surface (Fig. 2.4b), most likely due to parasite-induced reorganization of the erythrocyte cytoskeleton (Zuccala and Baum, 2011, Cowman *et al*., 2012). After invasion of the erythrocyte is complete, the posterior is sealed off followed by echinocytosis of the erythrocyte. The erythrocyte resumes its normal state within 10 minutes (Gilson and Crabb, 2009, Cowman *et al*., 2012).

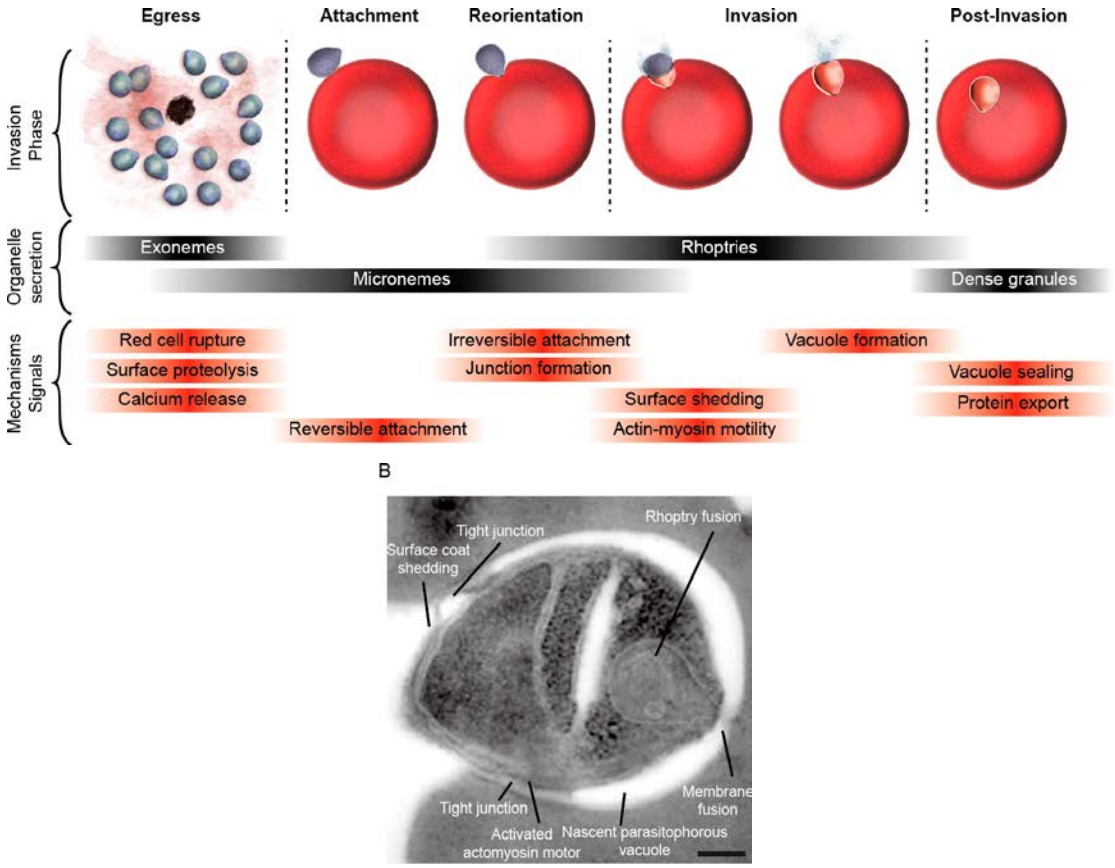


Figure 2.4: **a.** Schematic representation of the invasion of an erythrocyte by a merozoite from egress to post invasion. A tight junction between the merozoite and the erythrocyte is formed after reorientation of the merozoite (Cowman *et al*., 2012). Through the activity of proteases, proteins are shed into the supernatant as the tight junction moves across the merozoite surface. The parasite passes into the erythrocyte resulting in the ejection of the erythrocyte contents (Cowman *et al*., 2012). The membrane and parasitophorous vacuole form the space within the erythrocyte in which the parasite passes into and is fashioned out of some leftover erythrocyte cell membrane components as well as the rhoptries from the merozoite. As the tight junction moves to the posterior of the parasite the membranes seal off by an unknown mechanism (Cowman *et al*., 2012). **b.** A *P. falciparum* merozoite in the process of invading a human red blood cell (image S. Ralph, University of Melbourne, Australia). Bar, 200 nm (Cowman *et al*., 2012).

Once inside the erythrocyte, the parasite flattens into an apparent ring form (Grüring *et al*., 2011), growing and consuming the erythrocyte haemoglobin, changing the cell membrane in order to facilitate import of nutrients as well as disposing of toxic haem waste product through crystallization to biologically inert haemozoin (malaria pigment) (White *et al*., 2014). In a susceptible individual, the parasite population will expand between six and twenty times

per cycle (Simpson *et al*., 2002). The majority of contents inside of the erythrocytes will be consumed by the parasite at the end of the erythrocytic cycle.

As the parasite grows it forms trophozoites, with no fundamental structural difference except being larger in shape and size (Fig. 2.5). These trophozoites undergo repetitive mitosis within the erythrocyte forming an erythrocytic schizont. As the erythrocytic schizont grows in size it will eventually rupture, releasing between 6 and 30 daughter merozoites. Each of the daughter merozoites permeate an uninfected erythrocyte, therefore repeating the cycle (White *et al*., 2014). This asexual cycle is repeated almost indefinitely and is responsible for the clinical symptoms of the disease (Cox, 2010).

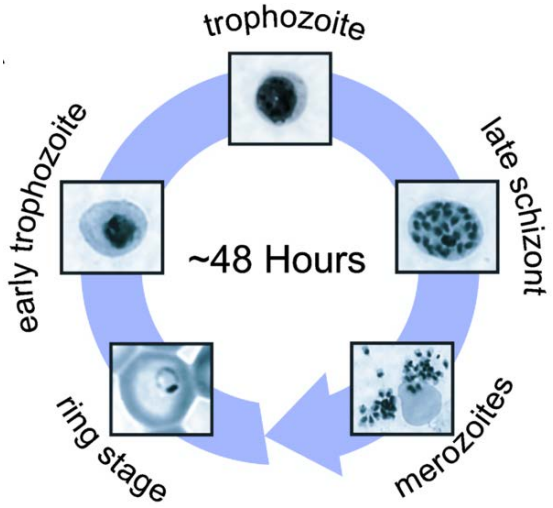


Figure 2.5: The major stages throughout the erythrocytic cycle of *P*. *falciparum* (Bozdech *et al*., 2003).

As the cycle continues, some young merozoites will develop into longer-lived, non-multiplying male and female gametocytes that flow freely in the peripheral circulation, signalling the start of the sexual phase of the parasites' life cycle (Kuehn *et al*., 2010, Cox, 2010, White *et al*., 2014). The catalyst for the production of macrogametocytes (female) and microgametocytes (male) is unknown (Kamchonwongpaisan *et al*., 1997). Mature male and female gametocytes are ingested by the female *Anopheline* mosquito when she takes a blood meal whereupon the gametocytes are transferred into the mosquito midgut (Fig. 2.6). The gametocytes then mature into gametes where the male gametocytes undergo rapid nuclear division and become motile by acquiring flagella (also known as microgametocyte exflagellation). This results in the production of haploid motile microgametes which move rapidly in order to fertilize female macrogametes. Fusion and meiosis then takes place to form zygotes (which takes about a day) that develop into motile ookinetes able to cross the

midgut epithelium of the mosquito. The ookinetes then travel to the extracellular space between the midgut epithelium and overlaying basal lamina to undergo meiosis which transforms them into oocysts. Nine to twelve days later the oocysts will rupture releasing thousands of sporozoites that will journey through the haemolymph into the salivary glands, where they await inoculation into the next human host, thereby restarting the life cycle (White, 2008a, White *et al*., 2014).

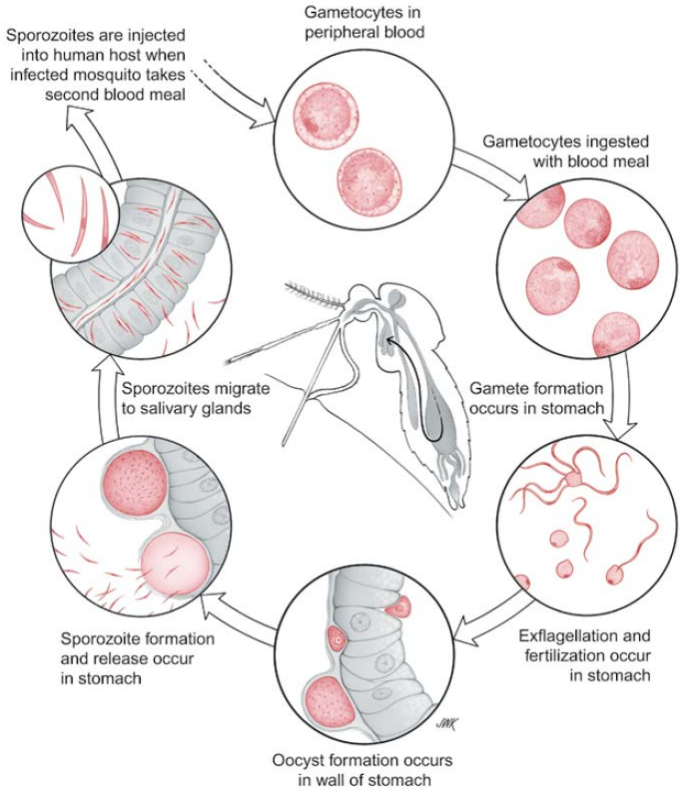


Figure 2.6: Sexual (sporogonic) phase in mosquito midgut (Cox, 2010).

2.4 Clinical features of malaria

Clinical symptoms of malaria are associated with the rupture of schizonts and the destruction of erythrocytes (Trampuz *et al*., 2003). Malaria symptoms often resemble those of common viral infections which can lead to a delay in diagnosis (Murphy and Oldfield, 1996). When malaria is contracted, the first symptoms are nonspecific and can include any of the following: headache, fatigue, a vague absence of well-being, abdominal discomfort, muscle aches and dizziness which are followed by irregular fever. Interestingly, malaria is the most common cause of fever in endemic areas (White *et al*., 2014). Physical signs can include splenomegaly, hepatomegaly, tachycardia, orthostatic hypotension, pallor and jaundice (Trampuz *et al*., 2003). Nausea and vomiting also occur frequently.

Most patients with uncomplicated malaria have few abnormal physical findings apart from fever, a palpable spleen and mild anaemia (White *et al*., 2014). Young children are more susceptible to liver enlargement and in areas with persistent transmission, recurrent infections will cause splenomegaly and chronic anaemia, while adults will suffer more from mild jaundice.

2.5 Pathogenesis

In *P*. *falciparum* malaria, protuberances or 'knobs' form from the surface of the infected erythrocyte some 12 – 15 h after invasion. These protuberances cause attachment of the infected erythrocyte to other infected erythrocytes (platelet-mediated agglutination), the vessel walls (cytoadherence) or uninfected erythrocytes (rosetting) (Doumbo *et al*., 2009) (Fig. 2.7).

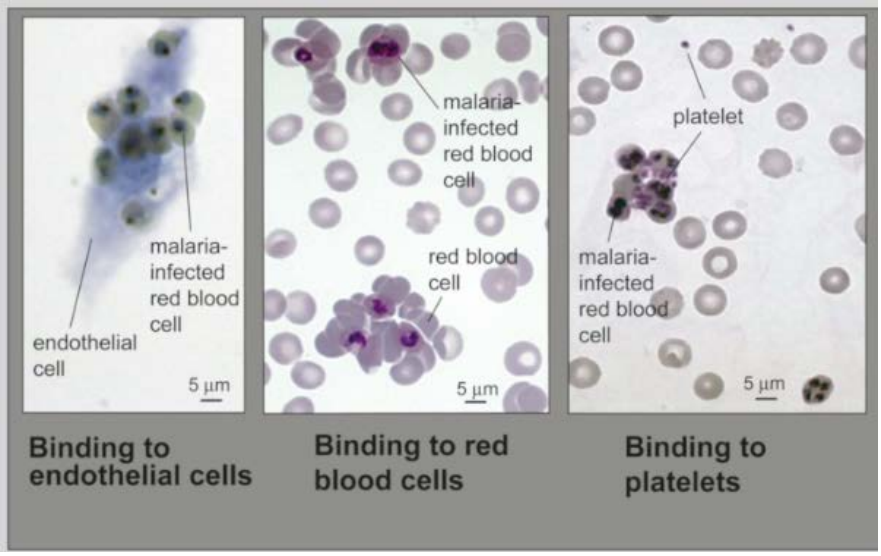


Figure 2.7: Types of adhesion involving erythrocytes infected with the malaria parasite (Doumbo *et al*., 2009).

This attachment causes sequestration (Fig. 2.8) of erythrocytes containing mature parasites which are carried into the vascular system of vital organs (especially the brain). The sequestered erythrocytes interfere with microcirculatory flow and metabolism as well as the functioning of the vascular endothelium (Pongponratn *et al*., 2003).

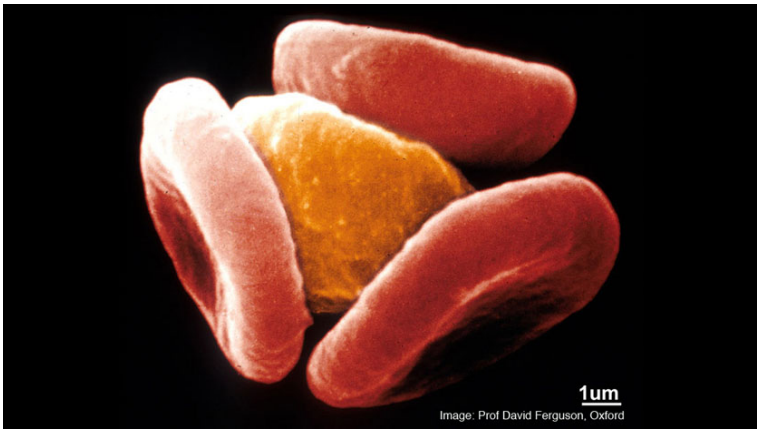


Figure 2.8: Microscopic image of sequestration of a red blood cell (Rowe, 2013).

Interestingly, different *Plasmodium* species can influence the shape of and have different preference to the erythrocytes. Whilst *P. vivax* only invades young erythrocytes, *P. falciparum* and *P. knowlesi* are less selective in invading erythrocytes at any stage; thus, very high parasite densities will be attained (Buffet *et al*., 2011). Also in *P. vivax* malaria the infected erythrocyte enlarge and deform (Suwanarusk *et al*., 2004), but in *P. falciparum* malaria the erythrocyte becomes more spherical and rigid (White *et al*., 2014).

The human host responds to the infection of erythrocytes by enhancing clearance by the spleen that accelerates removal of both infected and normal erythrocytes (Looareesuwan *et al*., 1987, Buffet *et al*., 2011). Rupture of the schizont releases the parasites as well as the host cellular material into the bloodstream thereby leading to activation of macrophages and monocytes. This also induces the release of pro-inflammatory cytokines, causing fever and other pathological effects associated with the disease (Karunaweera *et al*., 1992, Ayimba *et al*., 2011).

2.6 Severe malaria

P. falciparum is the main cause of severe malaria involving widespread erythrocyte sequestration and dysfunction of vital organs. The extent of microvascular obstruction parallels clinical severity and mortality rises when the proportion of infected erythrocytes exceeds 2% (White *et al*., 2014). Severe malaria is usually accompanied by major complications which can include cerebral malaria, pulmonary oedema, severe anaemia and acute renal failure. Acidosis and hypoglycaemia can also arise and are characteristically the most common metabolic complications (Trampuz *et al*., 2003). Severe malaria together with any of these complications can cause death within days (Trampuz *et al*., 2003). In many patients, several of these complications can evolve in rapid succession within a few hours.

The manifestation of severe malaria, however, depends on age (Dondorp *et al*., 2008). In children, hypoglycaemia and severe anaemia are common, whereas jaundice, acute kidney injury and acute pulmonary oedema are more common in adults. Coma (mostly prevalent in cerebral malaria) and acidosis occur in all age groups.

2.7 Diagnosis

As the symptomatic stage of malaria begins, parasite densities in the blood reach 50/µL which corresponds roughly to 100 million parasites in the blood of an infected adult. When this level is reached, the parasites become detectable by microscopy or rapid diagnostic tests (White *et al*., 2014). Light microscopy of thick and thin stained blood smears seems to be the golden standard for the diagnosis of malaria (Moody and Chiodini, 2000). Smears permit both species identification as well as quantification of parasites, which can be expressed as either a percentage of erythrocytes infected or as parasites per µL (Suh *et al*., 2004). Thick smears are used for screening of *Plasmodium* parasites and are 20 – 40 times more sensitive when compared to thin smears. Thin smears are used to identify the infecting parasite species (including simultaneous infections from different species), to quantify parasitaemia, and to measure the presence of malarial pigment in neutrophils and monocytes, and to identify gametocytes and schizonts (Trampuz *et al*., 2003). It is important that during diagnosis of fever that malaria should not be excluded until at least three negative blood smears have been obtained within 48 hours (Suh *et al*., 2004).

There are however, certain problems which arise when the processing and interpretation of malaria smears are carried out. Smears require the appropriate equipment as well as substantial training, factors that can limit their use in endemic regions. Fortunately, there are rapid malaria tests (which require minimal skill and effort) available that can overcome these problems. The most practical and widely used are the rapid detection tests (RDTs) which detect either *P*. *falciparum* histidine-rich protein 2 (*Pf*HRP2), aldolase antigens, species-specific lactate dehydrogenase, or pan-malaria in finger-prick blood samples (Moody, 2002, WHO, 2011). Diagnosing *P. falciparum* malaria with *Pf*HRP2-based RDTs are as good as routine microscopy. Newer diagnostic methods for *P*. *falciparum* and *P*. *vivax* are based on the detection of *Plasmodium* lactate dehydrogenase, given that sensitivity for *P*. *vivax* is low at parasite densities less than 200/µL. For non-falciparum species, aldolase-based tests are less sensitive (WHO, 2011). RDTs are valuable in epidemic investigations and surveys because of the simplicity and speed. However, they cannot be used to quantify, that is accurately assess the level of, parasitaemia and are quite expensive (White *et al*., 2014).

Measurement of *Pf*HRP2 concentrations in plasma can be used to estimate the sequestered parasite biomass in severe malaria (Dondorp *et al*., 2005).

2.8 Vector control and malaria prevention

Vector control is crucial to malaria prevention. The emergence of recent artemisinin combination therapy (ACT) resistance, lack of vaccines and unaffordable antimalarial drugs make vector control an important cornerstone in the control and prevention of malaria. There are various methods that are established and well-practiced throughout endemic areas in order to control and prevent malaria. In this section the most important methods of vector control and the implications of employing these methods have on the overall statistics of malaria will be discussed.

Indoor residual spraying (IRS)

In indoor residual spraying, a long-lasting insecticide is sprayed on the inside walls of houses and other structures where people sleep, resulting in killing of the mosquitoes as they enter houses and rest on sprayed surfaces (Karunamoorthi, 2011). Efficacy of IRS generally depends on the flying habits of the indigenous *Anopheline* vector species, that is, if the mosquitoes enter houses and rest there and the prevalence of insecticide resistance (White *et al*., 2014). IRS is frequently used in epidemic-prone, malaria endemic areas as well as areas where seasonal transmission takes place. Insecticides normally used for IRS include pyrethroids and dichlorodiphenyltrichloroethane (DDT) (WHO, 2010a, Karunamoorthi, 2011).

IRS as a vector control method has been widely adopted, especially in the African Region. Even so, a decreased protection rate of the global population at risk of contracting malaria has been reported from national control programmes, deteriorating from 5.7% in 2010 to 3.4% in 2014 (WHO, 2015a).

Insecticide treated nets (ITNs)

Insecticide treated nets (ITNs) are more effective when compared to IRS as their coverage is easier to sustain; therefore they are the most commonly used form of vector control (Karunamoorthi, 2011). Standard ITNs have to be retreated every year, whereas long-lasting ITNs retain their activity during the life-span of the net. Not only does the ITN protect the user against infectious bites, but also protects the public at large by killing *Anopheline*

mosquitoes (Moonen *et al*., 2010, Godfray, 2013). In some parts of Asia however, the chief mosquito vectors are active either in the early evening or early mornings hence, protection against mosquito bites are minimal (White *et al*., 2014).

That most malaria endemic countries have endorsed universal access to ITNs has resulted in an increase in the portion of the population able to sleep under an ITN over the past ten years. Most ITNs are issued to the population for free, especially in countries with on-going malaria transmission (WHO, 2015a). This has resulted in the fall in malaria mortality and morbidity.

Emergence of resistance to insecticides used with IRS and ITNs

As malaria mosquitoes develop resistance to insecticides used in IRS and ITNs, the efficiency of vector control is hampered. Currently reliance is placed on a solitary class of insecticides, namely pyrethroids. If resistance towards this class of insecticide develop it will clearly have an effect on vector control. In identifying this threat, the WHO released the *Global plan for insecticide resistance management in malaria vectors* (GPIRM) in 2012 (WHO, 2012). The GPIRM sets out five strategies: undertaking resistance monitoring, implementing insecticide resistance management strategies, filling knowledge gaps on mechanisms of insecticide resistance and the impact of resistance management, developing new vector control tools, and ensuring that key enabling mechanisms are in place. Although the GPIRM has been in place since 2012, there are several countries using insecticides for vector control that did not monitor insecticide resistance in 2013 – 2014. Among the 96 countries that implement ITNs or IRS for vector control, 86 reported that monitoring of insecticide resistance was undertaken, yet only 42 countries provided resistance data. Thus, it is clear that monitoring is not conducted every year as required by the GPIRM (WHO, 2012).

Other control methods

Other methods of control include ultra-low-volume (ULV) space spraying (fogging), repellents and larva control.

ULV space spraying – This is used globally for the control of adult mosquito populations. Space sprays can be utilized as a fog, wherein kerosene oil is implemented as a carrier that produces dense fog droplets for insecticides. This method of control is not very effective as the operational costs are high and residual effects are low (Karunamoorthi, 2011).

Repellents – Chemical repellents play a vital role in protecting people from mosquitoes and may therefore reduce the transmission of malaria. *N,N*-diethyl-*m*-toluamide, *N*,*N*-diethyl mandelic acid amide, allethrin, dimethyl phthalate, and *N*,*N*-diethylphenylacetamide are most commonly used in commercial repellents. However, synthetic repellents also have drawbacks, including reduced efficacy due to sweating and allergic reactions (Karunamoorthi, 2011).

Larva control – The emphasis is on the use of larvicides. Adult females lay between 50 and 200 eggs per oviposition. The eggs are laid singly and directly on water and are relatively small (*c*. 0.5 x 0.2 mm^2). Depending on the water temperature, mosquito larvae (also called 'wigglers') can live in water between 4 – 14 days (Enayati *et al*., 2009). Depending on the accessibility and size of the breeding site and whether or not breeding sites within the mosquito flight range can be pinpointed, larval control by either non-chemical or chemical means becomes a practical method of vector control (Karunamoorthi, 2011).

Vaccination – Malaria vaccines are an area of intensive research as much time, money and effort has gone into the development of a malaria vaccine. Especially with the emergence of artemisinin and multidrug-resistant strains of *P*. *falciparum*, this is the main driving force behind research. Various vaccines have reached clinical trials, but unfortunately so far most have demonstrated insufficient immunogenicity. However, the RTS,S subunit vaccine which targets the circumsporozoite protein from the pre-erythrocytic phase of *P*. *falciparum* shows some promise (White *et al*., 2014). The final Phase III results for the vaccine were published in April 2015. As per the recommendations, the vaccine will be evaluated as an addition to, not a replacement for, existing preventive, diagnostic and treatment measures. If RTS,S/AS01 becomes available for use artemisinin-based combination therapies as well as long-lasting insecticidal nets, and rapid diagnostic tests will still be used continuously as the vaccine is not sufficiently immunogenic for stand-alone use (WHO, 2015b).

Chemoprophylaxis for travellers

For travellers entering a malaria area, chemoprophylaxis has to be considered (Chen *et al*., 2006). The recommendation for chemoprophylaxis usually depends on length of the planned trip, type of travelling, the age of the traveller, host risks or pre-existing illness (del Prado *et al*., 2014). The higher the traveller's risk of succumbing to malaria and evolving complications, the greater the demand for chemoprophylaxis. Travellers at the highest risk of contracting malaria are babies and young children (< 5 years), the elderly, pregnant women and immuno-compromised patients (Nujum *et al*., 2014). There are a variety of drugs that can be used as chemoprophylaxis for travellers including the atovaquone-proguanil

combination, mefloquine, primaquine, doxycycline and chloroquine (CQ). Although CQ resistance occurs in almost all regions, it may still be used in areas where the risk of *P. falciparum* malaria is low. Details of further drug indications, classification, chemistry and comments are given in section 2.9.

2.9 Chemotherapy

Effective malaria chemotherapy aims at treating the patient as well as hindering the infectivity of the parasite to the vector *via* exploiting the differences in metabolism between the malaria parasite and the host (Murambiwa *et al*., 2011). Repeated administration of antimalarial drugs at sub-therapeutic levels can interrupt parasite transmission, but this mode of treatment may induce the development of drug resistance (Watkins and Mosobo, 1993). Currently, genes that have been identified as playing a role in the development of resistance include *Pf*CRT (*P. falciparum* chloroquine resistance transporter), *Pf*MDR1 (*P. falciparum* multidrug resistance transporter 1), *Pf*NHE (*P. falciparum* sodium/proton exchanger), and *Pf*MRP (*P. falciparum* multidrug resistance-associated protein).

Traditionally, the clinical action of antimalarial drugs is classified according to the life cycle stages of the parasite, where different drugs inhibit different life stages namely blood schizonticides, tissue schizonticides, hypnozoiticides and gametocytocides. In this review however, we discuss the different antimalarials according to their pharmacological classification. Currently used antimalarials can be divided into five main pharmacological classes, namely quinoline and quinoline-related antimalarials (which can be sub-classified further into aryl-amino alcohols, 4-aminoquinolines and 8-aminoquinolines), antifolates (including DHPS- and DHFR-inhibitors), antibiotics, hydroxynapthoquinones and the artemisinin class of compounds. As the development of malaria parasite resistance is becoming more and more relevant, drug resistance genes and parasite resistance mechanisms will also be included.

2.9.1 Quinoline and related antimalarials

2.9.1.1 Aryl-amino alcohols

Quinine and quinidine

Quinine **1** and quinidine **2** are diastereoisomers, with quinine being the preferred drug. Quinidine is cardiotoxic but has a higher intrinsic antiplasmodial activity (Ashley *et al*., 2006). Quinine **1** is one of the oldest antimalarial agents. It is the principal active ingredient in the pulverized bark of the cinchona tree native to South America that was used for centuries to treat fevers and chills. It was not until 1820 when the active alkaloid was isolated from the bark and named quinine (Butler *et al*., 2010). With the structural elucidation of quinine in the early 20th Century, synthetic alternatives to quinine were prepared. One of the most successful was resochin, which subsequently was called chloroquine (CQ). Because of its relative cheapness, and relative lack of toxicity, this ultimately became more popular than quinine for the standard treatment of uncomplicated malaria (Butler *et al*., 2010).

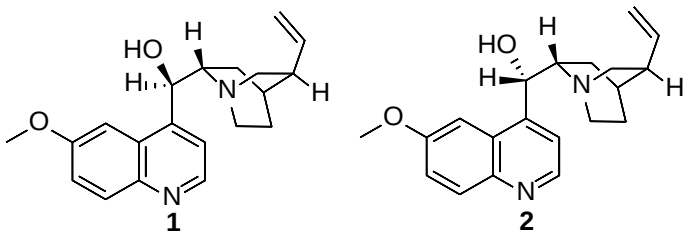


Figure 2.9: Quinine **1**, one of the first antimalarial drugs, and its diastereoisomer quinidine **2**.

Quinine is a blood schizonticide which is active against the asexual erythrocytic forms of *P*. *falciparum*, *P*. *vivax, P*. *ovale* and *P*. *malariae*. Quinine possesses no activity against the gametocytes of *P*. *falciparum*. However this drug has gametocytocidal activity against *P*. *malariae* as well as *P*. *vivax* (Murambiwa *et al*., 2011). Quinine has a short half-life of 8 – 10 h, which thus may have inhibited the general lack of development of predominant quinine resistance. Nevertheless, numerous reports do indicate the development of quinine resistance (Meng *et al*., 2010, Briolant *et al*., 2011). The mechanism by which quinine acts against *P*. *falciparum* is only partially understood, but it is demonstrated to work on the same principles as CQ. Quinine accumulates in the digestive vacuole (DV) of the parasite to inhibit the detoxification of haem, which is a vital process within the parasite (Fitch, 2004). Studies have shown that there are multiple genes present within the parasite that effect susceptibility, therefore making the genetic basis for resistance to quinine a complex mechanism. To date, three genes have been identified to influence resistance in quinine

namely: *Pf*CRT, *Pf*MDR1 and *Pf*NHE1. Interestingly, all of them are used to encode transporter proteins (Sidhu *et al*., 2002, Cooper *et al*., 2002, Cooper *et al*., 2007, Nkrumah *et al*., 2009). Quinine has been reserved for treatment against resistant malaria in combination with antibiotics and also to treat severe cases of malaria (Petersen *et al*., 2011).

Mefloquine

Mefloquine **3** is a 4-methanolquinoline which shares structural similarities with quinine. It inhibits the asexual stages of *P. vivax* as well as *P. falciparum*, making this drug a blood schizonticide (Murambiwa *et al*., 2011). Mefloquine has a long half-life of 14 – 18 days (Stepniewska and White, 2008).

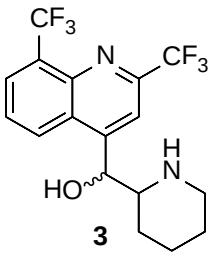


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Figure 2.10: Mefloquine **3**, a 4-methanolquinoline.

Resistance of mefloquine is facilitated by the amplification of *Pf*MDR1, which leads to the overexpression of this gene in the DV membrane transporter (Cowman *et al*., 1994). The exact mechanism of action for mefloquine is unclear, but *in vitro* experiments demonstrated that mefloquine binds to haem and exerts antimalarial activity *via* inhibiting haem detoxification (Fitch *et al*., 1979, Eastman and Fidock, 2009). A decrease of *Pf*MDR1-mediated influx into the DV is noted to occur with decreased parasite susceptibility to mefloquine (Rohrbach *et al*., 2006, Sidhu *et al*., 2006). Furthermore, it has been hypothesized that mefloquine might target the *Pf*MDR1 transport function as mefloquine prevents access of other solutes into the DV (Rohrbach *et al*., 2006). Mefloquine is used for both treatment and as prophylaxis in Africa and is especially useful as chemoprophylaxis in travellers from non-endemic countries (Schlagenhauf *et al*., 2011).

Halofantrine

Halofantrine **4** belongs to the phenanthrene class of compounds that includes lumefantrine, is a schizonticidal drug but with no apparent action on the sporozoite, gametocyte or hepatic

stages of infection. However, it is effective against both CQ sensitive and resistant strains of *Plasmodia* (Bilolikar *et al*., 1994).

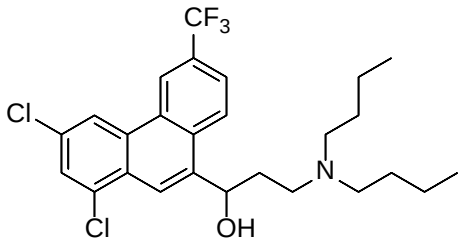

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Figure 2.11: Halofantrine **4**.

The mechanism of action appears to be due to inhibition formation of haemozoin although the exact mode of action is unknown (Nosten *et al*., 2012). Recent studies have revealed that halofantrine forms complexes with haematin (ferriprotoporphyrin IX), where inhibition of haemozoin formation takes place at the lipid-aqueous interface, an environment that is much more compatible with the crystal structure of halofantrine-ferriprotoporphyrin IX (De Villiers *et al*., 2008). Halofantrine has poor solubility and is therefore erratically absorbed. However, fat co-administration can enhance the absorption of this drug. When compared to mefloquine, halofantrine is readily eliminated; it has a half-life of ~4.7 days. Few studies have been done on the development of resistance of this drug. Altered halofantrine transport may be the consequence of *Pf*MDR1 mutations, which could signify a role for the efflux transport mechanism for development of resistance to this drug (Sanchez *et al*., 2008). Clinical studies in Southeast Asia have revealed cross-resistance between halofantrine and mefloquine (Ter Kuile *et al*., 1993). Halofantrine has been withdrawn from use due to its cardiotoxicity (Nosten *et al*., 2012).

Lumefantrine

Lumefantrine **5** is a racemic substituted fluorene derivative with a structure similar to that of halofantrine. Lumefantrine is active against the asexual stages of *P*. *falciparum* and *P*. *vivax*. The mechanism of action for this particular drug is not fully understood but it is believed to inhibit polymerisation of haem resulting in parasite death (Nosten *et al*., 2012).

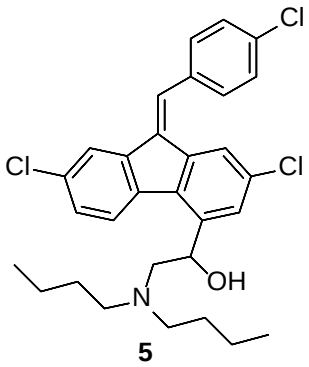

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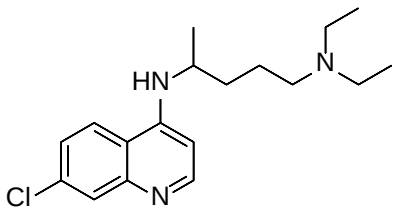
Figure 2.12: Lumefantrine **5**.

The terminal elimination half-life of lumefantrine is roughly 4.5 days, a much shorter half-life than that of mefloquine or piperaquine. This drug has a low and variable bioavailability and is highly lipophilic. However, bioavailability can be increased with the intake of fatty foods which notably enhance drug absorption (Ashley *et al*., 2007). Lumefantrine resistance is easily induced in both animal models and *in vitro* (Nosten et al., 2012). Single nucleotide polymorphisms in the *pfmdr1* gene have been linked with amplified IC_{50} values *in vitro* (Anderson *et al*., 2005). Lumefantrine is used in combination with artemether (Coartem®) as part of the ACT treatment regimens stipulated by the WHO.

2.9.1.2 4-Aminoquinolines

Chloroquine (CQ)

CQ **6** is a rapid-acting schizonticide against *P*. *falciparum* with activity against the asexual erythrocytic forms of *P*. *malariae*, *P*. *ovale* and *P*. *vivax* (Murambiwa *et al*., 2011). CQ has one of the longest half-life (approximately 60 days) of all antimalarials. This extreme half-life results in exposure the parasite to the drug for a prolonged period of time after which CQ falls below its therapeutic concentration. This also provides a chemoprophylactic effect during the elimination phase (Stepniewska and White, 2008). CQ resistant parasites emerged roughly around 10 years after its first official use, first along the Thai-Cambodian border and also in Columbia in the late 1950's (Mita *et al*., 2009). CQ resistant *P*. *falciparum* is now predominant in nearly all malaria endemic regions (Petersen *et al*., 2011).



6

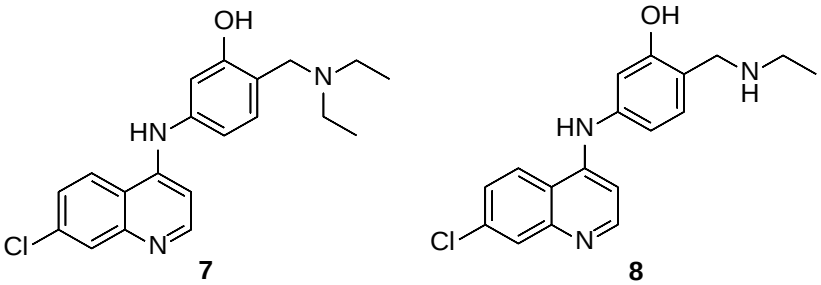
Figure 2.13: Chloroquine (CQ) **6**.

The mechanism of action of CQ involves the haem detoxification pathway in the DV. Normally, erythrocytic haemoglobin is broken down by the parasite followed by dimerization of toxic haem monomers to inert biocrystals of haemozoin (Egan, 2008); CQ has pK_a values of 8.1 and 10.2 making it a weak base; hence, at the neutral pH of blood a part of the drug remains uncharged (Martin *et al*., 2009). Neutral CQ can therefore migrate freely across membranes, but once CQ passes into the acidic DV, it is protonated and is unable to diffuse readily across the membrane (Martin *et al*., 2009). Thus, CQ accumulates in the DV and binds to haem (Fitch, 2004). This interaction prevents the dimerization of the free haem to haemozoin. The resulting build-up of free haem leads to eventual death of the parasite due to the toxicity of the free haem (Zhang *et al*., 1999).

Polymorphisms in *Pf*CRT seem to be the main determinant in CQ resistance (Sidhu *et al*., 2002). The extent of CQ resistance may be controlled by *Pf*MDR1 (Barnes *et al*., 1992), indicating that the concentration of CQ can increase inside the DV as a result of *Pf*MDR1 overexpression.

Amodiaquine

Amodiaquine **7**, although structurally related to CQ, has a very short half-life of 3 hours. It is likely that antimalarial activity is exerted principally by the primary metabolite, monodesethyl amodiaquine **8** which has a half-life of 9 – 18 days (Stepniewska and White, 2008).



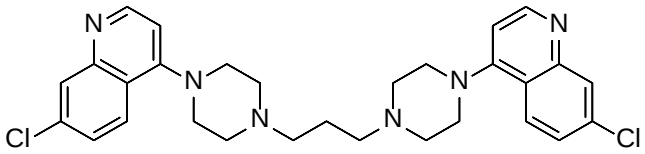
7 **8**

Figure 2.14: Amodiaquine **7** and monodesethyl amodiaquine **8**.

Because of the structural similarity of amodiaquine to CQ, the mechanism of action is hypothesized to be the inhibition haem detoxification by accumulating in the DV and binding to haem (Foley and Tilley, 1998, Hayeshi *et al*., 2008). Mutations in *Pf*CRT and *Pf*MDR1 may be the basis for cross resistance between CQ and amodiaquine (Sá *et al*., 2009).

Piperaquine

In order to overcome CQ resistance, two 4-aminoquinoline moieties attached via linkers of various lengths, namely the bisquinolines, were prepared. Piperaquine **9** is one such bisquinoline which is highly lipid soluble with a half-life of 16.5 days (Price *et al*., 2007).



9

Figure 2.15: Piperaquine, **9**.

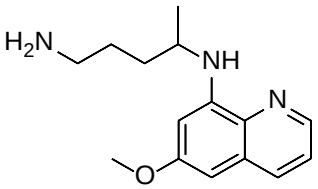
Piperaquine shows remarkably good activity against CQ-resistant strains. This was held to be due to the sterically demanding chemical structure that was presumed to prevent the drug from fitting into the substrate binding site of *Pf*CRT. However, piperaquine resistance duly emerged and this, coupled with the advent of artemisinin derivatives as effective antimalarial drugs, resulted in a marked decrease in the use of piperaquine during the 1980's (Held *et al*., 2015). However, its pharmacokinetic profile, efficacy, tolerability, and low cost make it a favourable partner dug for use with dihydroartemisinin in artemisinin combination therapy (O'Neill *et al*., 2012). This is discussed further below.

2.9.1.3 8-Aminoquinolines

Primaquine

Primaquine **10** has a half-life of 6 h (Edwards *et al*., 1993) and is one of the few antimalarial drugs approved for therapy against *P. vivax* liver stage hypnozoites (Wells *et al*., 2010). This antimalarial drug induces haemolytic anaemia in glucose-6-phosphate dehydrogenase (G6PD) deficient patients (Burgoine *et al*., 2010). However, limiting the dose to one treatment per week for 8 weeks might be safe and an effective alternative to the traditional 14-day course of the drug (Myat Phone *et al*., 1994). In a *P. falciparum* infection a single dose of 0.75 mg/kg is well tolerated in G6PD deficient patients excluding children younger

than 4 years and pregnant women. Primaquine also eradicates the primary exoerythrocytic stage of *Plasmodium* species as well as the gametocytocidal and sporontocidal phases (Murambiwa *et al*., 2011).



10

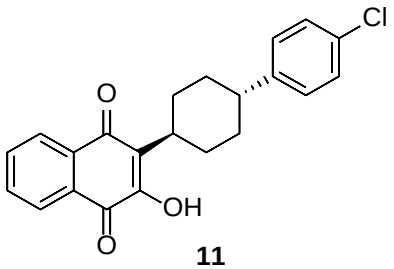
Figure 2.16: Primaquine **10**.

The mode of action for primaquine is largely unknown but it is thought that the mitochondria of the parasite could be the biological target. Structural changes within the inner membranes as well as swelling of the parasite mitochondria are observed upon administration of primaquine (Beaudoin and Aikawa, 1968, Aikawa and Beaudoin, 1970, Howells *et al*., 1970, Boulard *et al*., 1983, Lanners, 1991). These morphological changes may be associated with destruction of mitochondrial function by the drug (Rotmann, 1975, Peters *et al*., 1984, Krungkrai *et al*., 1999). Numerous active metabolites of primaquine appear to be responsible for antimalarial activity (Idowu *et al*., 1995, Vale *et al*., 2009). Several other studies indicate that primaquine can lead to the reversal of CQ resistance through binding to *Pf*CRT; this may lead to a synergistic action between CQ and primaquine (Sanchez *et al*., 2004, Bray *et al*., 2005, Egan, 2008).

2.9.2 Hydroxynaphthoquinones

Atovaquone

Atovaquone **11** is used with proguanil in a combination known as Malarone® for prophylactic medication for travellers visiting malaria endemic areas (Petersen *et al*., 2011). This combination is mainly used as such because of facile resistance arising to atovaquone from single nucleotide polymorphisms in the cytochrome β-gene (Gil *et al*., 2003).



11

Figure 2.17: Atovaquone **11**.

The antimalarial activity of atovaquone is due to the inhibition of the parasite mitochondrial function (Fry and Beesley, 1991, Fry and Pudney, 1992). Atovaquone collapses the mitochondrial membrane potential, which results in the interruption of pyrimidine biosynthesis (Srivastava *et al*., 1997, Painter *et al*., 2007). Evidence suggests that the cytochrome bc_1 complex, which is located in the inner mitochondrial membrane, is explicitly targeted by atovaquone which results in the suppression of the respiratory chain (Petersen *et al*., 2011).

2.9.3 Antifolates

Antifolates inhibit the biosynthesis and interconversion of folate derivatives which play a vital role in the production of deoxythymidylate (dTMP) and consequently the synthesis of DNA (Nzila, 2012). *P*. *falciparum* entirely depend on the *de novo* synthesis pathway of dTMP, making the folate pathway (Fig. 2.18) critical to the parasites' survival (Hyde, 2007).

The malaria parasite can synthesize as well as reclaim folate, where both of these pathways can increase the folate availability. Thus, the potency of antimalarial sulfa drugs can be attributed to the blockade of dihydropteroate synthase which results in the inhibition of the *de novo* folate synthesis pathway. Research demonstrates that adding of folate derivatives (e.g. folinic acid or folic acid) will decrease the activity of antifolate drugs *in vitro* as well as *in vivo* (Watkins *et al*., 1985, Van Hensbroek *et al*., 1995, Kinyanjui *et al*., 1999, Carter *et al*., 2005). Similarly, an improvement in the antimalarial activity of the antifolate drug is observed when the folate concentration in *in vivo* cell culture medium is decreased (Watkins *et al*., 1997, Wang *et al*., 1997). Therefore, a substantial contribution is made to antifolate drug efficacy through the uptake of folate. The foundation for the development of compounds that can enhance the activity of antifolate antimalarial drugs relies on the inhibition of this salvage pathway (Nzila, 2006).

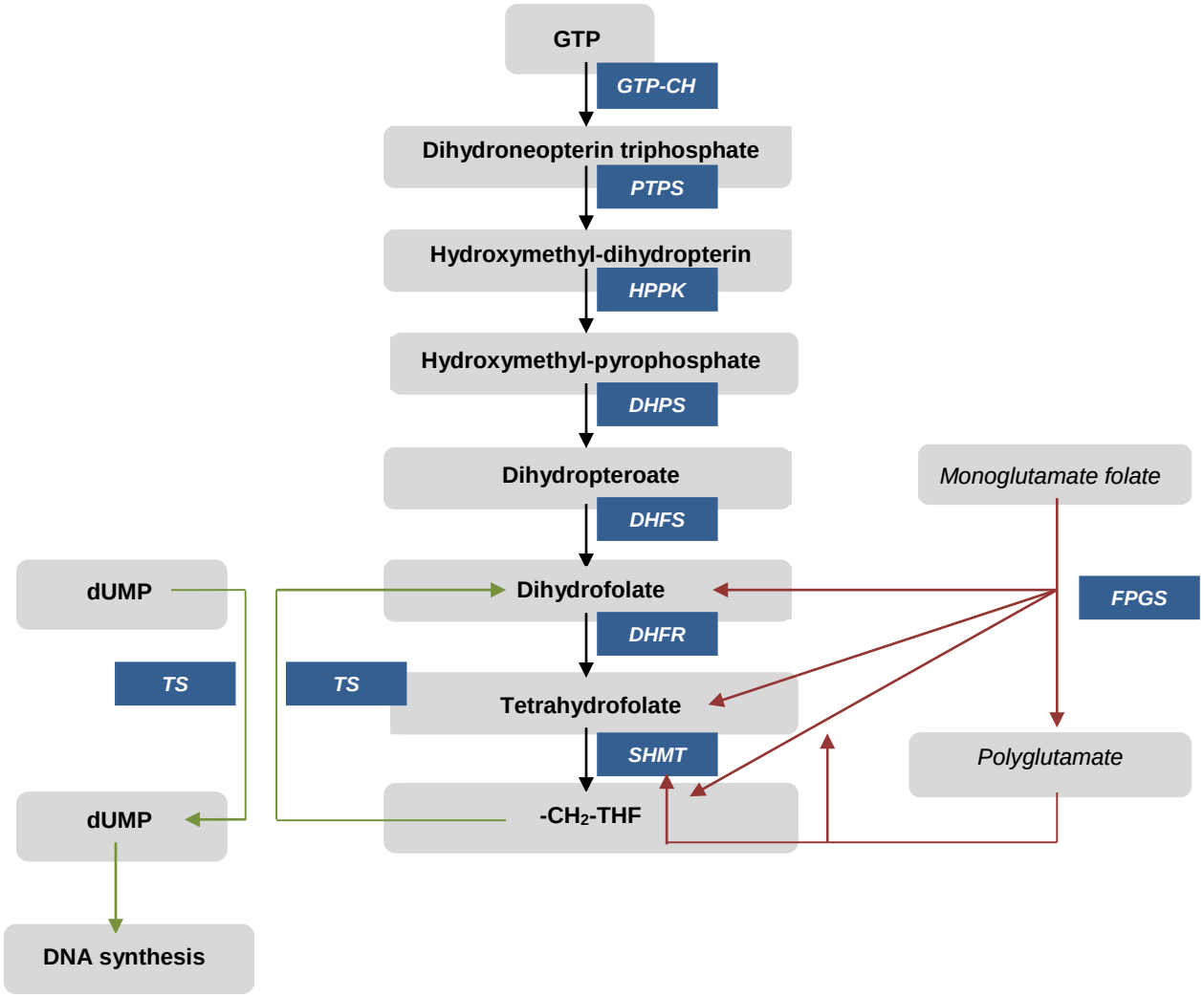


Figure 2.18: Folate biochemical pathway in *P. falciparum*. The following abbreviations are used: *GTPCH* GTP cyclohydrolase I, *PTPS* pyruvyl tetrahydropterin synthase III, *HPPK* hydroxymethyl dihydropterin pyrophosphokinase, *DHPS* dihydropteroate synthase, *DFHS* dihydrofolate synthase, *FPGS* folylpoly-gamma-glutamate synthase, *DHFR* dihydrofolate reductase, *SHMT* serine hydroxymethyltransferase, *TS* thymidylate synthase. *PTPS* has recently been characterized in *P. falciparum* (Dittrich *et al*., 2008).

Antifolates that are in use currently target two specific enzymes namely dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS). Therefore, antifolates used against malaria can be sub-divided into two classes, namely DHPS inhibitors (class I antifolates) and DHFR inhibitors (class II antifolates) (Nzila, 2006). Combining DHFR and DHPS inhibitors results in a synergistic effect and such combinations can be used when treating malaria patients.

2.9.3.1 DHPS inhibitors

DHPS inhibitors belong to two families: sulfone and sulfonamide with structures of representative drugs given in Fig. 2.19.

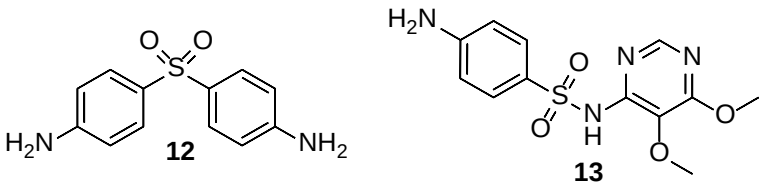


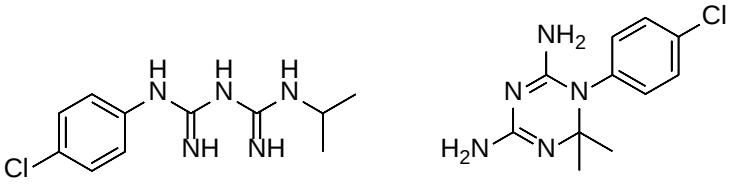
Figure 2.19: Class I antifolates dapsone **12** and sulfadoxine **13**.

Efforts to use drugs from this class of compound as monotherapy in the fight against malaria were made. These efforts had to be abandoned because of these drugs elicited severe toxicity and displayed low efficacy (Michel, 1968). However, once the discovery was made that DHPS inhibitors work synergistically with DFHR inhibitors interest in using these different components as combination treatment for malaria was rekindled. Dapsone **12** is the most potent DHPS inhibitor when compared to other compounds of the same class (Nzila, 2006). The major mechanism of sulfadoxine resistance stems from point mutations in the *dhps* domain of the *dhps-pppk* gene (Nzila, 2006).

2.9.3.2 DHFR inhibitors

Proguanil

The first reported antifolate antimalarial drug was proguanil and this is used, as mentioned above, in combination with atovaqoune. Proguanil **14** (a prodrug) is converted *in vivo* into its active metabolite cycloguanil **15**, an inhibitor of DHFR, *via* oxidative ring closure. The synergistic mechanism between atovaquone and cycloguanil is not fully understood. Proguanil and dapsone can also be used as combination therapy (Lapdap®). Unfortunately, this therapeutic combination can no longer be recommended as dapsone causes haemolysis in G6PD deficient patients (Luzzatto, 2010).

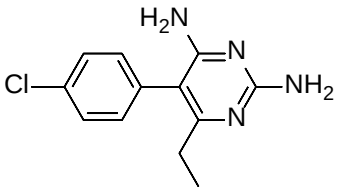


14 **15**

Figure 2.20: Proguanil **14** and its active metabolite cycloguanil **15**.

Pyrimethamine

Pyrimethamine **16** belongs to the 2,4-diaminopyrimidine class of compounds. Initially used for treatment of cancer, it was only discovered later that it is effective against malaria because of the structural resemblance to proguanil (Falco *et al*., 1951).



16

Figure 2.21: Pyrimethamine **16**.

Pyrimethamine is the most widely used antimalarial antifolate drug thus far, being first used in monotherapy (Peters, 1987) and later being combined with sulfadoxine (Fansidar®). This sulfadrug combination has a long elimination half-life of >80 h (Roper *et al*., 2004). Pyrimethamine is also used in combination with dapsone (Maloprim®), where dapsone has a short half-life of 24 h (Winstanley *et al*., 1997). Unfortunately, this drug combination has a very low efficacy that can be attributed to the shorter half-life of dapsone; as soon as the second day of treatment with this combination starts the drug efficacy decreases due to the reduction of synergy between dapsone and pyrimethamine (Nzila, 2006).

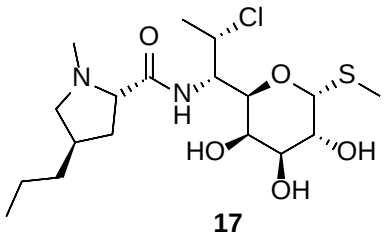
Point mutations in the *dhfr* domain of the *dhfr-ts* gene have been linked to the development of pyrimethamine resistance (Peterson *et al*., 1988, Peterson *et al*., 1990, Foote *et al*., 1990). A lot of effort has focussed on determination of *dhfr* and *dhps* genotypes that may predict resistance to pyrimethamine/sulfadoxine. Malaria parasites that have point mutations at codons 51, 59 and 108 of *dhfr* appear to have pyrimethamine/sulfadoxine resistance. Also, point mutations on codons 437 and/or 540 or 437 and/or 581 of the *dhps* gene may enhance resistance as well (Sibley *et al*., 2001, Gregson and Plowe, 2005). Mutation at

codon 164 of *dhfr* is linked to high levels of pyrimethamine/sulfadoxine resistance. Mutation on this codon is not usually found in Africa (Nzila, 2006).

2.9.4 Antibiotics

Clindamycin

Clindamycin **17** is the only semisynthetic lincosamide antibiotic prepared for clinical use (Krishna and Staines, 2012). Clindamycin binds to the 50S ribosomal unit which results in the inhibition of protein synthesis inside bacteria (Reusser, 1975). In apicomplexan parasites, however, clindamycin results in "delayed-death" as it interferes with the survival as well as function of organelles inside the parasite apicoplast after a few rounds of parasite replication has taken place.



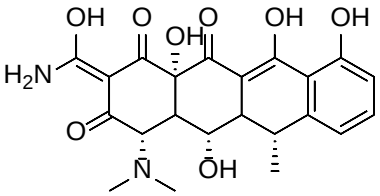
17

Figure 2.22: Clindamycin **17**.

Clindamycin has an elimination half-life of 2 – 3 hours (Lell and Kremsner, 2002). Clindamycin can be used in combination with artesunate in order to treat malaria. This drug is also safe to use in children (Lell and Kremsner, 2002).

Doxycycline

Doxycycline **18** is a broad spectrum bacteriostatic agent synthetically derived from naturally occurring tetracyclines (Tan *et al*., 2011). Doxycycline can either be used for prevention of malaria in travellers or in conjunction with another drug for malaria treatment. Uncomplicated malaria caused by CQ resistant *P*. *falciparum* or *P*. *vivax* can be treated with a combination of doxycycline with quinine. Doxycycline in combination with intravenous quinidine may be used to treat severe malaria (Tan *et al*., 2011).



18

Figure 2.23: Doxycycline **18**.

Having a half-life of 15 – 25 hours, doxycycline is a slow acting blood schizonticidal agent, killing the asexual, erythrocytic stages of the parasite, with no effect on the sporontocidal or gametocytocidal stages (Shamiss *et al*., 1996, Baudon *et al*., 1999). Although the exact mode of action of doxycycline is poorly defined, it is shown to inhibit the expression of apicoplast genes resulting in non-functional apicoplasts; it thus impedes the development of viable parasites (Dahl *et al*., 2006). The antimalarial activity can also be scribed to the inhibition of protein synthesis (Budimulja *et al*., 1997).

2.9.5 Artemisinins

2.9.5.1 Introduction

Like quinine, artemisinin is derived from traditional medicine involving a plant with fever-reducing properties (Willcox *et al*., 2004). Artemisinin is extracted from a plant known as *qinghao* (or sweet wormwood, *Artemisia annua*) that was used to treat fever and chills in China for over 2 millennia (Lusha, 1979, Klayman, 1985, Luo and Shen, 1987). In 1967 the Chinese government started an integrated programme in order to discover antimalarial activities in a number of medicinal herbs that included qinghao. In 1973 a chemical substance that was called *qinghaosu*, was isolated and shown to be highly active against malaria in malaria patients. This is now called artemisinin (White, 1994). Since the discovery of artemisinin, an array of semisynthetic derivatives has been developed with a variety of formulations.

Artemisinins kill all *Plasmodium* parasites that infect humans (Li *et al*., 1994, Same-Ekobo *et al*., 1998, Borrmann *et al*., 2002), with the asexual stage of infection being the most susceptible (White, 1997). Like other antimalarial drugs, artemisinins are predominantly active against the ring and trophozoite stages of the infection. Artemisinins inhibit the metabolism of parasites more quickly when compared to other antimalarials and they also reduce cytoadherence of infected cells (Udomsangpetch *et al*., 1996). Artemisinins have no

prophylactic value as they have no effect on parasite development in the hepatic stages (Woodrow and Bustamante, 2011).

2.9.5.2 Mechanism of action

A number of theories have been put forward to explain how artemisinins act as antimalarial drugs. These theories, together with their supportive biochemistry, are outlined at length in a number of reviews (Krishna *et al*., 2008, O'Neill *et al*., 2010, Li and Zhou, 2010, Haynes *et al*., 2012). In order to understand the mechanisms of action, a few considerations must be kept in mind. Firstly, any drug that is safe and effective should exert distinctive toxicity towards the pathogen with respect to the host (Karunajeewa, 2012). Therefore, in the case of the malaria parasite, drug activity may be due to the action on some process or metabolic pathway, favoured by, or essential to the existence of, the parasite (Karunajeewa, 2012). In the parasite DV detoxification of digested haem that rises from the breakdown of host-cell haemoglobin takes place. Therefore, this so-called haem pathway has been investigated at length in relation to the mode of action of artemisinin. *Pf*ATP6 plays a critical role in the oxidative metabolism inside the parasite, whereas in mammalian cells this enzyme is absent. Inhibition of *Pf*ATP6 has also been suggested as a mode of action and is also discussed below. There is also interest in the relationship between artemisinins, free haem and iron that occur in abnormal concentrations inside the parasite in an infected red blood cell. If this is responsible for the “activation” of artemisinin, it could possibly clarify the variance in toxicity between parasitic and host cells (Karunajeewa, 2012). Furthermore, the peroxide moiety is required for antimalarial activity. Therefore, the direct or indirect action of the peroxide moiety should be included in any proposed mechanism of action pathway for the artemisinins (Karunajeewa, 2012).

For the various theories that have been put forward, it seems that some of these theories are by no means exclusive and it is possible that the antimalarial activity of the artemisinins is a multi-faceted process (O'Neil *et al*., 2007). However, aspects of the chemistry of artemisinins as discussed in the review by Haynes (Haynes *et al*., 2013) would seem to negate aspects of some of the theories.

Haem pathway hypothesis

According to this theory, the haem activates the peroxide in artemisinin to generate free radicals that in turn alkylate the haem. The parasite evidently is unable to detoxify the

alkylated haem, for example by dimerization to form haemozoin, which is non-toxic (Karunajeewa, 2012). Results of some studies are interpreted as supporting the idea that parasites exposed to artemisinins and synthetic peroxides *in vitro* form haem-drug adducts (Meshnick, 2003, Creek *et al*., 2008). However, other studies (Haynes *et al*., 2003, Krishna *et al*., 2006) show otherwise. By using stable artemisinins that display potent antimalarial activities under carefully controlled conditions *in vitro*, haem alkylation, haem dimerization and consequently formation of haemozoin did not occur. Another study supporting these observations with rodent malaria models demonstrate the lack of formation of haem-artemisinin adducts, that is, alkylation does not occur as a mechanistically significant step (Krishna *et al*., 2006). Artemisinins are also toxic to other parasite species (*Babesia* and *Toxoplasma* spp.) which are either not exposed to substantial amounts of haem *in vivo*, or not exposed to haem at all (Karunajeewa, 2012).

Protein alkylation hypothesis

Parasite proteins responsible for important functions within the parasite may be changed through the formation of covalent bonds which is a result of free radical generation inside the parasite (Meshnick, 2002). Targets are assumed to include numerous cellular enzymes, proteases involved in haemoglobin degradation and even membrane transporters (Karunajeewa, 2012). However, the radicals generated from artemisinins are unlikely to act as alkylating agents, a conclusion that is supported by a wide body of evidence indicating that such radicals would not be sufficiently reactive to act as alkylating agents (Haynes *et al*., 2013).

Inhibition of PfATP6 hypothesis

The oxidative metabolism of the malaria parasite relies on the *Pf*ATP6 enzyme, a transmembrane calcium transporter associated with the sarco-endoplasmic reticulum. It was suggested that the inhibition of *Pf*ATP6 may be the basis for the mode of action of artemisinins (Eckstein-Ludwig *et al*., 2003). The simplistic connection was made between the structurally complex thapsigargin, a sesquiterpene lactone, and artemisinin, also a 'sesquiterpene lactone', but structurally very different. (Karunajeewa, 2012). Thapsigargin selectively inhibits the mammalian sarco-endoplasmic reticulum membrane calcium ATPase (SERCA), which is responsible for maintaining a low concentration of cytosolic free calcium, a cellular signalling agent essential for the survival of cells (Karunajeewa, 2012). Remarkably, artemisinin was reported to display similar activity on mammalian SERCA when compared to thapsigargin (Eckstein-Ludwig *et al*., 2003). Thus, it was suggested that

*Pf*ATP6 could be the molecular target of artemisinin, given that *Pf*ATP6 is the only enzyme orthologous to the mammalian SERCA. Experiments purported to demonstrate that thapsigargin and artemisinin did inhibit *Pf*ATP6. Also, there were no effects on non-SERCA Ca^{2+} ATPase or other transporters implying a rather specific mechanism of action. Furthermore, it was reported that the effect of artemisinin was eliminated when desferrioxamine (an iron chelator) was incubated with erythrocytes containing malaria parasites. This observation was held up to prove that enzyme inhibition is necessary through bioactivation of artemisinin by iron (Eckstein-Ludwig *et al*., 2003). However, the theory was discredited once it was shown essentially that the work could not be repeated and that thapsigargin does not antagonize the effect of artemisinin, that is, artemisinin cannot bind to *Pf*ATP6 (Arnou *et al*., 2011, Abiodun *et al*., 2013). It is more likely that any effect on *Pf*ATP6 is a downstream effect involving modulation of calcium levels by elevated oxidative stress mediated by artemisinins (Haynes *et al*., 2012).

Mitochondrial function hypothesis

Studies with enzymes expressed in yeast have demonstrated that artemisinin has an effect on the membrane potential of the mitochondria through the effects on the electron transport chain (Li *et al*., 2005). It was shown that artemisinin damages the malarial mitochondria through the swift production of reactive oxygen species (ROS), the actual cytotoxic agents. It would appear that this mechanism of toxicity is rather selective as ROS production takes place within malaria as well as in yeast but not in mammalian cells (Wang *et al*., 2010). These studies demonstrated that mitochondrial toxicity seems to be relying on the peroxide moiety as deoxyartemisinin derivatives (with no peroxide) fail to exhibit ROS production or toxicity within the mitochondria. The significance of this pathway has been supported by further evidence by using desferrioxamine to disrupt the electron transport chain of the mitochondria which result in the improvement of antimalarial activity (Hastings *et al*., 2002). However, studies in transgenic parasites (Vaidya and Mather, 2009) do not agree that parasites are killed by artemisinins through a mitochondrial-based mechanism (Karunajeewa, 2012).

Co-factor hypothesis

It was found by Haynes and co-workers that artemisinins rapidly oxidize reduced flavin cofactors such as flavin adenine dinucleotide ($FADH_2$), flavin mononucleotide ($FMNH_2$), reduced riboflavin and model reduced flavins at physiological pH under anaerobic conditions (Haynes *et al*., 2010). Intriguingly, under aerobic conditions it was found that the artemisinins

also induce rapid autoxidation of the reduced flavins. As such reduced flavin cofactors are critical for the functioning of flavoenzyme disulfide reductases such as glutathione reductase (GR), thioredoxin reductase (TrxR) and others that normally maintain redox homeostasis in the malaria parasites, it is likely that artemisinins and synthetic peroxide overwhelm redox control in the malaria parasite. Thus, rapid build-up of ROS ensues, resulting in death of the parasite. Also, as artemisinins have the ability to induce autoxidation of the reduced cofactors, an additional source of ROS is generated (Vennerstrom and Eaton, 1988, Hunt and Stocker, 1989, Berman and Adams, 1997, Del Pilar Crespo *et al*., 2008, Kumura *et al*., 2009, Haynes *et al*., 2010, Bousejra-El Garah *et al*., 2011). Therefore, the NADPH supplied *via* the rate-limiting glucose-6-phosphate dehydrogenase (G6PD)-catalyzed step of the hexose monophosphate shunt, already heightened within the stressed environment of the cytosol (Sarma *et al*., 2003, Buchholz *et al*., 2008), is unable to meet the drug-induced demand for additional reducing equivalents required to overcome the increased oxidative stress (Fig. 2.24) (Becker *et al*., 2003, Krauth-Siegel *et al*., 2005, Haynes *et al*., 2011).

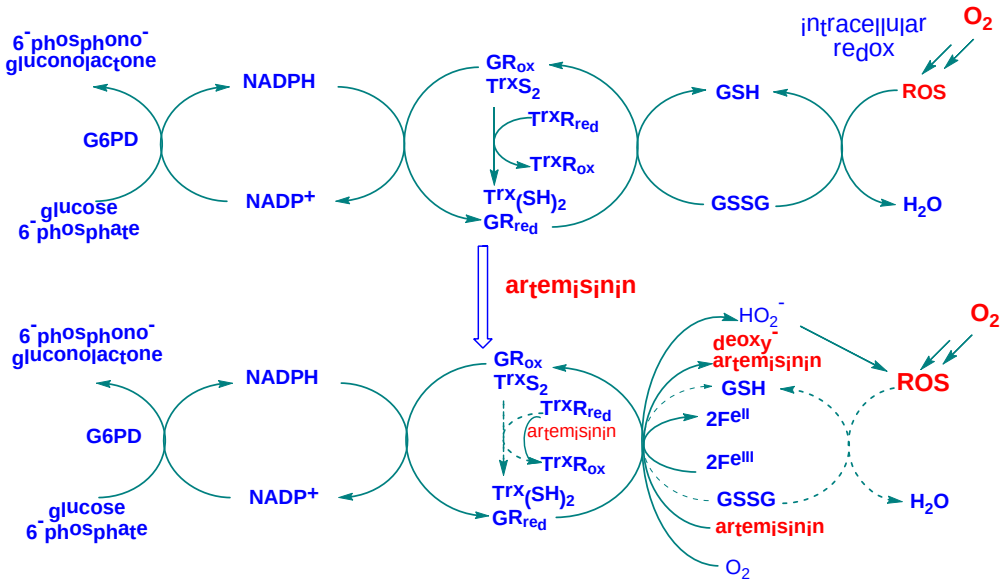


Figure 2.24: Proposed enhancement of oxidative stress facilitated by peroxidic antimalarial drugs such as the artemisinins which leads to a loss of redox homeostasis. GR glutathione reductase, oxidized FAD cofactor, red $FADH_2$ cofactor; TrxSH thioredoxin reduced, $TrxS_2$ thioredoxin oxidized; TrxR thioredoxin reductase, oxidized FAD, red $FADH_2$. Maintenance of GSH and reduced thioredoxin [$Trx(SH)_2$] requires NADPH provided by the rate-limiting hexose monophosphate shunt which is catalyzed by glucose-6-phosphate dehydrogenase (G6PD) (Haynes *et al*., 2012).

This hypothesis also originates from the demonstration that artemisinins are prone to both single-electron transfer as well as two-electron reduction processes in the presence of

methylene blue and ascorbic acid (Haynes *et al*., 2010). Also, this hypothesis does not corroborate the idea that artemisinins exert their antimalarial activity by chemically reacting in the parasite DV with Fe^{II}, either free or in the form of haem-Fe^{II} (O'Neill *et al*., 2010). The study itself (Haynes *et al*., 2012) obviates the necessity to speculate that a chemical reaction of the peroxide with iron takes place (Haynes *et al*., 2004, Coghi *et al*., 2009, Haynes *et al*., 2011) as iron itself contributes substantially to oxidative stress on its own (Cabantchik *et al*., 2002, Kakhlon and Cabantchik, 2002, Scholl *et al*., 2005, Mladenka *et al*., 2006, Kumar *et al*., 2007). Notably, the reduction of haem-Fe^{III} and non-haem-Fe^{III} to Fe^{II} *via* dihydroflavins is significantly faster than intracellular thiols (Pierre *et al*., 2002, Woodmansee and Imlay, 2002, Petrat *et al*., 2003, Kinoshita *et al*., 2007), which is applicable to the intraerythrocytic malaria parasite that creates haem-iron, and thereafter free iron, by catabolism of methaemoglobin. Haem-Fe^{III} is produced in the DV but is still able to enter the cytosol (Campanale *et al*., 2003, Fisher *et al*., 2007, Kumar *et al*., 2007) where it deteriorates to non-haem-Fe^{III}. Both species in the presence of redox enzymes and related cofactors undergo redox cycling. This supplementary method of ablation of flavin co-factor by iron must work to burden the production of NAD(P)H in the cytosol of the parasite as seen in Fig. 2.24. It is clear that sequestration of non-haem-Fe^{III} by iron chelators such as deferiprone or desferrioxamine will affect the Fe^{II}-Fe^{III} redox cycle as well as the ability of Fe^{III} to oxidize the reduced flavin cofactors. This is reflected in the reduction of the parasiticidal activity of artemisinins in the presence of the iron chelators (Stocks *et al*., 2007, Haynes *et al*., 2007b, Haynes *et al*., 2011).

The focal point for the mechanism of action for this hypothesis is not on the DV but rather the parasite cytosol as artemisinins are unlikely to exert their mechanism of action in the parasite DV (Haynes *et al*., 2010, Haynes *et al*., 2011).

According to Fig. 2.25, methylene blue and riboflavin are substrates for NADPH-glutathione reductase and for NAD(P)H-flavin oxidoreductase that are present in the cytosol of the parasite (Schirmer *et al*., 2003, Kinoshita *et al*., 2007, Haynes *et al*., 2011). The reduced conjugates including the $FADH_2$ cofactors of glutathione reductase, thioredoxin reductase, and other flavoenzymes are oxidized rapidly by artemisinins. Generally, the NAD(P)H consumption is significantly increased (as illustrated in Fig. 2.24). Therefore, treatment of reduced glutathione reductase ($FADH_2$ cofactor) under aerobic conditions with artemisinin enhances NADPH consumption. The cycle involving oxidation of all reduced conjugates by oxygen generates ROS. Pumping of haem *via* complexes with the quinoline and arylmethanols into the cytosol, and generation of free Fe^{III} leads to redox cycling of the Fe^{II}-Fe^{III} couple that matches the activity of the artemisinins and redox-active drugs.

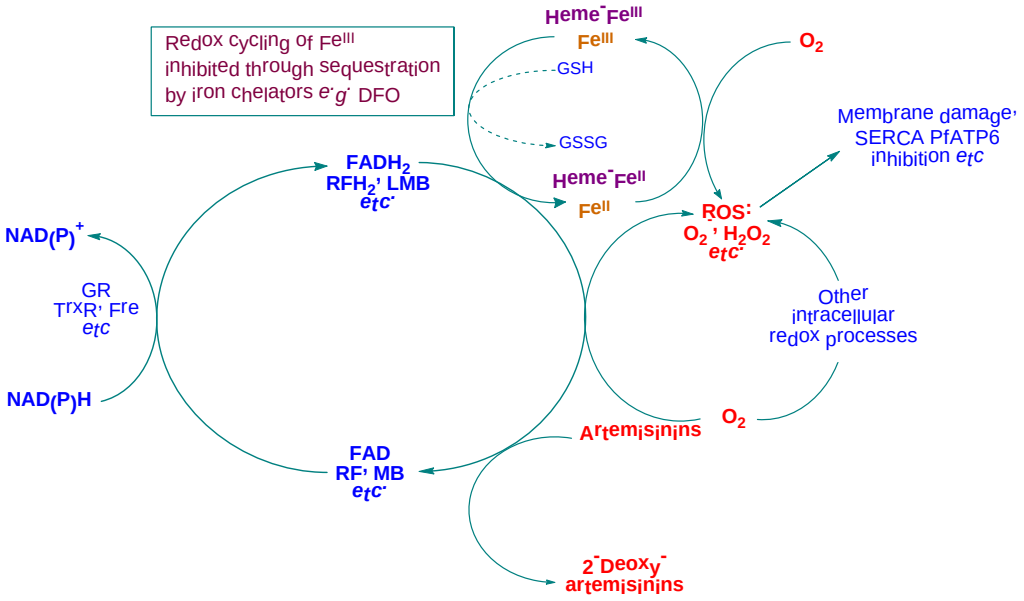


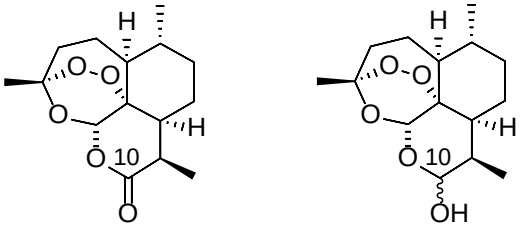
Figure 2.25: Proposal for cytosolic action of artemisinins. GR: glutathione reductase, TrxR: thioredoxin reductase, Fre: flavin oxidoreductase, RF and RFH_2: riboflavin and dihydroriboflavin, MB and LMB: methylene blue and leucomethylene blue, ROS: reactive oxygen species, DFO: desferrioxamine (Haynes *et al*., 2012).

Even if thiols like GSH reduce Fe^{III}, reduction by $FADH_2$ and other reduced flavin cofactors are significantly faster and involve single-electron transfer reactions (as noted in Fig. 2.24) (Pierre *et al*., 2002, Woodmansee and Imlay, 2002, Petrat *et al*., 2003, Kinoshita *et al*., 2007), such oxidation further draws on NAD(P)H demand. Also, re-oxidation of Fe^{II} by oxygen leads to ROS production as Fenton-like chemistry involving ROS logically accounts for membrane damage (Berman and Adams, 1997, Del Pilar Crespo *et al*., 2008, Kumura *et al*., 2009, Bousejra-El Garah *et al*., 2011) and the generation of ROS must have an effect on the *P. falciparum* SERCA PfATP6 (Eckstein-Ludwig *et al*., 2003) *via* modulation of calcium levels characteristic of ROS signalling (Haynes *et al*., 2012).

2.9.5.3 Artemisinin and its first generation semisynthetic peroxides

Artemisinin **19** is a sesquiterpene lactone and its activity against malaria is associated with the peroxide embedded in the 1,2,4-trioxane moiety (Haynes, 2001). It is a highly crystalline compound that cannot dissolve in either water or oil and therefore can only be given *via* the parenteral route. Artemisinin is converted into semisynthetic derivatives by modification at the C10 position. In this way, the dihydroartemisinin (DHA), the oil-soluble artemether and arteether, and the water soluble artesunate are prepared. However, after absorption of the artemisinin derivatives take place they are metabolized to DHA **20** which in turn, with the

help of hepatic cytochrome P450 and other enzyme systems, is converted into inactive metabolites (Haynes, 2001). The extent of conversion differs between derivatives (Li *et al*., 1998).

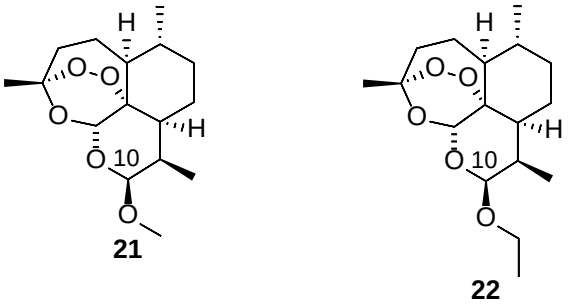


19 **20**

Figure 2.26: Artemisinin **19** and dihydroartemisinin (DHA) **20**.

Although DHA itself is a potent antimalarial, there are problems with this compound. DHA has an extremely short half-life of 45 minutes (Batty *et al*., 1998, Ilett *et al*., 2002) which promotes parasite recrudescence and reduced drug efficacy which ultimately leads to the development of parasite resistance. Additionally it has been shown that the metabolism of artesunate and the ethers to DHA is facile (Grace *et al*., 1998). Secondly, according to *in vivo* assays with animal models (Brewer *et al*., 1994, Kamchonwongpaisan *et al*., 1997, Nontprasert *et al*., 1998) and *in vitro* studies with neuronal cell cultures (Wesche *et al*., 1994, Fishwick *et al*., 1995), DHA is neurotoxic.

Artemisinin has nanomolar antimalarial activity but it suffers from low solubility in both oil and water (Barradell and Fitton, 1995). In order to overcome the solubility problems, the first semisynthetic analogues were prepared (China Cooperative Research Group, 1982). Following sodium borohydride reduction of artemisinin to DHA, the lactol could be converted to its ether (artemether **21** and arteether **22**) and ester (artesunate **23**) derivatives (Ploypradith, 2004).

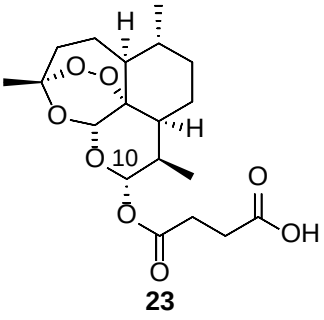


21 **22**

Figure 2.27: Artemether **21** and arteether **22**.

Arteether and artemether are rapidly metabolized to DHA which contributes to the antimalarial activities of these compounds (Woodrow *et al*., 2005). Oil based formulations of both drugs used in animals (Li *et al*., 1999) as well as humans (Teja-Isavadharm *et al*., 1996, Looareesuwan *et al*., 2002, Silamut *et al*., 2003) in pharmacokinetic studies proved to be slow releasing resulting in extended exposure times. Therefore, it is possible that the neurotoxicity is determined by the duration of exposure to the artemisinins rather than the maximum concentration reached by the artemisinins (Gordi and Lepist, 2004). Arteether is mainly used in India (Emal®) and in the Netherlands (Artemotil®) but the more prevalent substance is artemether (Artemos®) (Woodrow *et al*., 2005).

Artesunate **23**, also a semisynthetic analogue of artemisinin, is obtained from DHA in which the hemiacetal OH group is acetylated with succinic anhydride (Yang *et al*., 1982).



23

Figure 2.28: Artesunate **23**.

Artesunate is an unstable drug as the succinic ester linkage is rapidly cleaved, releasing DHA as the active agent. The antimalarial activity of artesunate is therefore largely mediated by DHA (Woodrow *et al*., 2005). Compared with DHA, artemisinin and artemether, artesunate has superior solubility in water (Krishna *et al*., 2008). Artesunate is also effective against clearing blood phase schizonts (Lin *et al*., 1987), but because of its high recrudescence rate (due to its very short pharmacological half-life of a mere few minutes) it is usually given in combination with mefloquine (Barradell and Fitton, 1995).

2.9.5.4 Artemisinin combination therapy (ACT)

Development of *P. falciparum* resistance against the quinoline and antifolate drugs led to the predominant usage of artemisinins for treatment of uncomplicated malaria. Consequently, the usage of artemisinins greatly increased (Karunajeewa, 2012). In addition, the side effects that were characteristically associated with CQ, quinine and mefloquine were not observed

when the artemisinins were used as antimalarial drugs. Also, when compared to other drugs the artemisinins relieved symptoms of malaria more efficiently (Hinton *et al*., 2007).

However, it was found during the early clinical development of artemisinins that a 5-day treatment with artemisinin monotherapy was linked to parasite recrudescence rates higher than 25% even if the cure rates were consistently close to 100% (Hien and White, 1993, McIntosh and Olliaro, 1999). This is very likely due to the extremely short half-lives of the artemisinins. Normally drug concentrations must remain above the therapeutically effective threshold for at least several complete life cycles of the malaria parasite in order to eliminate blood-stages of the parasite. As each cycle takes 48 h to complete, in order to achieve total elimination of the parasite burden and in doing so produce an effective cure (Karunajeewa, 2012), it was found necessary to treat at least for seven days with artemisinin monotherapy. Normally, malaria cure using drugs with longer half-lives such as CQ and mefloquine is possible with short-term treatment as the therapeutically effective drug concentrations endure long enough to eliminate parasites arising in successive life cycles. That is, the parasite recrudescence issue experienced with artemisinin use was addressed by utilizing artemisinin treatment that lasted up to seven days (Giao *et al*., 2001). Before the development of artemisinin resistance as reported from Cambodia, the 7 day treatment courses had cure rates higher than 95% (McIntosh and Olliaro, 1999). Unfortunately, a drawback is that the long-term treatment is likely to be undermined by poor patient adherence after subsidence of fever, but before all parasites were eliminated (Karunajeewa, 2012).

Thus, the concept of using artemisinin combination therapies (ACTs) was introduced wherein an artemisinin derivative was combined with a longer half-life drug such as mefloquine, lumefantrine, piperaquine or other drug. Within an ACT, the artemisinin component not only relieves the patient from malaria symptoms but is also responsible for the relatively rapid decrease in the parasite burden. Total elimination of the parasites occur several days after the last dose is taken as a result of the persistence of the partner drug eliminating remaining parasites (Karunajeewa, 2012). A great number of clinical trials have been carried out in order to evaluate clinical effectiveness of ACTs (Sinclair *et al*., 2009). Results of some of these trials suggest that it is the efficacy of the combination that is crucial rather than the choice of artemisinin or partner drug (Davis *et al*., 2005, Karunajeewa *et al*., 2008). However, it is important to note that progression of resistance in a specific area can be influenced if the artemisinin drug partner has pre-existing resistance (Karunajeewa *et al*., 2008). Therefore, the main idea behind an ACT is to suppress the emergence of resistance by using drugs with different mechanisms of action. Currently the WHO recommends one of

the following five approved ACTs: artesunate-mefloquine, artesunate-sulfadoxine-pyrimethamine, artesunate-amodiaquine, DHA-piperaquine and artemether-lumefantrine. According to recent estimates, the WHO advises that an ACT should be used when treating uncomplicated *P*. *falciparum* malaria (WHO, 2010b). *P*. *vivax* malaria should be treated with CQ, providing that the drug is still effective in the area associated with the *P. vivax* infection. However, *P*. *vivax* malaria can be treated with a suitable ACT if CQ resistance has been recorded. In order to avoid a relapse, 14-day treatment of primaquine should be combined with ACT as well as CQ, after the risk of haemolysis has been considered in G6PD deficient patients. It is recommended that a single dose of primaquine should be given to patients infected with *P*. *falciparum* in an area where resistance to artemisinins has been recorded and also if *P*. *falciparum* eradication is targeted (WHO, 2015a).

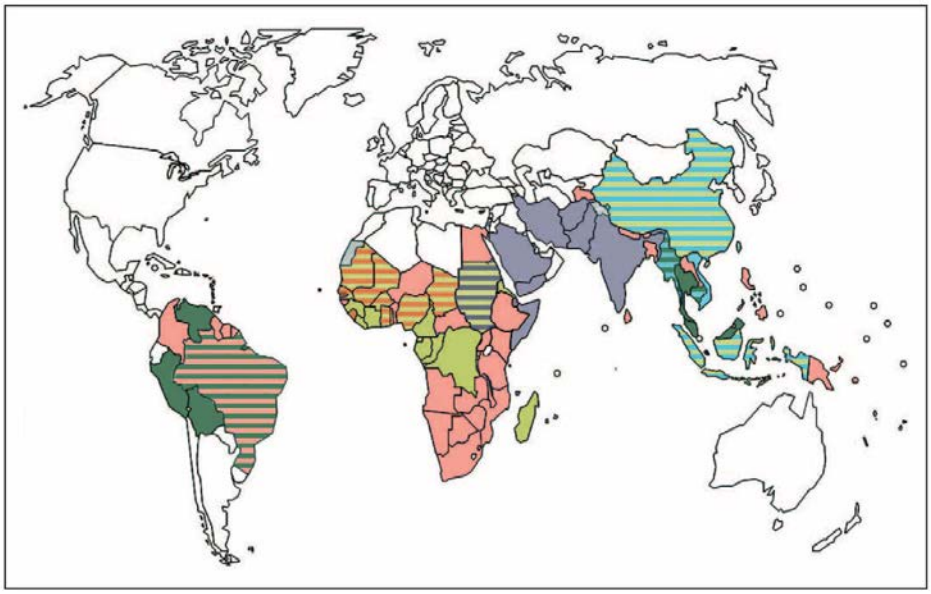


Figure 2.29: Global distribution of current artemisinin-based combination therapies used as first line treatment of uncomplicated *falciparum* malaria. This distribution was collated from maps of nationwide ACT use: ♦Artesunate-sulfadoxine-pyrimethamine; ♦Artesunate-mefloquine; ♦Artesunate-amodiaquine; ♦DHA-piperaquine; ♦Artemether-lumefantrine (O'Brien *et al*., 2011).

2.9.5.5 Artemisinin resistance

Theoretically, the development of artemisinn resistance should be limited due to their short half-lives (Hastings *et al*., 2002, Ilett and Batty, 2005, Stepniewska and White, 2008). However, resistance can also be triggered by a mutation affecting an individual enzymatic

pathway which is vital to the mode of action of the drug (Karunajeewa, 2012). Overall, however, the development of resistance towards artemisinins is so important that it sparked numerous debates as well as policy reconsiderations within the WHO (WHO, 2001, WHO, 2006, WHO, 2007). Accordingly, artemisinin was permitted to be used only in combination therapy (White, 1999a), and monotherapy was phased out (Rehwagen, 2006).

The use of an ACT relies on the idea that parasite mutations associated with resistance towards one drug will still allow the parasite to be vulnerable to the partner drug with its different mechanism of action. Thus, the parasite won't be able to survive long enough in order to pass on their resistance features to the succeeding generation (White *et al*., 1999b, White, 2004). With malaria, however, there are several limitations with regards to ACT usage (Kremsner and Krishna, 2004). The *P. falciparum* parasite has a genome (2.3×10^7 bp) that is larger and more complex when compared to those of other organisms such as the virus causing hepatitis B (3.2×10^3 bp) or *M. tuberculosis* (4.4×10^6 bp), there is an advantage of having a larger number of genetic loci that could facilitate resistance development (Karunajeewa, 2012). Thus, the development of multifaceted mechanisms of drug resistance when compared with viruses and bacteria is not surprising. For instance, drug transport mechanisms that are affected by resistance can result in resistance towards a variety of drugs with different mechanisms of action involving different molecular targets. *Pf*MDR1 is the best example in *P. falciparum* as it translates the P-glycoprotein homologue (Pgh) responsible for the efflux of drug into the parasite food vacuole (Cowman *et al*., 1994, Reed *et al*., 2000). Single nucleotide polymorphisms or increasing the gene copy number can facilitate resistance, where *Pf*MDR1 has been involved in resistance towards various antimalarial drugs such as CQ, lumefantrine, halofantrine, mefloquine, and amodiaquine (Price *et al*., 2004, Price *et al*., 2006).

Initial warnings of treatment failure with the ACT artesunate-mefloquine were first reported near the Thai-Cambodian border in the early 2000's (Vijaykadga *et al*., 2006, Denis *et al*., 2006). It was unclear at the time whether this was due to development of resistance either to artesunate or to mefloquine (O'Brien *et al*., 2011). The first clinically-significant artemisinin resistance was first described in Western Cambodia in 2008 (Noedl *et al*., 2008). The study identified artesunate-resistant infections in two patients who experienced parasite recrudescence following 7 days of artesunate monotherapy (4 mg/kg per day) and who showed delayed parasite clearance times (133 and 95 h, as compared with a median of 52 h for patients that were cured) in spite of maintaining acceptable drug levels. These times represent the interval from the start of the treatment to the time the parasitaemia (*i.e.* the percentage of parasitized erythrocytes) falls below the lower limit of parasite detection by

light microscopy (approximately 0.001%). Patient isolates with extended parasite clearance times displayed DHA half maximal inhibitory concentration (IC_{50}) values as determined by *ex vivo* drug dose-response measurements four times higher than the geometric mean for cured patients (Noedl *et a*l., 2010).

The emergence of artemisinin resistance is supported by the results of a study published in 2009 (Dondorp *et al*., 2009). The study established that following artesunate-mefloquine combination therapy or artesunate monotherapy, subjects displayed increased parasite clearance times. The average clearance time would increase in patients treated with 2 mg/kg of artesunate from 48 to 72 h. In patients treated with 4 mg/kg of the artesunate-mefloquine combination, clearance times also increased from 54 to 84 h. Neither the pharmacokinetics nor the differences in age between the two study populations could be held responsible for these variations (O'Brien *et al*., 2011).

The development of artemisinin resistance in western Cambodia can be attributed to different factors, namely the genetic background of the parasite displaying preference towards the emergence of multidrug resistance, the sub-optimal use of drugs, and 30 years of artemisinn monotherapy (Rathod *et al*., 1997, Newton *et al*., 2008, Dondorp *et al*., 2010). The genes of the malaria parasite contribute to 56% of parasite clearance discrepancies as determined by Pailin isolates in heritability studies, implying that determinants of resistance could expand geographically under drug selection (Anderson *et al*., 2010).

The Thai-Cambodian border is a historical focal point when it comes to multidrug resistance towards *P*. *falciparum* which adds to the concerns of weakening artemisinin efficacy (Enserink, 2010). Resistance towards CQ, sulfadoxine-pyrimethamine and mefloquine were the first to develop in this region; the sulfadoxine-pyrimethamine and mefloquine resistance spread to Africa (Wellems and Plowe, 2001*,* Wongsrichanalai *et al.,* 2002*,* Wootton *et al.,* 2002*,* Roper *et al*., 2004). In order to keep track of the rise and spread of artemisinin resistance substantial efforts are being made throughout the Greater Mekong Subregion (which is made up of Vietnam, the Yunnan Province of China, Thailand, Laos, Cambodia and Myanmar) to carefully map out and block the spread of resistant parasites (Fig. 2.30) (Cui *et al*., 2012).

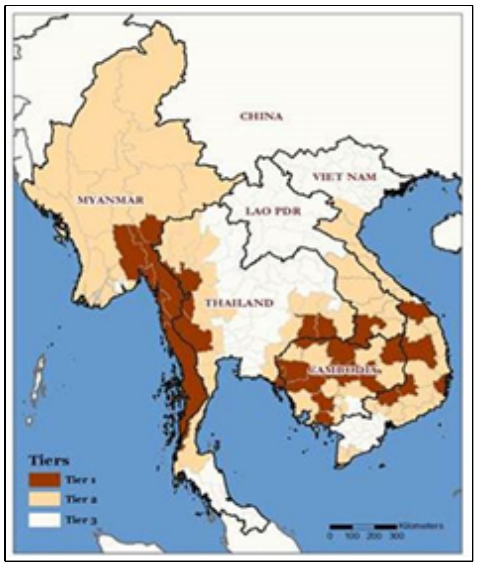


Figure 2.30: *P. falciparum* resistance towards artemisinin observed in the five countries in the Greater Mekong Subregion. **Tier 1**: areas with reliable evidence of resistance towards artemisinin. **Tier 2**: areas with a substantial inflow of people from Tier 1 areas, which includes those closely bordering Tier 1. **Tier 3**: areas with no evidence of artemisinin resistance and limited contact with Tier 1 areas (WHO, 2015a).

Between 1995 and 2007, some 3,200 patients were treated with the 3-day combination course of artesunate-mefloquine along the Thai-Myanmar border. Data collected from this study indicated that 96% of patients were clear of parasitaemia on the third day of treatment (Carrara *et al*., 2009). However, an increase in treatment failure rates (recrudescence of parasites observed during a 42-day follow-up) were associated with a small yet significant increase in parasite clearance times in this study. Gametocyte carriage, which increases the transmission risk of drug-resistant parasites, was also linked to decreased parasite clearance. Parasite clearance data was collected from 25 different countries (ranging from low to high transmission) making up a total of 18,000 patients in a 2010 study (Stepniewska *et al*., 2010), confirming the previous proposal from western Cambodia (Dondorp *et al*., 2009) that delayed parasite clearance time is a clear marker for resistance against artemisinin.

A more recent study published in 2014 identified *kelch13* as the molecular marker associated with mutations in southeast Asia (Ashley *et al*., 2014). The study involved 1,241 children as well as adults infected with acute, uncomplicated *P*. *falciparum* malaria in an open-label trial at 15 sites in 10 countries (7 in Asia and 3 in Africa). Patients received artesunate administered orally at a daily dose of either 2 mg/kg per day or 4 mg/kg, for 3 days, followed by a standard 3-day course of ACT. Results showed that the average parasite clearance half-lives varied from 1.9 h (Democratic Republic of Congo) to 7 h (Thai-

Cambodian border). Throughout Southeast Asia, from central Myanmar to southern Vietnam, an increase in parasite clearance times (half-life of parasite clearance > 5 h) were observed that were associated with a single point mutation in the “propeller” region of the *P. falciparum* kelch protein gene on chromosome 13 (*kelch13*). The occurrence of pre-treatment and post-treatment gametocytaemia was increased among patients with sluggish parasite clearance, signifying a superior potential for transmission. Of note, prolonged courses of ACTs are currently efficacious in areas where standard 3-day treatments are failing. Interestingly, emerging artemisinin resistance seems to be mainly limited to the Greater Mekong Subregion, as nothing conclusive has been reported yet from Africa (Kachur *et al*., 2010).

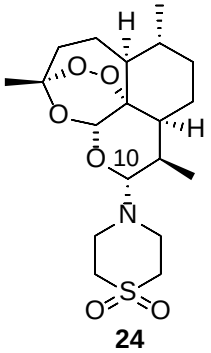
Another important factor that was identified for playing a role in artemisinin resistance was recrudescence where some studies suggested that the use of artemisinin can give rise to parasite recrudescence. *In vitro* studies have indicated that the *P. falciparum* parasite has a unique ability to become quiescent, or dormant, when exposed to artemisinins for short periods of time. This mechanism is believed to protect early ring-stage intraerythrocytic parasites from the drug; quiescence implies slowed turnover of intracellular enzymes, thus lowering susceptibility to the drug (Teuscher *et al*., 2010, Teuscher *et al*., 2011, Cheng *et al*., 2012). After the concentration of artemisinin has dropped to sub-therapeutic level, a portion of the dormant parasites continue their life cycle leading to parasite recrudescence, resistance and treatment failure. Another pressing issue is that of neurotoxicity. As previously mentioned, most of the artemisinins undergo facile metabolism to DHA which is neurotoxic in cell and animal assays. It stands to reason if parasite clearance is slowed *via* the ability of the parasite to become dormant in the presence of an artemisinin the length of treatment should be increased in order to reach 100% parasite clearance. Although it sounds like the logical step to take, it has some consequences. As the length of treatment increase, patients are at an increased risk of being exposed to higher neurotoxicity values. If the metabolic stability of an artemisinin derivative can be improved by, for example, not providing DHA *in vivo*, it would have a major advantage over its predecessors.

2.9.5.6 Other artemisinin derivatives

Artemisone

In order to prevent the metabolic transformation of first generation artemisinins to DHA as well as improving pharmacokinetics, several 10-(alkylamino)artemisinins were prepared. One such is artemisone **24** that in a Phase II clinical trial was shown to be three times as

efficacious as the comparator drug artesunate, and did not give DHA on metabolism (Vivas *et al*., 2007, Opsenica and Šolaja, 2012). Twelve different *P*. *falciparum* strains were used to determine the *in vitro* antimalarial activities of artemisone; when these activities were compared to other antimalarial drug classes they were independent of the drug-susceptibility profile of the different strains (Haynes *et al*., 2004, Vivas *et al*., 2007).



24

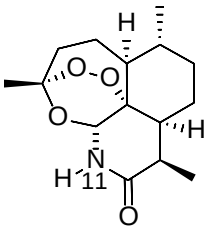
Figure 2.31: Artemisone **24**.

Artemisone has a minor antagonistic effect if used with CQ, pyrimethamine, atovaquone and amodiaquine and slight synergism with mefloquine as determined by evaluation of *in vitro* drug-drug interactions against multidrug-resistant strains of K1 and CQ sensitive 3D7 strains. Also, artemisone does not form DHA upon metabolism but rather forms mono-hydroxylated metabolites with syn-hydroxyl and peroxide groups (Haynes *et al*., 2006). This clearly distinguishes artemisone and analogues from the first generation artemisinins (Opsenica and Šolaja, 2012).

Artemisone is mainly metabolized by recombinant CYP3A4 as demonstrated through the incubation of 14 recombinant cytochrome P450 (CYP) isoforms together with microsomes and selective inhibitors of CYP. Remarkably, artemisone is not an inducer of its metabolism; this stands in contrast to the current clinical derivatives that induce their own metabolism (Ashton *et al*., 1998) involving the principal metabolizing enzyme CYP2B6 (Svensson and Ashton, 1999). These results indicate that although artemisinin and artemisone share structural similarities they have notably different metabolic profiles in *P*. *falciparum*. Compared with artesunate, artemisone demonstrated enhanced pharmacokinetics, improved efficacy as well as the absence of neurotoxicity *in vitro* and *in vivo* (Haynes *et al*., 2006, Nagelschmitz *et al*., 2008). The absence of neurotoxicity is noted as a decisive advantage, and this must become important if protracted treatment regimens involving artemisone and a combination partner against artemisinin-resistant parasites is eventually required. As noted above, neurotoxicity is an issue in current clinical artemisinins (Brewer *et al*., 1994).

11-Azaartemisinin

The first generation artemisinin derivatives artemether and artesunate discussed above are prepared from artemisinin by modification at the C10 position; all are converted into DHA *in vivo*. In contrast, 11-azaartemisinin **25** is a unique derivative prepared from artemisinin by replacement of O11 by nitrogen.



25

Figure 2.32: 11-Azaartemisinin **25**.

In 1995, Avery and co-workers synthesized the first azaartemisinin derivatives namely the *N*-alkyl-11-aza-9-desmethylartemisinins (Avery *et al*., 1995), with an elegant albeit complex synthesis. In the same year, Ziffer and co-workers also synthesized a series of *N*-substituted 11-azaartemisinins with promising *in vitro* and *in vivo* results (Torok and Ziffer, 1995). It was only relatively recently that Haynes and his co-workers were able to simplify and optimize the synthesis of 11-azaartemisinin (Haynes *et al*., 2007a).

11-Azaartemisinin **25** displays a number of advantages when compared to other artemisinins as it is easily obtained from artemisinin, contains a lactam moiety instead of the lactone of artemisinin. This is likely to make it more stable under acidic conditions (Bach and Dmitrenko, 2006). Therefore, it stands to reason that the azaartemisinins should have enhanced bioavailability when compared to artemisinin. Also, this compound cannot produce DHA *in vivo* as it is at a higher oxidation level than artemisinin.

An array of 11-azaartemisinin derivatives has been synthesized since 1995, many of which appear to have potential for treatment of malaria. As 11-azaartemisinin is the parent compound of the current study, a review discussing the synthesis, chemistry and biological evaluations of azaartemisinin and its derivatives is presented in Chapter 3.

REFERENCES

Abiodun, O. O., Brun, R. & Wittlin, S. (2013) *In vitro* interaction of artemisinin derivatives or the fully synthetic peroxidic antimalarial OZ277 with thapsigargin in *Plasmodium falciparum* strains. *Malaria Journal,* 12**,** 1-4.

Abkarian, M., Massiera, G., Berry, L., Roques, M. & Braun-Breton, C. (2011) A novel mechanism for egress of malarial parasites from red blood cells. *Blood,* 117**,** 4118-4124.

Aikawa, M. & Beaudoin, R. L. (1970) *Plasmodium fallax*: high-resolution autoradiography of exoerythrocytic stages treated with Primaquine *in vitro*. *Experimental Parasitology,* 27**,** 454-463.

Amino, R., Thiberge, S., Martin, B. A., Celli, S., Shorte, S., Frischknecht, F. & Ménard, R. (2006) Quantitative imaging of *Plasmodium* transmission from mosquito to mammal. *Nature Medicine,* 12**,** 220-224.

Anderson, T. J. C., Nair, S., Qin, H., Singlam, S., Brockman, A., Paiphun, L. & Nosten, F. O. (2005) Are transporter genes other than the chloroquine resistance locus (pfcrt) and multidrug resistance gene (pfmdr) associated with antimalarial drug resistance? *Antimicrobial Agents and Chemotherapy,* 49**,** 2180-2188.

Anderson, T. J. C., Nair, S., Nkhoma, S., Williams, J. T., Imwong, M., Yi, P., Socheat, D., Das, D., Chotivanich, K. & Day, N. P. J. (2010) High heritability of malaria parasite clearance rate indicates a genetic basis for artemisinin resistance in western Cambodia. *Journal of Infectious Diseases,* 201**,** 1326-1330.

Arnou, B., Montigny, C. D., Morth, J. P., Nissen, P., Jaxel, C., Møller, J. V. & Le Maire, M. (2011) The Plasmodium falciparum Ca^{2+}-ATPase PfATP6: insensitive to artemisinin, but a potential drug target. *Biochemical Society Transactions,* 39**,** 823-831.

Ashley, E., McGready, R., Proux, S. & Nosten, F. O. (2006) Malaria. *Travel Medicine and Infectious Disease,* 4**,** 159-173.

Ashley, E. A., Stepniewska, K., Lindegårdh, N., Annerberg, A., Kham, A., Brockman, A., Singhasivanon, P., White, N. J. & Nosten, F. (2007) How much fat is necessary to optimize lumefantrine oral bioavailability? *Tropical Medicine & International Health,* 12**,** 195-200.

Ashley, E. A., Dhorda, M., Fairhurst, R. M., Amaratunga, C., Lim, P., Suon, S., Sreng, S., Anderson, J. M., Mao, S. & Sam, B. (2014) Spread of artemisinin resistance in *Plasmodium falciparum* malaria. *New England Journal of Medicine,* 371**,** 411-423.

Ashton, M., Hai, T. N., Sy, N. D., Huong, D. X., Van Huong, N. & Nieu, N. T. (1998) Artemisinin pharmacokinetics is time-dependent during repeated oral administration in healthy male adults. *Drug Metabolism and Disposition,* 26**,** 25-27.

Avery, M. A., Bonk, J. D., Chong, W. K. M., Mehrotra, S., Miller, R., Milhous, W., Goins, D. K., Venkatesan, S. & Wyandt, C. (1995) Structure-activity relationships of the antimalarial agent artemisinin. 2. Effect of heteroatom substitution at O-11: synthesis and bioassay of N-Alkyl-11-aza-9-desmethylartemisinins. *Journal of Medicinal Chemistry,* 38**,** 5038-5044.

Ayimba, E., Hegewald, J., Segbena, A. Y., Gantin, R. G., Lechner, C. J., Agosssou, A., Banla, M. & Soboslay, P. T. (2011) Proinflammatory and regulatory cytokines and chemokines in infants with uncomplicated and severe *Plasmodium falciparum* malaria. *Clinical & Experimental Immunology,* 166**,** 218-226.

Bach, R. D. & Dmitrenko, O. (2006) The effect of carbonyl substitution on the strain energy of small ring compounds and their six-member ring reference compounds. *Journal of the American Chemical Society,* 128**,** 4598-4611.

Bannister, L. H., Mitchell, G. H., Butcher, G. A., Dennis, E. D. & Cohen, S. (1986) Structure and development of the surface coat of erythrocytic merozoites of *Plasmodium knowlesi*. *Cell and Tissue Research,* 245**,** 281-290.

Bannister, L. H., Hopkins, J. M., Fowler, R. E., Krishna, S. & Mitchell, G. H. (2000) A brief illustrated guide to the ultrastructure of *Plasmodium falciparum* asexual blood-stages. *Parasitology Today,* 16**,** 427-433.

Barnes, D. A., Foote, S. J., Galatis, D., Kemp, D. J. & Cowman, A. F. (1992) Selection for high-level chloroquine resistance results in deamplification of the pfmdr1 gene and increased sensitivity to mefloquine in *Plasmodium falciparum*. *The EMBO Journal,* 11**,** 3067.

Barradell, L. B. & Fitton, A. (1995) Artesunate. *Drugs,* 50**,** 714-741.

Batty, K. T., Thu, A., Thi, L., Davis, T. M. E., Ilett, K. F., Xuan Mai, T., Canh Hung, N., Phuc Tien, N., Powell, S. M. & Van Thien, H. (1998) A pharmacokinetic and

pharmacodynamic study of intravenous vs. oral artesunate in uncomplicated falciparum malaria. *British Journal of Clinical Pharmacology,* 45**,** 123-129.

Baudon, D., Martet, G., Pascal, B., Bernard, J., Keundjian, A. & Laroche, R. (1999) Efficacy of daily antimalarial chemoprophylaxis in tropical Africa using either doxycycline or chloroquine-proguanil; a study conducted in 1996 in the French Army. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 93**,** 302-303.

Beaudoin, R. L. & Aikawa, M. (1968) Primaquine-induced changes in morphology of exoerythrocytic stages of malaria. *Science,* 160**,** 1233-1234.

Becker, K., Rahlfs, S., Nickel, C. & Schirmer, R. H. (2003) Glutathione - functions and metabolism in the malarial parasite *Plasmodium falciparum*. *Biological Chemistry,* 384**,** 551-566.

Berman, P. A. & Adams, P. A. (1997) Artemisinin enhances heme-catalysed oxidation of lipid membranes. *Free Radical Biology and Medicine,* 22**,** 1283-1288.

Bilolikar, H. G., Bagade, A. C., Phadke, M. A. & Gambhir, P. S. (1994) Efficacy of halofantrine in malaria. *Indian Pediatrics,* 32**,** 77-79.

Borrmann, S., Szlezak, N., Binder, R. K., Missinou, M. A., Lell, B. & Kremsner, P. G. (2002) Evidence for the efficacy of artesunate in asymptomatic *Plasmodium malariae* infections. *Journal of Antimicrobial Chemotherapy,* 50**,** 751-754.

Boulard, Y., Landau, I., Miltgen, F., Ellis, D. S. & Peters, W. (1983) The chemotherapy of rodent malaria, XXXIV. Causal prophylaxis Part III: Ultrastructural changes induced in exo-erythrocytic schizonts of *Plasmodium yoelii* by primaquine. *Annals of Tropical Medicine and Parasitology,* 77**,** 555-568.

Bousejra-El Garah, F., Wong, M. H.-L., Amewu, R. K., Muangnoicharoen, S., Maggs, J. L., Stigliani, J.-L., Park, B. K., Chadwick, J., Ward, S. A. & O'Neill, P. M. (2011) Comparison of the reactivity of antimalarial 1, 2, 4, 5-tetraoxanes with 1, 2, 4-trioxolanes in the presence of ferrous iron salts, heme, and ferrous iron salts/phosphatidylcholine. *Journal of Medicinal Chemistry,* 54**,** 6443-6455.

Bozdech, Z., Llinás, M., Pulliam, B. L., Wong, E. D., Zhu, J. & Derisi, J. L. (2003) The transcriptome of the intraerythrocytic developmental cycle of *Plasmodium falciparum*. *PLoS Biology,* 1**,** e5.

Bray, P. G., Deed, S., Fox, E., Kalkanidis, M., Mungthin, M., Deady, L. W. & Tilley, L. (2005) Primaquine synergises the activity of chloroquine against chloroquine-resistant *P. falciparum*. *Biochemical Pharmacology,* 70**,** 1158-1166.

Brewer, T. G., Grate, S. J., Peggins, J. O., Weina, P. J., Petras, J. M., Levine, B. S., Heiffer, M. H. & Schuster, B. G. (1994) Fatal neurotoxicity of arteether and artemether. *The American Journal of Tropical Medicine and Hygiene,* 51**,** 251-259.

Briolant, S. B., Pelleau, S. P., Bogreau, H., Hovette, P., Zettor, A. S., Castello, J., Baret, E., Amalvict, R. M., Rogier, C. & Pradines, B. (2011) *In vitro* susceptibility to quinine and microsatellite variations of the *Plasmodium falciparum* Na+/H+ exchanger (Pfnhe-1) gene: the absence of association in clinical isolates from the Republic of Congo. *Malaria Journal,* 10**,** 1.

Brown, M. & Hebert, A. A. (1997) Insect repellents: an overview. *Journal of the American Academy of Dermatology,* 36**,** 243-249.

Bruce-Chwatt, L. J. (1981) Alphonse Laveran's discovery 100 years ago and today's global fight against malaria. *Journal of the Royal Society of Medicine,* 74**,** 531.

Buchholz, K., Schirmer, R. H., Eubel, J. K., Akoachere, M. B., Dandekar, T., Becker, K. & Gromer, S. (2008) Interactions of methylene blue with human disulfide reductases and their orthologues from *Plasmodium falciparum*. *Antimicrobial Agents and Chemotherapy,* 52**,** 183-191.

Budimulja, A. S., Tapchaisri, P., Wilairat, P. & Marzuki, S. (1997) The sensitivity of *Plasmodium* protein synthesis to prokaryotic ribosomal inhibitors. *Molecular and Biochemical Parasitology,* 84**,** 137-141.

Buffet, P. A., Safeukui, I., Deplaine, G., Brousse, V., Prendki, V., Thellier, M., Turner, G. D. & Mercereau-Puijalon, O. (2011) The pathogenesis of *Plasmodium falciparum* malaria in humans: insights from splenic physiology. *Blood,* 117**,** 381-392.

Burgoine, K. L., Bancone, G. & Nosten, F. O. (2010) The reality of using primaquine. *Malaria Journal,* 9**,** 376.

Butler, A. R., Khan, S. & Ferguson, E. (2010) A brief history of malaria chemotherapy. *The Journal of the Royal College of Physicians of Edinburgh,* 40**,** 172-177.

Cabantchik, Z. I., Kakhlon, O. R., Epsztejn, S., Zanninelli, G. & Breuer, W. (2002) Intracellular and extracellular labile iron pools. IN HERSHKO, C. (Ed.) *Iron Chelation Therapy.* 1 ed. United States, Springer. p. 55-75.

Campanale, N., Nickel, C., Daubenberger, C. A., Wehlan, D. A., Gorman, J. J., Klonis, N., Becker, K. & Tilley, L. (2003) Identification and characterization of heme-interacting proteins in the malaria parasite, *Plasmodium falciparum*. *Journal of Biological Chemistry,* 278**,** 27354-27361.

Carrara, V. I., Zwang, J., Ashley, E. A., Price, R. N., Stepniewska, K., Barends, M., Brockman, A., Anderson, T., McGready, R. & Phaiphun, L. (2009) Changes in the treatment responses to artesunate-mefloquine on the north-western border of Thailand during 13 years of continuous deployment. *PLoS One,* 4**,** e4551.

Carter, J. Y., Loolpapit, M. P., Lema, O. E., Tome, J. L., Nagelkerke, N. J. D. & Watkins, W. M. (2005) Reduction of the efficacy of antifolate antimalarial therapy by folic acid supplementation. *The American Journal of Tropical Medicine and Hygiene,* 73**,** 166-170.

Chen, L. H., Wilson, M. E. & Schlagenhauf, P. (2006) Prevention of malaria in long-term travellers. *Jama,* 296**,** 2234-2244.

Cheng, Q., Kyle, D. E. & Gatton, M. L. (2012) Artemisinin resistance in *Plasmodium falciparum*: A process linked to dormancy? *International Journal for Parasitology: Drugs and Drug Resistance,* 2**,** 249-255.

China Cooperative Research Group. (1982) The chemistry and synthesis of qinghaosu derivatives. *Journal of Traditional Chinese Medicine* 2**,** 1-16.

Coghi, P., Basilico, N., Taramelli, D., Chan, W. C., Haynes, R. K. & Monti, D. (2009) Interaction of artemisinins with oxyhemoglobin Hb-Fe^{II}, Hb-Fe^{II}, CarboxyHb-Fe^{II}, Heme-Fe^{II}, and carboxyheme Fe^{II}: significance for mode of action and implications for therapy of cerebral malaria. *ChemMedChem,* 4**,** 2045-2053.

Cooper, R. A., Ferdig, M. T., Su, X.-Z., Ursos, L. M. B., Mu, J., Nomura, T., Fujioka, H., Fidock, D. A., Roepe, P. D. & Wellems, T. E. (2002) Alternative mutations at position 76 of the vacuolar transmembrane protein PfCRT are associated with chloroquine resistance and unique stereospecific quinine and quinidine responses in *Plasmodium falciparum*. *Molecular Pharmacology,* 61**,** 35-42.

Cooper, R. A., Lane, K. D., Deng, B., Mu, J., Patel, J. J., Wellems, T. E., Su, X. & Ferdig, M. T. (2007) Mutations in transmembrane domains 1, 4 and 9 of the *Plasmodium falciparum* chloroquine resistance transporter alter susceptibility to chloroquine, quinine and quinidine. *Molecular Microbiology,* 63**,** 270-282.

Cowman, A. F., Galatis, D. & Thompson, J. K. (1994) Selection for mefloquine resistance in *Plasmodium falciparum* is linked to amplification of the pfmdr1 gene and cross-resistance to halofantrine and quinine. *Proceedings of the National Academy of Sciences,* 91**,** 1143-1147.

Cowman, A. F., Berry, D. & Baum, J. (2012) The cellular and molecular basis for malaria parasite invasion of the human red blood cell. *The Journal of Cell Biology,* 198**,** 961-971.

Cox, F. E. G. (2010) History of the discovery of the malaria parasites and their vectors. *Parasite Vectors,* 3**,** 5.

Creek, D. J., Charman, W. N., Chiu, F. C. K., Prankerd, R. J., Dong, Y., Vennerstrom, J. L. & Charman, S. A. (2008) Relationship between antimalarial activity and heme alkylation for spiro-and dispiro-1, 2, 4-trioxolane antimalarials. *Antimicrobial Agents and Chemotherapy,* 52**,** 1291-1296.

Cui, L., Yan, G., Sattabongkot, J., Cao, Y., Chen, B., Chen, X., Fan, Q., Fang, Q., Jongwutiwes, S. & Parker, D. (2012) Malaria in the Greater Mekong Subregion: heterogeneity and complexity. *Acta Tropica,* 121**,** 227-239.

Dahl, E. L., Shock, J. L., Shenai, B. R., Gut, J., Derisi, J. L. & Rosenthal, P. J. (2006) Tetracyclines specifically target the apicoplast of the malaria parasite *Plasmodium falciparum*. *Antimicrobial Agents and Chemotherapy,* 50**,** 3124-3131.

Davis, T. M. E., Karunajeewa, H. A. & Ilett, K. F. (2005) Artemisinin-based combination therapies for uncomplicated malaria. *Medical Journal of Australia,* 182**,** 181.

De Villiers, K. A., Marques, H. M. & Egan, T. J. (2008) The crystal structure of halofantrine-ferriprotoporphyrin IX and the mechanism of action of arylmethanol antimalarials. *Journal of Inorganic Biochemistry,* 102**,** 1660-1667.

Del Pilar Crespo, M., Avery, T. D., Hanssen, E., Fox, E., Robinson, T. V., Valente, P., Taylor, D. K. & Tilley, L. (2008) Artemisinin and a series of novel endoperoxide antimalarials

exert early effects on digestive vacuole morphology. *Antimicrobial Agents and Chemotherapy,* 52**,** 98-109.

Del Prado, G. R. L., García, C. H. N., Cea, L. M., Espinilla, V. F. N., Moreno, M. F. M. O., Marquez, A. D., Polo, M. J. P. & García, I. A. S. (2014) Malaria in developing countries. *The Journal of Infection in Developing Countries,* 8**,** 001-004.

Dembele, L., Gego, A., Zeeman, A.-M., Franetich, J.-F. O., Silvie, O., Rametti, A., Le Grand, R., Dereuddre-Bosquet, N., Sauerwein, R. & Van Gemert, G.-J. (2011) Towards an *in vitro* model of *Plasmodium* hypnozoites suitable for drug discovery. *PLoS One,* 6**,** e18162.

Denis, M. B., Tsuyuoka, R., Poravuth, Y., Narann, T. S., Seila, S., Incardona, S., Lim, P., Sem, R., Socheat, D. & Christophel, E. M. (2006) Surveillance of the efficacy of artesunate and mefloquine combination for the treatment of uncomplicated falciparum malaria in Cambodia. *Tropical Medicine & International Health,* 11**,** 1360-1366.

Dittrich, S., Mitchell, S. L., Blagborough, A. M., Wang, Q., Wang, P., Sims, P. F. G. & Hyde, J. E. (2008) An atypical orthologue of 6-pyruvoyltetrahydropterin synthase can provide the missing link in the folate biosynthesis pathway of malaria parasites. *Molecular Microbiology,* 67**,** 609-618.

Dondorp, A. M., Desakorn, V., Pongtavornpinyo, W., Sahassananda, D., Silamut, K., Chotivanich, K., Newton, P. N., Pitisuttithum, P., Smithyman, A. M. & White, N. J. (2005) Estimation of the total parasite biomass in acute falciparum malaria from plasma PfHRP2. *PLoS Med,* 2**,** e204.

Dondorp, A. M., Lee, S. J., Faiz, M. A., Mishra, S., Price, R., Tjitra, E., Than, M., Htut, Y., Mohanty, S. & Yunus, E. B. (2008) The relationship between age and the manifestations of and mortality associated with severe malaria. *Clinical Infectious Diseases,* 47**,** 151-157.

Dondorp, A. M., Nosten, F. O., Yi, P., Das, D., Phyo, A. P., Tarning, J., Lwin, K. M., Ariey, F., Hanpithakpong, W. & Lee, S. J. (2009) Artemisinin resistance in *Plasmodium falciparum* malaria. *New England Journal of Medicine,* 361**,** 455-467.

Dondorp, A. M., Yeung, S., White, L., Nguon, C., Day, N. P. J., Socheat, D. & Von Seidlein, L. (2010) Artemisinin resistance: current status and scenarios for containment. *Nature Reviews Microbiology,* 8**,** 272-280.

Doolan, D. L., Doban, C. & Baird, J. K. (2009) Acquired immunity to malaria. *Clinical Microbiology Reviews,* 22**,** 13-36.

Doumbo, O. K., Thera, M. A., Kone, A. K., Raza, A., Tempest, L. J., Lyke, K. E., Plowe, C. V. & Rowe, J. A. (2009) High levels of *Plasmodium falciparum* rosetting in all clinical forms of severe malaria in African children. *The American Journal of Tropical Medicine and Hygiene,* 81**,** 987-993.

Eastman, R. T. & Fidock, D. A. (2009) Artemisinin-based combination therapies: a vital tool in efforts to eliminate malaria. *Nature Reviews Microbiology,* 7**,** 864-874.

Eckstein-Ludwig, U., Webb, R. J., Van Goethem, I. D. A., East, J. M., Lee, A. G., Kimura, M., O'Neill, P. M., Bray, P. G., Ward, S. A. & Krishna, S. (2003) Artemisinins target the SERCA of *Plasmodium falciparum*. *Nature,* 424**,** 957-961.

Edwards, G., Mcgrath, C. S., Ward, S. A., Supanaranond, W., Pukrittayakamee, S., Davis, T. M. & White, N. J. (1993) Interactions among primaquine, malaria infection and other antimalarials in Thai subjects. *British Journal of Clinical Pharmacology,* 35**,** 193-198.

Egan, T. J. (2008) Haemozoin formation. *Molecular and Biochemical Parasitology,* 157**,** 127-136.

Enayati, A., Lines, J., Maharaj, R. & Hemingway, J. (2009) Suppressing the vector. IN FEACHEM, R. G. A., PHILLIPS, A. A. & TARGETT, G. A. (Eds.) *Shrinking the Malaria Map: A Prospectus on Malaria Elimination.* San Francisco, The Global Health Group. 140-154p.

Enserink, M. (2010) Malaria's drug miracle in danger. *Science,* 328**,** 844-846.

Falco, E. A., Goodwin, L. G., Hitchings, G. H., Rollo, I. M. & Russell, P. B. (1951) 2:4-diaminopyrimidines – a new series of antimalarials. *British Journal of Pharmacology and Chemotherapy,* 6**,** 185-200.

Fisher, N., Bray, P. G., Ward, S. A. & Biagini, G. A. (2007) The malaria parasite type II NADH: quinone oxidoreductase: an alternative enzyme for an alternative lifestyle. *Trends in Parasitology,* 23**,** 305-310.

Fishwick, J., Mclean, W. G., Edwards, G. & Ward, S. A. (1995) The toxicity of artemisinin and related compounds on neuronal and glial cells in culture. *Chemico-Biological Interactions,* 96**,** 263-271.

Fitch, C. D. (2004) Ferriprotoporphyrin IX, phospholipids, and the antimalarial actions of quinoline drugs. *Life Sciences,* 74**,** 1957-1972.

Fitch, C. D., Chan, R. L. & Chevli, R. (1979) Chloroquine resistance in malaria: accessibility of drug receptors to mefloquine. *Antimicrobial Agents and Chemotherapy,* 15**,** 258-262.

Foley, M. & Tilley, L. (1998) Quinoline antimalarials: mechanisms of action and resistance and prospects for new agents. *Pharmacology & Therapeutics,* 79**,** 55-87.

Foote, S. J., Galatis, D. & Cowman, A. F. (1990) Amino acids in the dihydrofolate reductase-thymidylate synthase gene of *Plasmodium falciparum* involved in cycloguanil resistance differ from those involved in pyrimethamine resistance. *Proceedings of the National Academy of Sciences,* 87**,** 3014-3017.

Fry, M. & Beesley, J. E. (1991) Mitochondria of mammalian *Plasmodium spp*. *Parasitology,* 102**,** 17-26.

Fry, M. & Pudney, M. (1992) Site of action of the antimalarial hydroxynaphthoquinone, 2-[trans-4-(4'-chlorophenyl) cyclohexyl]-3-hydroxy-1, 4-naphthoquinone (566C80). *Biochemical Pharmacology,* 43**,** 1545-1553.

Fujioka, H. & Aikawa, M. (2002) Structure and life cycle. IN PERLMANN, P. & TROYE-BLOMBERG, M. (Eds.) *Malaria Immunology.* Basel, Karger. 80:1-26p.

Gething, P. W., Patil, A. P., Smith, D. L., Guerra, C. A., Elyazar, I. R. F., Johnston, G. L., Tatem, A. J. & Hay, S. I. (2011a) A new world malaria map: *Plasmodium falciparum* endemicity in 2010. *Malaria Journal,* 10**,** 1-16.

Gething, P. W., Elyazar, I. R. F., Moyes, C. L., Smith, D. L., Battle, K. E., Guerra, C. A., Patil, A. P., Tatem, A. J., Howes, R. E. & Myers, M. F. (2011b) A long neglected world malaria map: *Plasmodium vivax* endemicity in 2010. *PLoS Neglected Tropical Diseases,* 6**,** e1814.

Giao, P. T., Binh, T. Q., Kager, P. A., Long, H. P., Van Thang, N., Van Nam, N. & De Vries, P. J. (2001) Artemisinin for treatment of uncomplicated falciparum malaria: is there a place for monotherapy? *The American Journal of Tropical Medicine and Hygiene,* 65**,** 690-695.

Gil, J. P., Nogueira, F., Stromberg-Norklit, J., Lindberg, J., Carrolo, M., Casimiro, C., Lopes, D., Arez, A. P., Cravo, P. V. & Rosario, V. E. (2003) Detection of atovaquone and Malarone - resistance conferring mutations in *Plasmodium falciparum* cytochrome b gene (cytb). *Molecular and Cellular Probes,* 17**,** 85-89.

Gilson, P. R. & Crabb, B. S. (2009) Morphology and kinetics of the three distinct phases of red blood cell invasion by *Plasmodium falciparum* merozoites. *International Journal for Parasitology,* 39**,** 91-96.

Glushakova, S., Yin, D., Li, T. & Zimmerberg, J. (2005) Membrane transformation during malaria parasite release from human red blood cells. *Current Biology,* 15**,** 1645-1650.

Godfray, H. C. J. (2013) Mosquito ecology and control of malaria. *Journal of Animal Ecology,* 82**,** 15-25.

Gordi, T. & Lepist, E.-I. (2004) Artemisinin derivatives: toxic for laboratory animals, safe for humans? *Toxicology Letters,* 147**,** 99-107.

Grace, J. M., Aguilar, A. J., Trotman, K. M. & Brewer, T. G. (1998) Metabolism of beta-arteether to dihydroqinghaosu by human liver microsomes and recombinant cytochrome P450. *Drug Metabolism and Disposition,* 26**,** 313-317.

Gregson, A. & Plowe, C. V. (2005) Mechanisms of resistance of malaria parasites to antifolates. *Pharmacological Reviews,* 57**,** 117-145.

Grüring, C., Heiber, A., Kruse, F., Ungefehr, J., Gilberger, T.-W. & Spielmann, T. (2011) Development and host cell modifications of *Plasmodium falciparum* blood-stages in four dimensions. *Nature Communications,* 2**,** 165.

Hastings, I. M., Watkins, W. M. & White, N. J. (2002) The evolution of drug-resistant malaria: the role of drug elimination half-life. *Philosophical Transactions of the Royal Society of London B: Biological Sciences,* 357**,** 505-519.

Hastings, I. M. (2006) Gametocytocidal activity in antimalarial drugs speeds the spread of drug resistance. *Tropical Medicine & International Health,* 11**,** 1206-1217.

Hayeshi, R., Masimirembwa, C., Mukanganyama, S. & Ungell, A. L. B. (2008) Lysosomal trapping of amodiaquine: impact on transport across intestinal epithelia models. *Biopharmaceutics & Drug Disposition,* 29**,** 324-334.

Haynes, R. K. (2001) Artemisinin and derivatives: the future for malaria treatment? *Current Opinion in Infectious Diseases,* 14**,** 719-726.

Haynes, R. K., Monti, D., Taramelli, D., Basilico, N., Parapini, S. & Olliaro, P. (2003) Artemisinin antimalarials do not inhibit hemozoin formation. *Antimicrobial Agents and Chemotherapy,* 47**,** 1175-1175.

Haynes, R. K., Ho, W. Y., Chan, H. W., Fugmann, B., Stetter, J. R., Croft, S. L., Vivas, L., Peters, W. & Robinson, B. L. (2004) Highly antimalarial-active artemisinin derivatives: biological activity does not correlate with chemical reactivity. *Angewandte Chemie International Edition,* 43**,** 1381-1385.

Haynes, R. K., Fugmann, B., Stetter, J. R., Rieckmann, K., Heilmann, H. D., Chan, H. W., Cheung, M. K., Lam, W. L., Wong, H. N. & Croft, S. L. (2006) Artemisone - a highly active antimalarial drug of the artemisinin class. *Angewandte Chemie,* 118**,** 2136-2142.

Haynes, R. K., Wong, H. N., Lee, K. W., Lung, C. M., Shek, L. Y., Williams, I. D., Croft, S. L., Vivas, L., Rattray, L. & Stewart, L. (2007a) Preparation of *N*-Sulfonyl- and *N*-Carbonyl-11-azaartemisinins with greatly enhanced thermal stabilities: *in vitro* antimalarial activities. *ChemMedChem,* 2**,** 1464-1479.

Haynes, R. K., Chan, W. C., Lung, C. M., Uhlemann, A. C., Eckstein, U., Taramelli, D., Parapini, S., Monti, D. & Krishna, S. (2007b) The Fe^{2+}-mediated decomposition, PfATP6 binding, and antimalarial activities of artemisone and other artemisinins: the unlikelihood of C-centered radicals as bioactive intermediates. *ChemMedChem,* 2**,** 1480-1497.

Haynes, R. K., Chan, W.-C., Wong, H.-N., Li, K.-Y., Wu, W.-K., Fan, K.-M., Sung, H. H. Y., Williams, I. D., Prosperi, D. & Melato, S. (2010) Facile oxidation of leucomethylene blue and dihydroflavins by artemisinins: relationship with flavoenzyme function and antimalarial mechanism of action. *ChemMedChem,* 5**,** 1282-1299.

Haynes, R. K., Cheu, K.-W., Tang, M. M.-K., Chen, M.-J., Guo, Z.-F., Guo, Z.-H., Coghi, P. & Monti, D. (2011) Reactions of antimalarial peroxides with each of leucomethylene blue and dihydroflavins: flavin reductase and the cofactor model exemplified. *ChemMedChem,* 6**,** 279-291.

Haynes, R. K., Cheu, K. W., Chan, H. W., Wong, H. N., Li, K. Y., Tang, M. M. K., Chen, M. J., Guo, Z. F., Guo, Z. H. & Sinniah, K. (2012) Interactions between artemisinins and

other antimalarial drugs in relation to the cofactor model - a unifying proposal for drug action. *ChemMedChem,* 7**,** 2204-2226.

Haynes, R. K., Cheu, K.-W., N'da, D., Coghi, P. & Monti, D. (2013) Considerations on the mechanism of action of artemisinin antimalarials: Part 1-The'carbon radical' and 'heme' hypotheses. *Infectious Disorders-Drug Targets (Formerly Current Drug Targets-Infectious Disorders),* 13**,** 217-277.

Held, J., Supan, C., Salazar, C. L., Tinto, H., Bonkian, L. N., Nahum, A., Moulero, B., Sié, A., Coulibaly, B., Sirima, S. B., Siribie, M., Otsyula, N., Otieno, L., Abdallah, A. M., Kimutai, R., Bouyou-Akotet, M., Kombila, M., Cantalloube, C., Din-Bell, C., Djeriou, E., Waitumbi, J., Mordmuller, B., Ter-Minassian, D., Lell, B. & Kremsner, P. G. (2015) Ferroquine and artesunate in African adults and children with *Plasmodium falciparum* malaria: a phase 2, multicentre, randomised, double-blind, dose-ranging, non-inferiority study. *Lancet,* 15**,** 1409-1419.

Hien, T. T. & White, N. J. (1993) Qinghaosu. *The Lancet,* 341**,** 603-608.

Hinton, R. L., Auwun, A., Pongua, G., Davis, T. M., Karunajeewa, H. A. & Reeder, J. C. (2007) Caregivers' acceptance of using artesunate suppositories for treating childhood malaria in Papua New Guinea. *The American Journal of Tropical Medicine and Hygiene,* 76**,** 634-640.

Howells, R. E., Peters, W. & Fullard, J. (1970) The chemotherapy of rodent malaria, XIII. Fine structural changes observed in the erythrocytic stages of *Plasmodium berghei* following exposure to primaquine and menoctone. *Annals of Tropical Medicine and Parasitology,* 64**,** 203-7.

Hunt, N. H. & Stocker, R. (1989) Oxidative stress and the redox status of malaria-infected erythrocytes. *Blood Cells,* 16**,** 499-526; discussion 527-30.

Hyde, J. E. (2007) Targeting purine and pyrimidine metabolism in human apicomplexan parasites. *Current Drug Targets,* 8**,** 31.

Idowu, O. R., Peggins, J. O. & Brewer, T. G. (1995) Side-chain hydroxylation in the metabolism of 8-aminoquinoline antiparasitic agents. *Drug Metabolism and Disposition,* 23**,** 18-27.

Ilett, K. & Batty, K. (2005) Artemisinin and its derivatives. IN MCKINNON, P. S. & PELOQUIN, C. A. (Eds.) *Antimicrobial Therapy and Vaccines.* Pitsburg, ESun Technologies. 981-1002p.

Ilett, K. F., Batty, K. T., Powell, S. M., Binh, T. Q., Thu, L. T. A., Phuong, H. L., Hung, N. C. & Davis, T. M. E. (2002) The pharmacokinetic properties of intramuscular artesunate and rectal dihydroartemisinin in uncomplicated falciparum malaria. *British Journal of Clinical Pharmacology,* 53**,** 23-30.

Kachur, S. P., Macarthur, J. R. & Slutsker, L. (2010) A call to action: addressing the challenge of artemisinin-resistant malaria. *Expert Review of Anti-Infective Therapy,* 8**,** 365-366.

Kakhlon, O. & Cabantchik, Z. I. (2002) The labile iron pool: characterization, measurement, and participation in cellular processes. *Free Radical Biology and Medicine,* 33**,** 1037-1046.

Kamchonwongpaisan, S., Mckeever, P., Hossler, P., Ziffer, H. & Meshnick, S. R. (1997) Artemisinin neurotoxicity: neuropathology in rats and mechanistic studies *in vitro*. *The American Journal of Tropical Medicine and Hygiene,* 56**,** 7-12.

Karunajeewa, H. A. (2012) Artemisinins: artemisinin, dihydroartemisinin, artemether and artesunate. IN STAINES, H. M. & KRISHNA, S. (Eds.) *Treatment and Prevention of Malaria.* Basel, Springer. 157-190p.

Karunajeewa, H. A., Mueller, I., Senn, M., Lin, E., Law, I., Gomorrai, P. S., Oa, O., Griffin, S., Kotab, K. & Suano, P. (2008) A trial of combination antimalarial therapies in children from Papua New Guinea. *New England Journal of Medicine,* 359**,** 2545-2557.

Karunamoorthi, K. (2011) Vector control: a cornerstone in the malaria elimination campaign. *Clinical Microbiology and Infection,* 17**,** 1608-1616.

Karunaweera, N. D., Grau, G. E., Gamage, P., Carter, R. & Mendis, K. N. (1992) Dynamics of fever and serum levels of tumor necrosis factor are closely associated during clinical paroxysms in *Plasmodium vivax* malaria. *Proceedings of the National Academy of Sciences,* 89**,** 3200-3203.

Kinoshita, A., Nakayama, Y., Kitayama, T. & Tomita, M. (2007) Simulation study of methemoglobin reduction in erythrocytes. *FEBS Journal,* 274**,** 1449-1458.

Kinyanjui, S. M., Mberu, E. K., Winstanley, P. A., Jacobus, D. P. & Watkins, W. M. (1999) The antimalarial triazine WR99210 and the prodrug PS-15: folate reversal of *in vitro* activity against *Plasmodium falciparum* and a non-antifolate mode of action of the prodrug. *The American Journal of Tropical Medicine and Hygiene,* 60**,** 943-947.

Klayman, D. L. (1985) Qinghaosu (artemisinin): an antimalarial drug from China. *Science,* 228**,** 1049-1055.

Krauth-Siegel, R. L., Bauer, H. & Schirmer, R. H. (2005) Dithiol proteins as guardians of the intracellular redox milieu in parasites: old and new drug targets in Trypanosomes and malaria-causing Plasmodia. *Angewandte Chemie International Edition,* 44**,** 690-715.

Kremsner, P. G. & Krishna, S. (2004) Antimalarial combinations. *The Lancet,* 364**,** 285-294.

Krishna, S., Bustamante, L., Haynes, R. K. & Staines, H. M. (2008) Artemisinins: their growing importance in medicine. *Trends in Pharmacological Sciences,* 29**,** 520-527.

Krishna, S. & Staines, H. M. (2012) Non-Antifolate Antibiotics: Clindamycin, Doxycycline, Azithromycin and Fosmidomycin. IN STAINES, H. M. & KRISHNA, S. (Eds.) *Treatment and Prevention of Malaria.* Basel, Springer. 141-156p.

Krishna, S., Woodrow, C. J., Staines, H. M., Haynes, R. K. & Mercereau-Puijalon, O. (2006) Re-evaluation of how artemisinins work in light of emerging evidence of *in vitro* resistance. *Trends in Molecular Medicine,* 12**,** 200-205.

Krungkrai, J., Burat, D., Kudan, S., Krungkrai, S. & Prapunwattana, P. (1999) Mitochondrial oxygen consumption in asexual and sexual blood-stages of the human malarial parasite, *Plasmodium falciparum*. *Southeast Asian Journal of Tropical Medicine and Public Health,* 30**,** 636-642.

Kuehn, A., Simon, N. & Pradel, G. (2010) Family members stick together: multi-protein complexes of malaria parasites. *Medical Microbiology and Immunology,* 199**,** 209-226.

Kumar, S., Guha, M., Choubey, V., Maity, P. & Bandyopadhyay, U. (2007) Antimalarial drugs inhibiting hemozoin (β-hematin) formation: A mechanistic update. *Life Sciences,* 80**,** 813-828.

Kumura, N., Furukawa, H., Onyango, A. N., Izumi, M., Nakajima, S., Ito, H., Hatano, T., Kim, H.-S., Wataya, Y. & Baba, N. (2009) Different behavior of artemisinin and tetraoxane

in the oxidative degradation of phospholipid. *Chemistry and Physics of Lipids,* 160**,** 114-120.

Lanners, H. N. (1991) Effect of the 8-aminoquinoline primaquine on culture-derived gametocytes of the malaria parasite *Plasmodium falciparum*. *Parasitology Research,* 77**,** 478-481.

Lell, B. & Kremsner, P. G. (2002) Clindamycin as an antimalarial drug: review of clinical trials. *Antimicrobial Agents and Chemotherapy,* 46**,** 2315-2320.

Li, G.-Q., Guo, X.-B., Fu, L.-C., Jian, H.-X. & Wang, X.-H. (1994) Clinical trials of artemisinin and its derivatives in the treatment of malaria in China. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 88**,** 5-6.

Li, Q. G., Peggins, J. O., Fleckenstein, L. L., Masonic, K., Heiffer, M. H. & Brewer, T. G. (1998) The pharmacokinetics and bioavailability of dihydroartemisinin, arteether, artemether, artesunic acid and artelinic acid in rats. *Journal of Pharmacy and Pharmacology,* 50**,** 173-182.

Li, Q. G., Brueckner, R. P., Peggins, J. O., Trotman, K. M. & Brewer, T. G. (1999) Arteether toxicokinetics and pharmacokinetics in rats after 25 mg/kg/day single and multiple doses. *European Journal of Drug Metabolism and Pharmacokinetics,* 24**,** 213-223.

Li, J. & Zhou, B. (2010) Biological actions of artemisinin: insights from medicinal chemistry studies. *Molecules,* 15**,** 1378-1397.

Li, W., Mo, W., Shen, D., Sun, L., Wang, J., Lu, S., Gitschier, J. M. & Zhou, B. (2005) Yeast model uncovers dual roles of mitochondria in the action of artemisinin. *PLoS Genetics,* 1**,** e36.

Lin, A. J., Klayman, D. L. & Milhous, W. K. (1987) Antimalarial activity of new water-soluble dihydroartemisinin derivatives. *Journal of Medicinal Chemistry,* 30**,** 2147-2150.

Looareesuwan, S., Ho, M., Wattanagoon, Y., White, N. J., Warrell, D. A., Bunnag, D., Harinasuta, T. & Wyler, D. J. (1987) Dynamic alteration in splenic function during acute *falciparum* malaria. *New England Journal of Medicine,* 317**,** 675-679.

Looareesuwan, S., Oosterhuis, B., Schilizzi, B. M., Sollie, F. A. E., Wilairatana, P., Krudsood, S., Lugt, C. B., Peeters, P. A. M. & Peggins, J. O. (2002) Dose-finding and efficacy study for IM artemotil (beta-arteether) and comparison with IM artemether in

acute uncomplicated *P. falciparum* malaria. *British Journal of Clinical Pharmacology,* 53**,** 492-500.

Luo, X. D. & Shen, C. C. (1987) The chemistry, pharmacology, and clinical applications of qinghaosu (artemisinin) and its derivatives. *Medicinal Research Reviews,* 7**,** 29-52.

Lusha, X. (1979) A new drug for malaria. *China Reconstructs,* 28**,** 48-49.

Luzzatto, L. (2010) The rise and fall of the antimalarial Lapdap: a lesson in pharmacogenetics. *The Lancet,* 376**,** 739-741.

Martin, R. E., Marchetti, R. V., Cowan, A. I., Howitt, S. M., Bröer, S. & Kirk, K. (2009) Chloroquine transport via the malaria parasite's chloroquine resistance transporter. *Science,* 325**,** 1680-1682.

Mcfadden, G. I. (1996) Plastid in human parasites. *Nature,* 381**,** 482.

McIntosh, H. M. & Olliaro, P. (1999) Artemisinin derivatives for treating uncomplicated malaria. *Cochrane Database of Systematic Reviews,* 2.

Ménard, R., Heussler, V., Yuda, M. & Nussenzweig, V. (2008) *Plasmodium* pre-erythrocytic stages: what's new? *Trends in Parasitology,* 24**,** 564-569.

Meng, H., Zhang, R., Yang, H., Fan, Q., Su, X., Miao, J., Cui, L. & Yang, Z. (2010) *In vitro* sensitivity of *Plasmodium falciparum* clinical isolates from the China-Myanmar border area to quinine and association with polymorphism in the Na+/H+ exchanger. *Antimicrobial Agents and Chemotherapy,* 54**,** 4306-4313.

Meshnick, S. R. (2002) Artemisinin: mechanisms of action, resistance and toxicity. *International Journal for Parasitology,* 32**,** 1655-1660.

Meshnick, S. R. (2003) Artemisinin and heme. *Antimicrobial Agents and Chemotherapy,* 47**,** 2712-2713.

Michel, R. (1968) Comparative study of the association of sulfalene and pyrimethamine and of sulfalene alone in mass chemoprophylaxis of malaria. *Médecine Tropicale (Mars),* 28**,** 488.

Mladenka, P. E., Simunek, T. Å., Hübl, M. & Hrdina, R. R. (2006) The role of reactive oxygen and nitrogen species in cellular iron metabolism. *Free Radical Research,* 40**,** 263-272.

Moody, A. (2002) Rapid diagnostic tests for malaria parasites. *Clinical Microbiology Reviews,* 15**,** 66-78.

Moody, A. H. & Chiodini, P. L. (2000) Methods for the detection of blood parasites. *Clinical & Laboratory Haematology,* 22**,** 189-201.

Moonen, B., Cohen, J. M., Snow, R. W., Slutsker, L., Drakeley, C., Smith, D. L., Abeyasinghe, R. R., Rodriguez, M. H., Maharaj, R. & Tanner, M. (2010) Operational strategies to achieve and maintain malaria elimination. *The Lancet,* 376**,** 1592-1603.

Morrissette, N. S. & Sibley, L. D. (2002) Cytoskeleton of apicomplexan parasites. *Microbiology and Molecular Biology Reviews,* 66**,** 21-38.

Murambiwa, P., Masola, B., Govender, T., Mukaratirwa, S. & Musabayane, C. T. (2011) Antimalarial drug formulations and novel delivery systems: a review. *Acta Tropica,* 118**,** 71-79.

Murphy, G. S. & Oldfield, E. C. (1996) *Falciparum* malaria. *Infectious Disease Clinics of North America,* 10**,** 747-775.

Myat Phone, K., Myint, O., Aung, N. & Aye Lwin, H. (1994) The use of primaquine in malaria infected patients with red cell glucose-6-phosphate dehydrogenase (G6PD) deficiency in Myanmar. *Southeast Asian Journal of Tropical Medicine and Public Health,* 25**,** 710-713.

Nagelschmitz, J., Voith, B., Wensing, G., Roemer, A., Fugmann, B., Haynes, R. K., Kotecka, B. M., Rieckmann, K. H. & Edstein, M. D. (2008) First assessment in humans of the safety, tolerability, pharmacokinetics, and *ex vivo* pharmacodynamic antimalarial activity of the new artemisinin derivative artemisone. *Antimicrobial Agents and Chemotherapy,* 52**,** 3085-3091.

Newton, P. N., Fernández, F. M., Plancon, A., Mildenhall, D. C., Green, M. D., Ziyong, L., Christophel, E. M., Phanouvong, S., Howells, S. & McIntosh, E. (2008) A collaborative epidemiological investigation into the criminal fake artesunate trade in South East Asia. *PLoS Med,* 5**,** e32.

Nkrumah, L. J., Riegelhaupt, P. M., Moura, P., Johnson, D. J., Patel, J., Hayton, K., Ferdig, M. T., Wellems, T. E., Akabas, M. H. & Fidock, D. A. (2009) Probing the multifactorial basis of *Plasmodium falciparum* quinine resistance: evidence for a strain-specific

contribution of the sodium-proton exchanger PfNHE. *Molecular and Biochemical Parasitology,* 165**,** 122-131.

Noedl, H., Se, Y., Schaecher, K., Smith, B. L., Socheat, D. & Fukuda, M. M. (2008) Evidence of artemisinin-resistant malaria in western Cambodia. *New England Journal of Medicine,* 359**,** 2619-2620.

Noedl, H., Se, Y., Sriwichai, S., Schaecher, K., Teja-Isavadharm, P., Smith, B., Rutvisuttinunt, W., Bethell, D., Surasri, S. & Fukuda, M. M. (2010) Artemisinin resistance in Cambodia: a clinical trial designed to address an emerging problem in Southeast Asia. *Clinical Infectious Diseases,* 51**,** e82-e89.

Nontprasert, A., Nosten-Bertrand, M., Pukrittayakamee, S., Vanijanonta, S., Angus, B. J. & White, N. J. (1998) Assessment of the neurotoxicity of parenteral artemisinin derivatives in mice. *The American Journal of Tropical Medicine and Hygiene,* 59**,** 519-522.

Nosten, F., Phillips-Howard, P. A. & Ter Kuile, F. O. (2012) Other 4-methanolquinolines, amyl alcohols and phenanthrenes: mefloquine, lumefantrine and halofantrine. IN STAINS, H. M. & KRISHNA, S. (Eds.) *Treatment and Prevention of Malaria.* Basel, Springer. 95-111p.

Nujum, Z. T., Sabarinadh, M. G., Mohan, A. & Praveen, P. S. (2014) A narrative review of use of drugs for prevention of malaria. *International Journal of Preventive and Therapeutic Medicine,* 4, 21-26.

Nzila, A. (2006) The past, present and future of antifolates in the treatment of *Plasmodium falciparum* infection. *Journal of Antimicrobial Chemotherapy,* 57**,** 1043-1054.

Nzila, A. (2012) Antifolates: Pyrimethamine, Proguanil, Sulfadoxine and Dapsone. IN STAINS, H. M. & KRISHNA, S. (Eds.) *Treatment and Prevention of Malaria.* Basel, Springer. 113-125p.

Nzila, A., Ward, S. A., Marsh, K., Sims, P. F. G. & Hyde, J. E. (2005a) Comparative folate metabolism in humans and malaria parasites (part I): pointers for malaria treatment from cancer chemotherapy. *Trends in Parasitology,* 21**,** 292-298.

Nzila, A., Ward, S. A., Marsh, K., Sims, P. F. G. & Hyde, J. E. (2005b) Comparative folate metabolism in humans and malaria parasites (part II): activities as yet untargeted or specific to *Plasmodium*. *Trends in Parasitology,* 21**,** 334-339.

O'Brien, C., Henrich, P. P., Passi, N. & Fidock, D. A. (2011) Recent clinical and molecular insights into emerging artemisinin resistance in *Plasmodium falciparum*. *Current Opinion in Infectious Diseases,* 24**,** 570-585.

O'Neil, M. T., Korsinczky, M. L. J., Gresty, K. J., Auliff, A. & Cheng, Q. (2007) A novel *Plasmodium falciparum* expression system for assessing antifolate resistance caused by mutant *P. vivax* dihydrofolate reductase-thymidylate synthase. *Journal of Infectious Diseases,* 196**,** 467-474.

O'Neill, P. M., Barton, V. E. & Ward, S. A. (2010) The molecular mechanism of action of artemisinin - the debate continues. *Molecules,* 15**,** 1705-1721.

O'Neill, P. M., Barton, V. E., Ward, S. A. & Chadwick, J. (2012) 4-Aminoquinolines: chloroquine, amodiaquine and next-generation analogues. IN STAINS, H. M. & KRISHNA, S. (Eds.) *Treatment and Prevention of Malaria.* Basel, Springer. 19-44p.

Opsenica, D. M. & Šolaja, B. A. (2012) Artemisinins and synthetic peroxides as highly efficient antimalarials. *Macedonian Journal of Chemistry and Chemical Engineering,* 31**,** 137-182.

Osman, M. E., Mockenhaupt, F. P., Bienzle, U., Elbashir, M. I. & Giha, H. A. (2007) Field-based evidence for linkage of mutations associated with chloroquine (pfcrt/pfmdr1) and sulfadoxine-pyrimethamine (pfdhfr/pfdhps) resistance and for the fitness cost of multiple mutations in *P. falciparum*. *Infection, Genetics and Evolution,* 7**,** 52-59.

Painter, H. J., Morrisey, J. M., Mather, M. W. & Vaidya, A. B. (2007) Specific role of mitochondrial electron transport in blood-stage *Plasmodium falciparum*. *Nature,* 446**,** 88-91.

Peters, W. (1987) Experimental resistance IV: dihydrofolate reductase inhibitors and drugs with related activities. IN PETERS, W. (Ed.) *Chemotherapy and Drug Resistance in Malaria.* London, Academic Press Ltd. 481-521p.

Peters, W., Ellis, D., Boulard, Y. & Landau, I. (1984) The chemotherapy of rodent malaria. Part IV. The activity of a new 8-aminoquinoline, WR 225,448 against exo-erythrocytic schizonts of *Plasmodium yoelii Annals of Tropical Medicine and Parasitology,* 78**,** 467-478.

Petersen, I., Eastman, R. & Lanzer, M. (2011) Drug-resistant malaria: molecular mechanisms and implications for public health. *FEBS Letters,* 585**,** 1551-1562.

Peterson, D. S., Walliker, D. & Wellems, T. E. (1988) Evidence that a point mutation in dihydrofolate reductase-thymidylate synthase confers resistance to pyrimethamine in *falciparum* malaria. *Proceedings of the National Academy of Sciences,* 85**,** 9114-9118.

Peterson, D. S., Milhous, W. K. & Wellems, T. E. (1990) Molecular basis of differential resistance to cycloguanil and pyrimethamine in *Plasmodium falciparum* malaria. *Proceedings of the National Academy of Sciences,* 87**,** 3018-3022.

Petrat, F., Paluch, S., Dogruöz, E., Dörfler, P., Kirsch, M., Korth, H.-G., Sustmann, R. & De Groot, H. (2003) Reduction of Fe (III) ions complexed to physiological ligands by lipoyl dehydrogenase and other flavoenzymes *in vitro*. Implications for an enzymatic reduction of Fe(III) ions of the labile iron pool. *Journal of Biological Chemistry,* 278**,** 46403-46413.

Pierre, J. L., Fontecave, M. & Crichton, R. R. (2002) Chemistry for an essential biological process: the reduction of ferric iron. *Biometals,* 15**,** 341-346.

Plebanski, M. & Hill, A. V. S. (2000) The immunology of malaria infection. *Current Opinion in Immunology,* 12**,** 437-441.

Ploypradith, P. (2004) Development of artemisinin and its structurally simplified trioxane derivatives as antimalarial drugs. *Acta Tropica,* 89**,** 329-342.

Pongponratn, E., Turner, G. D. H., Day, N. P. J., Phu, N. H., Simpson, J. A., Stepniewska, K., Mai, N. T. H., Viriyavejakul, P., Looareesuwan, S. & Hien, T. T. (2003) An ultrastructural study of the brain in fatal *Plasmodium falciparum* malaria. *The American Journal of Tropical Medicine and Hygiene,* 69**,** 345-359.

Price, R. N., Uhlemann, A.-C., Brockman, A., McGready, R., Ashley, E., Phaipun, L., Patel, R., Laing, K., Looareesuwan, S. & White, N. J. (2004) Mefloquine resistance in *Plasmodium falciparum* and increased pfmdr1 gene copy number. *The Lancet,* 364**,** 438-447.

Price, R. N., Uhlemann, A.-C., Van Vugt, M., Brockman, A., Hutagalung, R., Nair, S., Nash, D., Singhasivanon, P., Anderson, T. J. C. & Krishna, S. (2006) Molecular and pharmacological determinants of the therapeutic response to artemether-lumefantrine in multidrug-resistant *Plasmodium falciparum* malaria. *Clinical Infectious Diseases,* 42**,** 1570-1577.

Price, R. N., Hasugian, A. R., Ratcliff, A., Siswantoro, H., Purba, H. L. E., Kenangalem, E., Lindegardh, N., Penttinen, P., Laihad, F. & Ebsworth, E. P. (2007) Clinical and pharmacological determinants of the therapeutic response to dihydroartemisinin-piperaquine for drug-resistant malaria. *Antimicrobial Agents and Chemotherapy,* 51**,** 4090-4097.

Protopopoff, N., Van Bortel, W., Speybroeck, N., Van Geertruyden, J.-P., Baza, D., D'alessandro, U. & Coosemans, M. (2009) Ranking malaria risk factors to guide malaria control efforts in African highlands. *PLoS One,* 4**,** e8022.

Rathod, P. K., Mcerlean, T. & Lee, P.-C. (1997) Variations in frequencies of drug resistance in *Plasmodium falciparum*. *Proceedings of the National Academy of Sciences,* 94**,** 9389-9393.

Reed, M. B., Saliba, K. J., Caruana, S. R., Kirk, K. & Cowman, A. F. (2000) Pgh1 modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum*. *Nature,* 403**,** 906-909.

Rehwagen, C. (2006) WHO ultimatum on artemisinin monotherapy is showing results. *The British Medical Journal,* 332**,** 1176.

Reusser, F. (1975) Effect of lincomycin and clindamycin on peptide chain initiation. *Antimicrobial Agents and Chemotherapy,* 7**,** 32-37.

Rohrbach, P., Sanchez, C. P., Hayton, K., Friedrich, O., Patel, J., Sidhu, A. B. S., Ferdig, M. T., Fidock, D. A. & Lanzer, M. (2006) Genetic linkage of pfmdr1 with food vacuolar solute import in *Plasmodium falciparum*. *The EMBO Journal,* 25**,** 3000-3011.

Roos, D. S., Crawford, M. J., Donald, R. G. K., Kissinger, J. C., Klimczak, L. J. & Striepen, B. (1999) Origin, targeting, and function of the apicomplexan plastid. *Current Opinion in Microbiology,* 2**,** 426-432.

Roper, C., Pearce, R., Nair, S., Sharp, B., Nosten, F. O. & Anderson, T. (2004) Intercontinental spread of pyrimethamine-resistant malaria. *Science,* 305**,** 1124-1124.

Ross, R. (1897) On some peculiar pigmented cells found in two mosquitos fed on malarial blood. *British Medical Journal,* 2**,** 1786.

Rotmann, A. (1975) Genetics of a primaquine-resistant yeast. *Journal of General Microbiology,* 89**,** 1-10.

Rowe, A. (2013) Malaria parasite adhesion. Edinburgh, The University of Edinburgh.

Sá, J. M., Twu, O., Hayton, K., Reyes, S., Fay, M. P., Ringwald, P. & Wellems, T. E. (2009) Geographic patterns of *Plasmodium falciparum* drug resistance distinguished by differential responses to amodiaquine and chloroquine. *Proceedings of the National Academy of Sciences,* 106**,** 18883-18889.

Same-Ekobo, A., Lohoue, J., Essono, E., Ravinet, L. & Ducret, J. P. (1998) Rapid resolution of *Plasmodium ovale* malarial attacks using artesunate (Arsumax). *Medecine Tropicale: Revue du Corps de Sante Colonial,* 59**,** 43-45.

Sanchez, C. P., Mclean, J. E., Stein, W. & Lanzer, M. (2004) Evidence for a substrate specific and inhibitable drug efflux system in chloroquine resistant *Plasmodium falciparum* strains. *Biochemistry,* 43**,** 16365-16373.

Sanchez, C. P., Rotmann, A., Stein, W. D. & Lanzer, M. (2008) Polymorphisms within PfMDR1 alter the substrate specificity for antimalarial drugs in *Plasmodium falciparum*. *Molecular Microbiology,* 70**,** 786-798.

Sarma, G. N., Savvides, S. N., Becker, K., Schirmer, M., Schirmer, R. H. & Karplus, P. A. (2003) Glutathione reductase of the malarial parasite *Plasmodium falciparum*: crystal structure and inhibitor development. *Journal of Molecular Biology,* 328**,** 893-907.

Schirmer, R. H., Coulibaly, B., Stich, A., Scheiwein, M., Merkle, H., Eubel, J., Becker, K., Becher, H., Müller, O. & Zich, T. (2003) Methylene blue as an antimalarial agent. *Redox Report,* 8**,** 272-275.

Schlagenhauf, P., Adamcova, M., Regep, L., Schaerer, M. T., Bansod, S. & Rhein, H.-G. (2011) Use of mefloquine in children-a review of dosage, pharmacokinetics and tolerability data. *Malaria Journal,* 10**,** 292.

Schlagenhauf, P., Blumentals, W. A., Suter, P., Regep, L., Vital-Durand, G., Schaerer, M. T., Boutros, M. S., Rhein, H.-G. & Adamcova, M. (2012) Pregnancy and fetal outcomes after exposure to mefloquine in the pre-and periconception period and during pregnancy. *Clinical Infectious Diseases,* 54**,** e124-e131.

Scholl, P. F., Tripathi, A. K. & Sullivan, D. J. (2005) Bioavailable iron and heme metabolism in *Plasmodium falciparum*. *Malaria: Drugs, Disease and Post-Genomic Biology.* Springer.

Shamiss, A., Atar, E., Zohar, L. & Cain, Y. (1996) Mefloquine versus doxycycline for malaria prophylaxis in intermittent exposure of Israeli Air Force aircrew in Rwanda. *Aviation, Space, and Environmental Medicine,* 67**,** 872-873.

Sibley, C. H., Hyde, J. E., Sims, P. F. G., Plowe, C. V., Kublin, J. G., Mberu, E. K., Cowman, A. F., Winstanley, P. A., Watkins, W. M. & Nzila, A. M. (2001) Pyrimethamine-sulfadoxine resistance in *Plasmodium falciparum*: what next? *Trends in Parasitology,* 17**,** 570-571.

Sidhu, A. B. S., Verdier-Pinard, D. & Fidock, D. A. (2002) Chloroquine resistance in *Plasmodium falciparum* malaria parasites conferred by pfcrt mutations. *Science,* 298**,** 210-213.

Sidhu, A. B. S., Uhlemann, A.-C., Valderramos, S. G., Valderramos, J.-C., Krishna, S. & Fidock, D. A. (2006) Decreasing pfmdr1 copy number in *Plasmodium falciparum* malaria heightens susceptibility to mefloquine, lumefantrine, halofantrine, quinine, and artemisinin. *Journal of Infectious Diseases,* 194**,** 528-535.

Silamut, K., Newton, P. N., Teja-Isavadharm, P., Suputtamongkol, Y., Siriyanonda, D., Rasameesoraj, M., Pukrittayakamee, S. & White, N. J. (2003) Artemether bioavailability after oral or intramuscular administration in uncomplicated *falciparum* malaria. *Antimicrobial Agents and Chemotherapy,* 47**,** 3795-3798.

Simpson, J. A., Aarons, L., Collins, W. E., Jeffery, G. M. & White, N. J. (2002) Population dynamics of untreated *Plasmodium falciparum* malaria within the adult human host during the expansion phase of the infection. *Parasitology,* 124**,** 247-263.

Sinclair, D., Zani, B., Donegan, S., Olliaro, P. & Garner, P. (2009) Artemisinin-based combination therapy for treating uncomplicated malaria (Review). *Cochrane Database of Systematic Reviews***,** CD007483.

Sinka, M. E., Bangs, M. J., Manguin, S., Rubio-Palis, Y., Chareonviriyaphap, T., Coetzee, M., Mbogo, C. M., Hemingway, J., Patil, A. P. & Temperley, W. H. (2012) A global map of dominant malaria vectors. *Parasite Vectors,* 5**,** 69.

Srivastava, I. K., Rottenberg, H. & Vaidya, A. B. (1997) Atovaquone, a broad spectrum antiparasitic drug, collapses mitochondrial membrane potential in a malarial parasite. *Journal of Biological Chemistry,* 272**,** 3961-3966.

Stepniewska, K., Ashley, E., Lee, S. J., Anstey, N., Barnes, K. I., Binh, T. Q., D'alessandro, U., Day, N. P. J., De Vries, P. J. & Dorsey, G. (2010) *In vivo* parasitological measures of artemisinin susceptibility. *Journal of Infectious Diseases,* 201**,** 570-579.

Stepniewska, K. & White, N. J. (2008) Pharmacokinetic determinants of the window of selection for antimalarial drug resistance. *Antimicrobial Agents and Chemotherapy,* 52**,** 1589-1596.

Stocks, P. A., Bray, P. G., Barton, V. E., Al-Helal, M., Jones, M., Araujo, N. C., Gibbons, P., Ward, S. A., Hughes, R. H. & Biagini, G. A. (2007) Evidence for a common non-heme chelatable-iron-dependent activation mechanism for semisynthetic and synthetic endoperoxide antimalarial drugs. *Angewandte Chemie International Edition,* 46**,** 6278-6283.

Suh, K. N., Kain, K. C., Jay, S. & Keystone, J. S. (2004) Malaria. *Canadian Medical Association Journal,* 170**,** 1693-1702.

Suwanarusk, R., Cooke, B. M., Dondorp, A. M., Silamut, K., Sattabongkot, J., White, N. J. & Udomsangpetch, R. (2004) The deformability of red blood cells parasitized by *Plasmodium falciparum* and *P. vivax*. *Journal of Infectious Diseases,* 189**,** 190-194.

Svensson, U. S. H. & Ashton, M. (1999) Identification of the human cytochrome P450 enzymes involved in the *in vitro* metabolism of artemisinin. *British Journal of Clinical Pharmacology,* 48**,** 528-535.

Tan, K. R., Magill, A. J., Parise, M. E. & Arguin, P. M. (2011) Doxycycline for malaria chemoprophylaxis and treatment: report from the CDC expert meeting on malaria chemoprophylaxis. *The American Journal of Tropical Medicine and Hygiene,* 84**,** 517-531.

Teja-Isavadharm, P., Nosten, F., Kyle, D. E., Luxemburger, C., Kuile, F. T., Peggins, J. O., Brewer, T. G. & White, N. J. (1996) Comparative bioavailability of oral, rectal, and intramuscular artemether in healthy subjects: use of simultaneous measurement by high performance liquid chromatography and bioassay. *British Journal of Clinical Pharmacology,* 42**,** 599-604.

Ter Kuile, F. O., Nosten, F., Chongsuphajaisiddhi, T., White, N. J., Dolan, G., Luxemburger, C., Phaipun, L., Edstein, M. D. & Webster, H. K. (1993) Halofantrine versus mefloquine in treatment of multidrug-resistant *falciparum* malaria. *The Lancet,* 341**,** 1044-1049.

Teuscher, F., Gatton, M. L., Chen, N., Peters, J., Kyle, D. E. & Cheng, Q. (2010) Artemisinin-induced dormancy in *Plasmodium falciparum*: duration, recovery rates, and implications in treatment failure. *Journal of Infectious Diseases,* 202**,** 1362-1368.

Teuscher, F., Chen, N., Kyle, D. E., Gatton, M. L. & Cheng, Q. (2011) Phenotopic changes in artemisinn-resistant *Plasmodium falciparum* line *in vitro*: Evidence for decreased sensitivity to dormancy and growth inhibition. *Antimicrobial Agents and Chemotherapy,* 12**,** 428-431.

Torok, D. S. & Ziffer, H. (1995) Synthesis and reactions of 11-azaartemisinin and derivatives. *Tetrahedron Letters,* 36**,** 829-832.

Trampuz, A., Jereb, M., Muzlovic, I. & Prabhu, R. M. (2003) Clinical review: Severe malaria. *Critical Care-London-,* 7**,** 315-323.

Udomsangpetch, R., Pipitaporn, B., Krishna, S., Angus, B., Pukrittayakamee, S., Bates, I., Suputtamongkol, Y., Kyle, D. E. & White, N. J. (1996) Antimalarial drugs reduce cytoadherence and rosetting of *Plasmodium falciparum*. *Journal of Infectious Diseases,* 173**,** 691-698.

Vaidya, A. B. & Mather, M. W. (2009) Mitochondrial evolution and functions in malaria parasites. *Annual Review of Microbiology,* 63**,** 249-267.

Vale, N., Moreira, R. & Gomes, P. (2009) Primaquine revisited six decades after its discovery. *European Journal of Medicinal Chemistry,* 44**,** 937-953.

Van Hensbroek, M. L. B., Morris-Jones, S., Meisner, S., Jaffar, S., Bayo, L., Dackour, R., Phillips, C. & Greenwood, B. M. (1995) Iron, but not folic acid, combined with effective antimalarial therapy promotes haematological recovery in African children after acute *falciparum* malaria. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 89**,** 672-676.

Vennerstrom, J. L. & Eaton, J. W. (1988) Oxidants, oxidant drugs, and malaria. *Journal of Medicinal Chemistry,* 31**,** 1269-1277.

Vijaykadga, S., Rojanawatsirivej, C., Cholpol, S., Phoungmanee, D., Nakavej, A. & Wongsrichanalai, C. (2006) *In vivo* sensitivity monitoring of mefloquine monotherapy and artesunate-mefloquine combinations for the treatment of uncomplicated *falciparum* malaria in Thailand in 2003. *Tropical Medicine & International Health,* 11**,** 211-219.

Vivas, L., Rattray, L., Stewart, L. B., Robinson, B. L., Fugmann, B., Haynes, R. K., Peters, W. & Croft, S. L. (2007) Antimalarial efficacy and drug interactions of the novel semisynthetic endoperoxide artemisone *in vitro* and *in vivo*. *Journal of Antimicrobial Chemotherapy,* 59**,** 658-665.

Wang, J., Huang, L., Li, J., Fan, Q., Long, Y., Li, Y. & Zhou, B. (2010) Artemisinin directly targets malarial mitochondria through its specific mitochondrial activation. *PLoS One,* 5**,** e:9582.

Wang, P., Sims, P. F. G. & Hyde, J. E. (1997) A modified *in vitro* sulfadoxine susceptibility assay for *Plasmodium falciparum* suitable for investigating Fansidar resistance. *Parasitology,* 115**,** 223-230.

Watkins, W. M., Sixsmith, D. G., Chulay, J. D. & Spencer, H. C. (1985) Antagonism of sulfadoxine and pyrimethamine antimalarial activity *in vitro* by *p*-aminobenzoic acid, *p*-aminobenzoylglutamic acid and folic acid. *Molecular and Biochemical Parasitology,* 14**,** 55-61.

Watkins, W. M. & Mosobo, M. (1993) Treatment of *Plasmodium falciparum* malaria with pyrimethamine-sulfadoxine: selective pressure for resistance is a function of long elimination half-life. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 87**,** 75-78.

Watkins, W. M., Mberu, E. K., Winstanley, P. A. & Plowe, C. V. (1997) The efficacy of antifolate antimalarial combinations in Africa: a predictive model based on pharmacodynamic and pharmacokinetic analyses. *Parasitology Today,* 13**,** 459-464.

Wellems, T. E. & Plowe, C. V. (2001) Chloroquine-resistant malaria. *Journal of Infectious Diseases,* 184**,** 770-776.

Wells, T. N. C., Burrows, J. N. & Baird, J. K. (2010) Targeting the hypnozoite reservoir of *Plasmodium vivax*: the hidden obstacle to malaria elimination. *Trends in Parasitology,* 26**,** 145-151.

Wernsdorfer, W. H. (2012) Global challenges of changing epidemiological patterns of malaria. *Acta Tropica,* 121**,** 158-165.

Wesche, D. L., Decoster, M. A., Tortella, F. C. & Brewer, T. G. (1994) Neurotoxicity of artemisinin analogs *in vitro*. *Antimicrobial Agents and Chemotherapy,* 38**,** 1813-1819.

White, N. J. (1994) Clinical pharmacokinetics and pharmacodynamics of artemisinin and derivatives. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 88**,** 41-43.

White, N. J. (1997) Assessment of the pharmacodynamic properties of antimalarial drugs *in vivo*. *Antimicrobial Agents and Chemotherapy,* 41**,** 1413.

White, N. J. (1999a) Antimalarial drug resistance and combination chemotherapy. *Philosophical Transactions of the Royal Society of London B: Biological Sciences,* 354**,** 739-749.

White, N. J., Nosten, F. O., Looareesuwan, S., Watkins, W. M., Marsh, K., Snow, R. W., Kokwaro, G., Ouma, J., Hien, T. T. & Molyneux, M. E. (1999b) Averting a malaria disaster. *The Lancet,* 353**,** 1965-1967.

White, N. J. (2004) Antimalarial drug resistance. *Journal of Clinical Investigation,* 113**,** 1084.

White, N. J. (2008) Malaria. IN MANSON, G. C. & ZUMLA, A. I. (Eds.) *Manson's Tropical Diseases.* 22 ed. Philadelphia, W.B. Saunders Company. 1201-1297p.

White, N. J., Pukrittayakamee, S., Hien, T. T., Faiz, M. A., Mokuolu, O. A. & Dondorp, A. M. (2014) Malaria. *The Lancet,* 383**,** 723-735.

Who (2001) Roll Back Malaria: Antimalarial drug combination therapy. Report of a technical consultation. Geneva, World Health Organization. 4-32p.

Who (2006) Global Malaria Programme: Guidelines for the treatment of malaria. Geneva, World Health Organization. 3-33p.

Who (2007) Report of a WHO Informal Consultation: Use of rectal artemisinin based suppositories in the management of severe malaria. Geneva, World Health Organization. 26-27p.

Who (2010a) Roll Back Malaria. World malaria day: Africa update. Progress & Impact Series Geneva, World Health Organization. 29-43p.

Who (2010b) Guidelines for the treatment of malaria, Second edition. Geneva, World Health Organization. 248p.

Who (2011) Malaria rapid diagnostic test performance — results of WHO product testing of malaria RDTs: round 3 (2010–2011). Geneva, World Health Organization. 133p.

Who (2012) Global plan for insecticide resistance management in malaria vectors. Geneva, World Health Organization. 176p.

Who (2015a) Global Malaria Programme: World Malaria Report. Geneva, World Health Organization. 8-17p.

Who (2015b) Malaria Vaccine Development: Summary of the October 2015 meeting of the Strategic Advisory Group of Experts on immunization (SAGE). Geneva, Switzerland, World Health Organization. 1-2p.

Willcox, M., Bodeker, G., Bourdy, G. V., Dhingra, V., Falquet, J., Ferreira, J. F. S., Graz, B., Hirt, H.-M. & Hsu, E. (2004) Artemisia annua as a traditional herbal antimalarial. IN WILLCOX, M., BODEKER, G. & RAOANAIVO, P. (Eds.) *Traditional Medicinal Plants and Malaria.* Boca Raton. 43-59p., CRC.

Winstanley, P., Watkins, W., Muhia, D., Szwandt, S., Amukoye, E. & Marsh, K. (1997) Chlorproguanil/dapsone for uncomplicated *Plasmodium falciparum* malaria in young children: pharmacokinetics and therapeutic range. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 91**,** 322-327.

Wongsrichanalai, C., Pickard, A. L., Wernsdorfer, W. H. & Meshnick, S. R. (2002) Epidemiology of drug-resistant malaria. *The Lancet Infectious Diseases,* 2**,** 209-218.

Woodmansee, A. N. & Imlay, J. A. (2002) Reduced flavins promote oxidative DNA damage in non-respiring *Escherichia coli* by delivering electrons to intracellular free iron. *Journal of Biological Chemistry,* 277**,** 34055-34066.

Woodrow, C. J., Haynes, R. K. & Krishna, S. (2005) Artemisinins. *Postgraduate Medical Journal,* 81**,** 71-78.

Woodrow, C. J. & Bustamante, L. Y. (2011) Mechanisms of artemisinin action and resistance: wider focus is needed. *Proceedings of the Natural Academy of Sciences, USA,* 94**,** 10931-10936.

Wootton, J. C., Feng, X., Ferdig, M. T., Cooper, R. A., Mu, J., Baruch, D. I., Magill, A. J. & Su, X.-Z. (2002) Genetic diversity and chloroquine selective sweeps in *Plasmodium falciparum*. *Nature,* 418**,** 320-323.

Yang, Q. C., Shi, W. Z., Li, R. & Gan, J. (1982) The antimalarial and toxic effect of artesunate on animal models. *Journal of Traditional Chinese Medicine = Chung i Tsa*

Chih Ying Wen Pan/sponsored by All-China Association of Traditional Chinese Medicine, Academy of Traditional Chinese Medicine, 2**,** 99.

Zhang, J., Krugliak, M. & Ginsburg, H. (1999) The fate of ferriprotorphyrin IX in malaria infected erythrocytes in conjunction with the mode of action of antimalarial drugs. *Molecular and Biochemical Parasitology,* 99**,** 129-141.

Zuccala, E. S. & Baum, J. (2011) Cytoskeletal and membrane remodelling during malaria parasite invasion of the human erythrocyte. *British Journal of Haematology,* 154**,** 680-689.

CHAPTER 3
The case for development of 11-azaartemisinins for malaria – Article 1

Chapter 3 contains the manuscript of an article submitted to Current Medicinal Chemistry. The article contains the background, aim, experimental details and *in vitro* biological results of synthesized compounds of this study. This article is prepared according to the author's guidelines available in the author information pack at the journal homepage: http://benthamscience.com/journals/current-medicinal-chemistry/author-guidelines/

The case for development of 11-azaartemisinins against malaria

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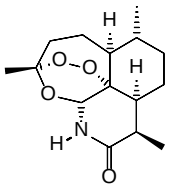
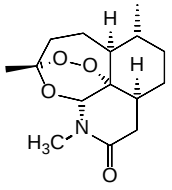
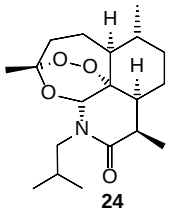
A critical overview of 11-azaartemisinin and its functionalized derivatives that has been synthesized over the past 20 years are examined and discussed with emphasis placed on the compounds synthetic accessibility as well as biological activities. Ultimately, to evaluate the suitability of the compounds to serve as new derivatives for treatment of malaria.



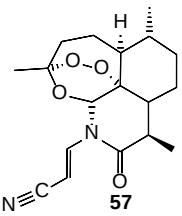
7
IC_{50} nM:
Pf. W2: 6.14
Pf. D6: 9.24



9
IC_{50} nM relative to artemisinin **1**:
Pf. W2: 5
Pf. D6: 2.13



24
IC_{50} nM relative to artemisinin **1**:
Pf. FCR3: 9



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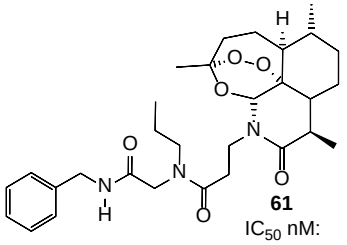
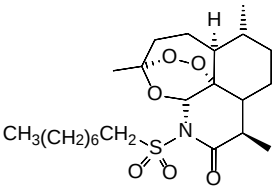
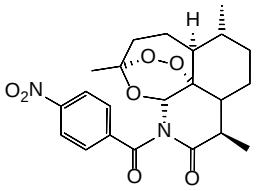
IC_{50} nM relative to artemisinin **1**:
Pf. W2: 1.7
Pf. D6: 2.6



61
IC_{50} nM:
Pf. FcB1: 0.3



67
IC_{50} nM:
Pf. 3D7: 2.4
Pf. K1: 4.6



74
IC_{50} nM:
Pf. 3D7: 1.0
Pf. K1: 1.4

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The Case for Development of 11-Azaartemisinins for Malaria

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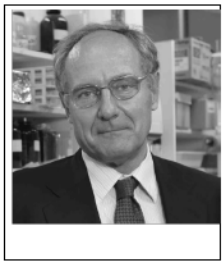

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Abstract: The current treatment regimens for uncomplicated malaria comprise an artemisinin in combination with another drug (ACT). However, the recent emergence of resistance to ACTs in South East Asia dramatically emphasizes the need for new artemisinins. The current artemisinins have been in use since the late 1970s and have relatively poor thermal, chemical and metabolic stabilities - all are metabolized or hydrolyzed *in vivo* to dihydroartemisinin (DHA) that itself undergoes facile decomposition *in vivo*. The current artemisinins possess neurotoxicity as demonstrated in animal models, an issue that mandates increased vigilance in view of trends to use of protracted treatment regimens involving sequential administration of different ACTs against the resistant disease. As artemisinins induce the most rapid reduction in parasitaemia of any drug, common sense dictates that any *new* artemisinin derivative, selected on the bases of more robust chemical and thermal stability, metabolic stability with respect to the generation of DHA *in vivo*, and relatively benign neurotoxicity should be used in any new ACT whose components are *rationally* chosen in order to counter resistant malaria and inhibit transmission. 11-Azaartemisinin and its *N*-substituted derivatives attract because of overall ease of preparation from artemisinin. Some derivatives also possess notable thermal stabilities and although metabolic pathways of the derivatives are as yet unknown, none can provide DHA. The azaartemisinins synthesized over the past 20 years are critically discussed on the basis of their synthetic accessibility and biological activities with the view to assessing suitability to serve as new artemisinin derivatives for treatment of malaria.

Keywords: Artemisinin, azaartemisinin, dihydroartemisinin, malaria, metabolism, resistance, stability.

1. INTRODUCTION

Malaria due to infection largely by *Plasmodium falciparum* and *P. vivax* is a serious health problem that creates a tremendous burden of morbidity and mortality. Globally, an estimated 3.2 billion people are at risk of being infected with the malaria parasite and developing the disease. According to the most recent estimates, 198 million cases of malaria occurred globally in 2013 and there were 584,000 deaths. The burden is heaviest in Africa, where an estimated 90% of all malaria deaths occur, with the majority (78%) of such deaths occurring in children under 5 years of age [1]. Following on from the global threat posed by the emergence of chloroquine (CQ)-resistant malaria in the 1960s, new chemotherapeutic agents based on artemisinin and its derivatives (Fig. **1**), collectively known as artemisinins, were first introduced in the late 1970s and early 1980s in China and used principally for treatment of CQ resistant falciparum malaria. The source of artemisinin is *Artemisia annua*, a plant known in China as qing hao whose use for treatment of fevers and chills in Chinese folk medicine dates back over 2000 years [2,3]. Artemisinin is a highly crystalline compound that is essentially insoluble in both water and oil, and is thus not easily formulated. Indeed artemisinin itself was first administered to malaria patients via the naso-gastric route. In order to acquire derivatives that were more easily formulated, the Chinese reduced artemisinin **1** to dihydroartemisinin **2** (DHA) that was converted into artemether **3**, arteether **4**, and artesunate **5** and other derivatives [4]. As artemether is soluble in sesame or peanut oil, it was

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0929-8673/15 $58.00+.00

able to be administered via intramuscular injection. Artesunate is relatively soluble in water at pH 7 and this property allows for intravenous administration or intramuscular injection; however, it is so unstable at pH 7 that such formulations need to be prepared immediately prior to administration. Although arteether **4** was not further examined in China, its development was actively pursued in the West on the basis of fallacies relating to the presence of the ethyl group vis-à-vis that of the methyl group in artemether **3** [5].

It was established early on that monotherapy with artemisinins resulted in recrudescence, that is, re-emergence of parasitaemia in the absence of external infection [6]. Thus, protracted treatment regimens (~7 days) had to be used. The phenomenon arises as a consequence of the short half-lives of artemisinins and poor bioavailability when administered for non-severe malaria. It is now known that artemether **3** and artesunate **5** are converted primarily into DHA **2** after administration. Human plasma concentrations reach a maximum usually within 1-3 hours based on the DHA formed from these drugs [7, 8]. The facile metabolism of artemether to DHA is reflected in the detection of the latter compound in subjects administered with artemether **3** [9]**.** Artesunate **5** is a prodrug for DHA; the latter has a half-life of 40-60 minutes that is similar to artemether **3**. Notably, DHA is labile under aqueous conditions at pH 7.4, wherein it rearranges completely and irreversibly into the peroxyhemiacetal **6** (Fig. **1**) [10]. The compound also forms from arteether **4** under conditions that simulate stomach acidity [11] or from DHA by heating [12]. It also has been detected as a circulating metabolite in patients treated with artesunate-mefloquine [13]. Analysis of patient plasma samples collected between 1-4 hours after drug administration indicated the presence of DHA and the peroxyhemiacetal (approximately 20-40% of DHA concentration). Overall, the problems of obtaining consistent biological assays of DHA that arise through its evidently facile decomposition in biological fluids have been highlighted [14]. Neurotoxicity of the current clinical artemisinins was demonstrated in extensive investigations. Artemisinins, especially DHA, elicit neurotoxicity in cell and animal assays [15]. The neurotoxicity of DHA reaches the level of activity that the drug displays *in vitro* against *P. falciparum*. However, as indicated below, the resistance of the malaria parasite to artemisinin therapy now necessitates administration of more protracted treatment regimens. This absolutely mandates renewed vigilance of the neurotoxicity [16].

It was recognized early in the development of artemisinins as antimalarial drugs that because of the low half-life of artemisinin, combination with a longer half-life drug would provide substantial benefit, and additionally suppress resistance emerging to either drug in the combination [6]. Thus, in a classic publication, Arnold, and Li and co-workers at the Guangzhou College of Traditional Chinese Medicine revealed the conceptualization and application of a combination of artemisinin with mefloquine and other drugs for treatment of malaria [17]. The concept was adopted later by others [3], leading eventually to the recommendation by the World Health Organization (WHO) that such combinations must be used for treatment of malaria [18]. The essence of the rationale behind the use of such artemisinin combination therapies (ACTs) is that the fast acting artemisinin derivative rapidly clears a large proportion of the parasites within its short pharmacological half-life, whilst the longer half-life partner drug then continues the clearance as the artemisinin concentration falls to sub-therapeutic levels [17]. As is the case with oral administration, artemisinins also display good activities when administered via the parenteral route or via intramuscular injection. Thus, even though artesunate rapidly decomposes to DHA at neutral pH, it is currently the drug of choice for intramuscular treatment of cerebral/complicated malaria [19]. The proviso is that because of the instability of artesunate in aqueous media with respect to formation of DHA, the dual pack formulation comprising solid artesunate and saline aqueous bicarbonate must be mixed to provide a solution that then has to be injected immediately. Nevertheless, the administration of artesunate in this fashion has raised concerns about delayed haemolysis [20].

Even though ACTs were originally prescribed on the basis of inhibiting development of resistance, it is starkly apparent that development of resistance is now taking place. Greatly increased parasite clearance times in patients treated with ACTs have been reported from Cambodia and other countries in South East Asia [21] and genetic markers of the resistant phenotype have been identified [22]. The phenomenon has necessitated at the least a twofold increase in the artemisinin loading in ACT treatment regimens so as to prolong exposure of blood-stage parasites to the artemisinin [23]. Such prescription must mandate enhanced pharmacovigilance in relation to the established neurotoxicity of the current clinical artemisinins. Irrespective of this, the historical precedent provided by the demise of CQ may well be

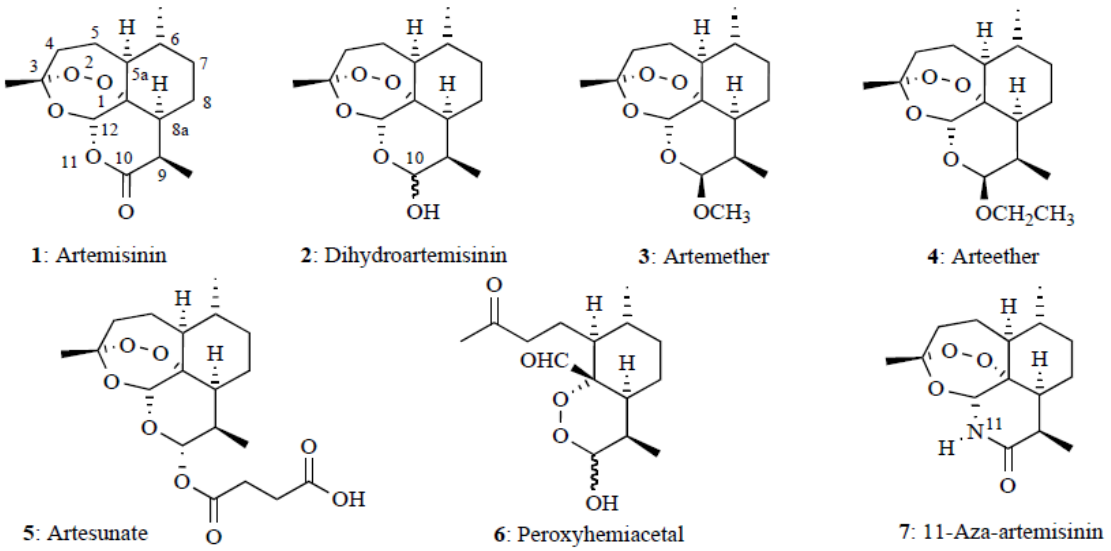


Fig. (1). Structures of artemisinin **1** and clinically used derivatives dihydroartemisinin (DHA) **2**, artemether **3** and artesunate **5** developed in China in the 1970s and 1980s. Arteether **4** was first prepared in China, but development was subsequently pursued in the West based on reasoning purporting to find benefit in its relatively greater lipophilicity with respect to artemether and presumed metabolism of the ethyl group to ethanol. DHA **2** is converted relatively rapidly into the rearranged peroxide **6** under neutral conditions, a compound that is also detected in patients treated with artesunate. 11-Aza-artemisinin **7** is formally derived from artemisinin by replacement of the lactone oxygen atom (O-11) by nitrogen to generate a lactam, an entity that is chemically more robust than the lactone in artemisinin.

repeated in the case of artemisinins. It would seem that the single minded perseverance with the current clinical artemisinin derivatives, all in use since the late 1970s and early 1980s, have led to the current situation. As it is the derivatives artemether **3** and artesunate **5** that decompose rapidly to DHA **2** and DHA itself that are used as the artemisinin component in ACTs, we are dealing with predominant exposure of the malaria parasite *in vivo* to DHA in all cases. As pointed out above, DHA is an unstable, flawed drug [12, 24]. It decomposes completely in solution at 100 °C over the course of 24 h, and at 40 °C at a relative humidity of 70%, solid DHA undergoes 2% decomposition after one month and 2.9% after three months [12]. Thus, the poor thermal stabilities of these drugs and of DHA in particular raise concerns over decomposition of formulated material in countries with hot and humid weather. Nevertheless, it may well be that formulation plays a role in stabilizing such compounds - thus artemether co-formulated with lumefantrine when stored under ambient tropical conditions is reported not to undergo decomposition over a one year period relative to the formulation stored under controlled conditions [25]. In general, the International Conference of Harmonization (ICH) and the WHO have guidelines prescribing accelerated thermal stress testing by heating the formulated drug at 40 ± 2 °C at a relative humidity of 75 ± 5% for six months [26]. The threshold of unknown decomposition products based on a daily dose of 100 mg should not exceed 0.2%, with less than 1.5% decomposition to known degradants the toxicity and efficacy profiles of which have been quantified as for the parent drug. Thus, application of the guidelines to development of artemisinin drugs is indeed problematic.

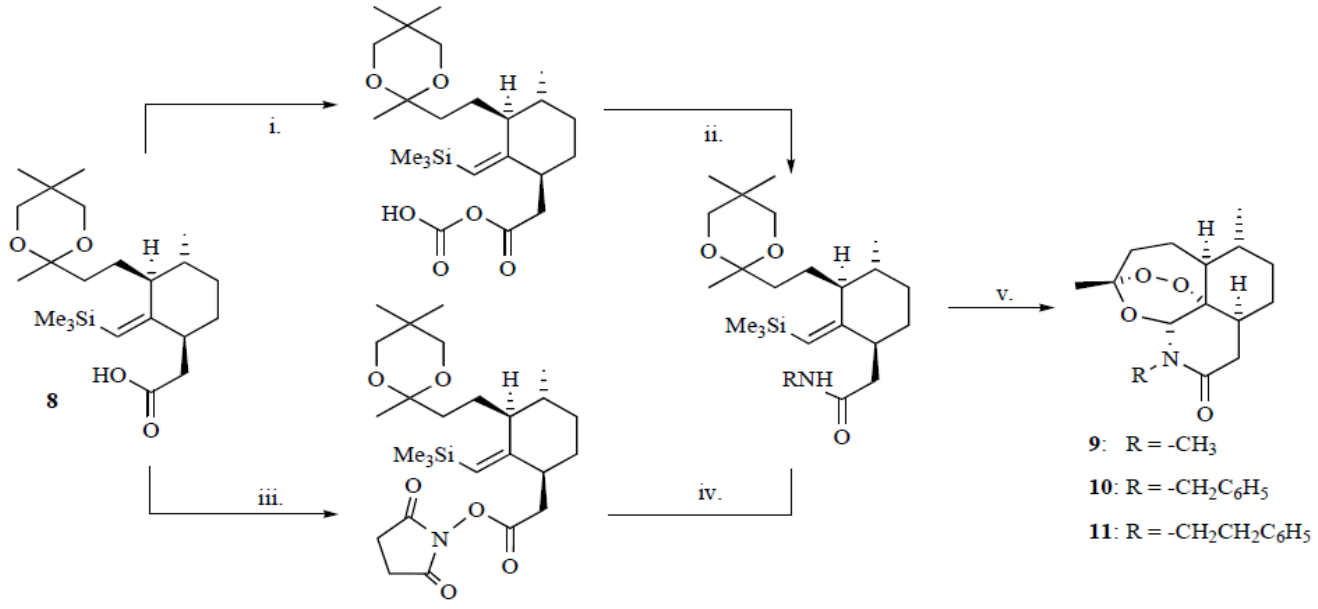
These shortcomings decisively justify the search for new artemisinin-based antimalarial drugs. As artemisinins induce the most rapid reduction in parasitaemia of any drug, *common sense* dictates that any *new* artemisinin derivative, selected on the bases of more robust chemical and thermal stability, metabolic stability with respect to generation of DHA *in vivo*, and relatively benign neurotoxicity should be used in any new ACT whose components are *rationally* chosen in order to counter resistant malaria and inhibit transmission of the resistant parasites. We have described elsewhere the development of newer artemisinin derivatives that have the oxygen atom of DHA at C-10 substituted by an amino group. Thus, one such compound artemisone [27] does not provide DHA on metabolism [28], displays no neurotoxicity in established models [29], is substantially more efficacious than the current clinical artemisinins, and elicits cure in patients with non-severe malaria at one-third the dose of currently the

most active artemisinin derivative artesunate. Given that dose loadings of current artemisinins in treatment regimens involving resistant malaria must be increased for effective therapy, the greater efficacy and lack of neurotoxicity of the newer artemisinins become critically important. However, there are clearly other derivatives that must have different pharmacokinetic profiles to the current artemisinins, and in particular have the potential not to provide DHA on metabolism or decomposition. Thus, it is in our common interests to highlight such compounds, and to evaluate the feasibility of introducing such compounds to supplant the current artemisinins.

In this review, we discuss the preparation and properties of derivatives of artemisinin known as 11-azaartemisinins. The parent compound 11-azaartemisinin **7** (Fig. **1**) is readily obtained from artemisinin and contains the six-membered lactam unit as opposed to the lactone unit of artemisinin. From a chemical viewpoint, the lactam is substantially more stable under acidic or basic conditions than is the lactone by virtue of lower ring strain [30] and the reduced electrophilicity at the carbonyl carbon atom bestowed by the adjacent electron-releasing nitrogen atom. They are expected to be more stable in acidic conditions such as in the stomach [31] and will be more stable in the blood at pH 7.4. Thus, azaartemisinins should have a superior bioavailability with respect to that of artemisinin. Azaartemisinin is at a higher oxidation level than dihydroartemisinin, and it is not possible for this compound or its derivatives to provide DHA *in vivo*, either via hydrolysis or metabolism.

2. TOTAL SYNTHESES OF N-ALKYL-11-AZA-9-DESMETHYLARTEMISININS

Fully synthetic 9-desmethyl azaartemisinins were prepared by Avery and co-workers as part of their extensive and elegant synthetic efforts leading to various functionalized artemisinin derivatives [32]. These were obtained from the intermediate carboxylic acid **8** to which the group already had access from a previous study [33]. An earlier cyclization protocol was adapted here wherein the intermediate was converted to the corresponding mixed anhydride (Scheme **1**) followed by reaction with the appropriate primary amines to give the corresponding amides. Exposure of the last compounds to ozone according to a method established earlier by Avery and others and subsequent acid-catalyzed cyclization gave the *N*-alkyl-11-aza-9-desmethylartemisinins, such as those **9-11** illustrated in Scheme **1**. The synthetic azaartemisinins were tested *in vitro* against chloroquine (CQ) resistant W2 and CQ sensitive and mefloquine resistant D6 strains of *P. falciparum* (*Pf*). A summary of the most active derivatives is given in Table **1**. As is the case for several of the compounds discussed in this review, the



Scheme 1. Total synthesis of 9-desmethyl-11-azaartemisinin derivatives according to Avery and co-workers from carboxylic acid **8** [32]. i. Ethyl chloroformate, Et_3N, CH_2Cl_2, 0 °C; ii. primary amine RNH_2, R = alkyl (except methyl), alkylaryl, *etc.*; iii. *N*-hydroxysuccinimide, dicyclohexylcarbodiimide, CH_2Cl_2, room temperature; iv. CH_3NH_2 aqueous; O_3, -78 °C, then silica gel-H_2SO_4 (15%); overall yields for compounds **9-11** and other compounds (12 examples) 20-65%.

IC_{50} values for the compounds are not given in the original publications, but rather the potencies relative to artemisinin **1** (Table **1**) [31].

Table 1. ***In vitro*** **activities expressed as relative potencies (%) of selected *N*-alkyl-9-desmethyl-11-aza-artemisinins against *P. falciparum* relative to artemisinin 1 [32].**[a]

Compound	*Pf* W2	*Pf* D6
1 Artemisinin	100	100
9 R = -CH_3	500	213
10 R = -$CH_2C_6H_5$	217	189
11 R = -$CH_2CH_2C_6H_5$	143	197

[a]Relative potency = [(IC_{50}artemisinin/IC_{50}analogue)(MW analogue/MW artemisinin)][100%]

Several other such derivatives were prepared, but these by and large proved to be less active than artemisinin. It is clear that whereas the *N*-methyl derivative **9** is relatively potent, activity falls away as the length and bulkiness of the alkyl chain increases [31]. It is interesting to note that the fully synthetic 9-desmethylartemisinin (compound **12**, Fig. **2**) [34] is approximately 6-fold more active than artemisinin against the W2 strain, and approximately 2-fold more active against the D6 strain [35, 36]. Thus, whilst activities of the 9-desmethyl-11-azaartemisinins are better than artemisinin **1**, they would seem to be equipotent or less active than 9-desmethyl artemisinin **12** itself.

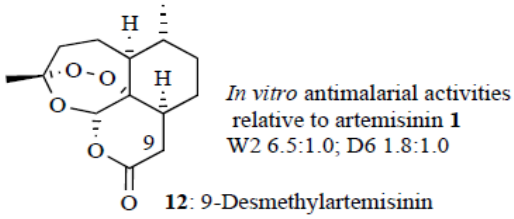


Fig. (2). 9-Desmethylartemisinin **12** first prepared by total synthesis by Avery and co-workers [33]; relative antimalarial activities as compared to artemisinin are from Table **1** in [34].

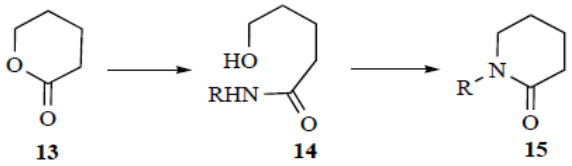
Log*P* values were determined for most of the compounds but no apparent correlation with the *in vitro* activity was found. *In vivo* efficacy, or toxicity, stability, solubility and bioavailability unfortunately were not ascertained for any of the compounds. However, analysis of the antimalarial data assisted in construction of a comparative molecular field analysis (CoMFA) model by Avery and co-workers in their efforts to provide a cohesive SAR model for artemisinin and its analogues.

However, it is clear that totally synthetic routes are not practical for obtaining drugs based on azaartemisinin, and the direct conversion of artemisinin by reaction with ammonia to produce the parent 11-azaartmisinin **7** or with primary amines to produce *N*-alkyl substituted azaartemisinins is by far the more attractive option.

3. DIRECT CONVERSION OF ARTEMISININ INTO AZAARTEMISININS

3.1. Preparation of 11-Azaartemisinin 7 from Artemisinin 1 and Ammonia

Ammonolysis of lactones is well-established, and in the case of the six-membered lactone δ-valerolactone **13**, treatment with amines generate hydroxyamide of general structure **14** in varying yields, depending upon the conditions used (Scheme **2**) [37]. Conversion into the lactam **15** requires forcing conditions, and thus it appears a moot point if artemisinin **1** could be converted into azaartemisinin **7** using conditions that may be compatible with the peroxide group.



Scheme 2. Putative conversion of the six membered lactone δ-valerolactone **13** by primary amines via the δ-hydroxy amide **14** into the *N*-substituted δ-valerolactam **15**. Extensive literature indicates forcing conditions (high pressure and temperature and/or metal catalysts) are required for effecting the overall conversion.

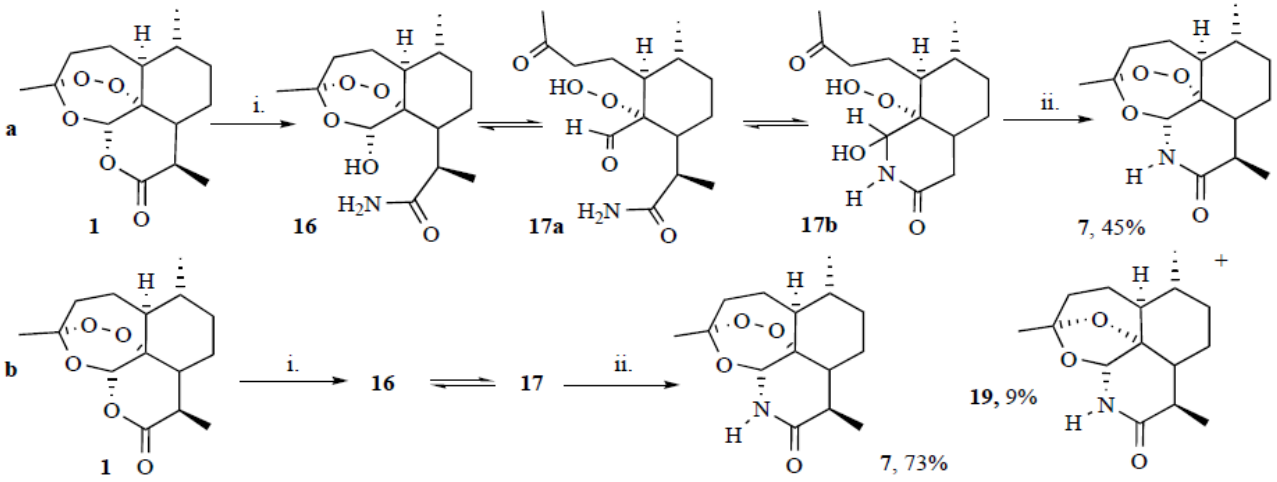
However, Ziffer and co-workers were able to effect the conversion under remarkably mild conditions. Artemisinin **1** was treated with ammonia in methanol at room temperature to produce an intermediate tentatively identified as the hydroxyamide **16** in apparent equilibrium with the ring-opened free hydroperoxides **17a** and **17b** as established by means of a 1H NMR spectroscopic examination of the reaction mixture. After evaporation of the reaction mixture, the residue was treated with dilute sulfuric acid in the presence of silica gel and the free radical inhibitor 2,6-di-*tert*-butyl-4-methylphenol (BHT) in dichloromethane to reform the tetracyclic ring system

of the artemisinin now incorporating the lactam (Scheme **3a**) [31]. The process is noteworthy for its relative economy, although the ammonolysis is markedly sensitive both to reaction temperature and concentration of reagents, with yields of the azaartemisinin varying between 40-65% [38]. The 11-aza-deoxyartemisinin **19** formally derived by reduction of azaartemisinin was formed as a by-product of this reaction; this compound has no antimalarial activity. Interestingly, if Amberlyst-15, an acid ion-exchange resin, rather than sulfuric acid was used, the deoxy product **19** did not form. A simpler process developed by the Haynes group involved treatment of artemisinin with 33% aqueous ammonia in a mixture of tetrahydrofuran-methanol (10:3) followed by direct evaporation of the reaction mixture. The residue was taken up into dichloromethane and treated with *p*-toluenesulfonic acid in the absence of any free radical inhibitor [38]. However, here also the temperature of the first step, the ammonolysis reaction, is important (Scheme **3b**). Yields of the product were improved to over 70% for product obtained by direct crystallization of the crude reaction mixture on a multigram scale [38].

Notably, 11-azaartemisinin **7** is a highly crystalline compound with a Log*P* of 2.32, has an aqueous solubility at pH 7 of >1000 mg/L [38] a value which renders this compound suitable for examination as an intravenous antimalarial, a purpose for which it would distinctly be more suited than artesunate. Although artesunate possesses a solubility of 565 mg/L at pH 7.2 [27] it decomposes rapidly at this pH. Azaartemisinin is over 15-fold more soluble than artemisinin, which possesses a solubility of 63 mg/L at pH 7.2 [27]. It possesses antimalarial activity essentially identical with that of artemisinin [31]. When screened against the CQ-resistant strain W2 and the CQ sensitive D6 strains, IC_{50} values are 6.1 and 9.2 nM, respectively.

3.2. The Problem of Competing Formation of 11-Azadeoxyartemisinin 19

The competing formation of azadeoxyartemisinin **19** in the sulfuric acid mediated cyclization detracts from the efficiency of generating azaartemisinin **7**. Logically, it derives by reduction of the open hydroperoxide **17a** or **17b** to the alcohol **18** that undergoes closure to **19** under the acidic conditions of the final step (step ii, Scheme **3a**). The reductant is likely to be ammonia: electrophilic oxygen transfer from the hydroperoxide to ammonia takes place wherein the ammonia converted into hydroxylamine (Fig. **3a**), in line with the well-established oxidation of amines by peroxides. It is significant that if the reaction mixture is treated with Amberlyst 15 according to the Ziffer process (Scheme **3a**), reduction does not occur; presumably the Amberlyst 15 absorbs the excess of ammonia, essentially removing it from thereaction mixture. In order to pinpoint the origin of **19**, artemisinin **1** was treated with the primary amine benzylamine in the presence of the tertiary amine *N*-methylmorpholine in the non-nucleophilic solvent



Scheme 3. a. Conversion of artemisinin **1** into 11-azaartemisinin **7** according to Ziffer [31]. i. **1**, excess NH_3, CH_3OH, room temperature, 1.5 h to **16** + **17**; ii. evaporation of solvent, then H_2SO_4 (15%)-silica gel, 2,6-di-*tert*-butyl-4-methylphenol, CH_2Cl_2, overnight: **7** 45% + **19** 8% or Amberlyst 15: **7** 65% . **b.** Modified method for conversion of artemisinin into 11-azaartemisinin **7** according to Haynes [38]. i. **1**, NH_4OH (aq., 33%), THF- CH_3OH, -10 to -15 °C. 10 hr; ii. evaporation of solvent, then *p*-TsOH, CH_2Cl_2, 12 h: **7** 73% by direct crystallization.

dichloromethane. The rationale is that if ammonia is reacting to form hydroxylamine in the original reaction of Fig. (**3**), the tertiary amine should intercept the hydroperoxide **20** generated by nucleophilic ring opening of the lactone by the primary amine, in which case it will be converted into the *N*-oxide **21**. In the event, a smooth reaction took place to generate the *N*-oxide **21** and the deoxy product **22** in good yields (Fig. **3b**) [39]. Other tertiary alkyl amines in the presence of benzylamine display similar behaviour. In the absence of the primary amine required for triggering nucleophilic ring opening of the lactone that leads to the hydroperoxide, no reaction of the *N*-methylmorpholine took place and the artemisinin was recovered intact. Thus the closed peroxide is incapable of initiating any oxidation. In the absence of the tertiary amine, the product **23** is formed from artemisinin and benzylamine. This product must arise via initial oxidation of benzylamine by the hydroperoxide **20** to benzylhydroxylamine, dehydration of the latter to the imine, and condensation of the imine with unreacted benzylamine (Fig. **3c**).

In summary, the preparation of azaartemisinin and its derivatives by reaction of ammonia or substituted primary amines with artemisinin to be described below may be affected by the reduction of the intermediate hydroperoxide by the amine. The outcome must reflect the balance between the ability of open hydroperoxides such as **17** or **20** (Scheme **3** and Fig. **3**) to undergo cyclization and facility of the intermolecular reduction by an excess of the amine. It is likely also that the temperature sensitivity of the overall process reflects this dichotomy.

Irrespective of the detailed mechanisms of the reactions, it is apparent the hydroperoxide **17** in an intracellular environment will have a lifetime that may be significant. Thus, the parasiticidal activity of artemisinins at large could be considered to reside in nucleophile-triggered formation of such ring opened hydroperoxides *in situ*. The hydroperoxides would be capable of oxidizing endogenous amines or other substrates, or producing hydroxyl radicals via the Fenton reaction [39, 40]. However, the generality of the ring-opening-intermolecular oxidation reactions

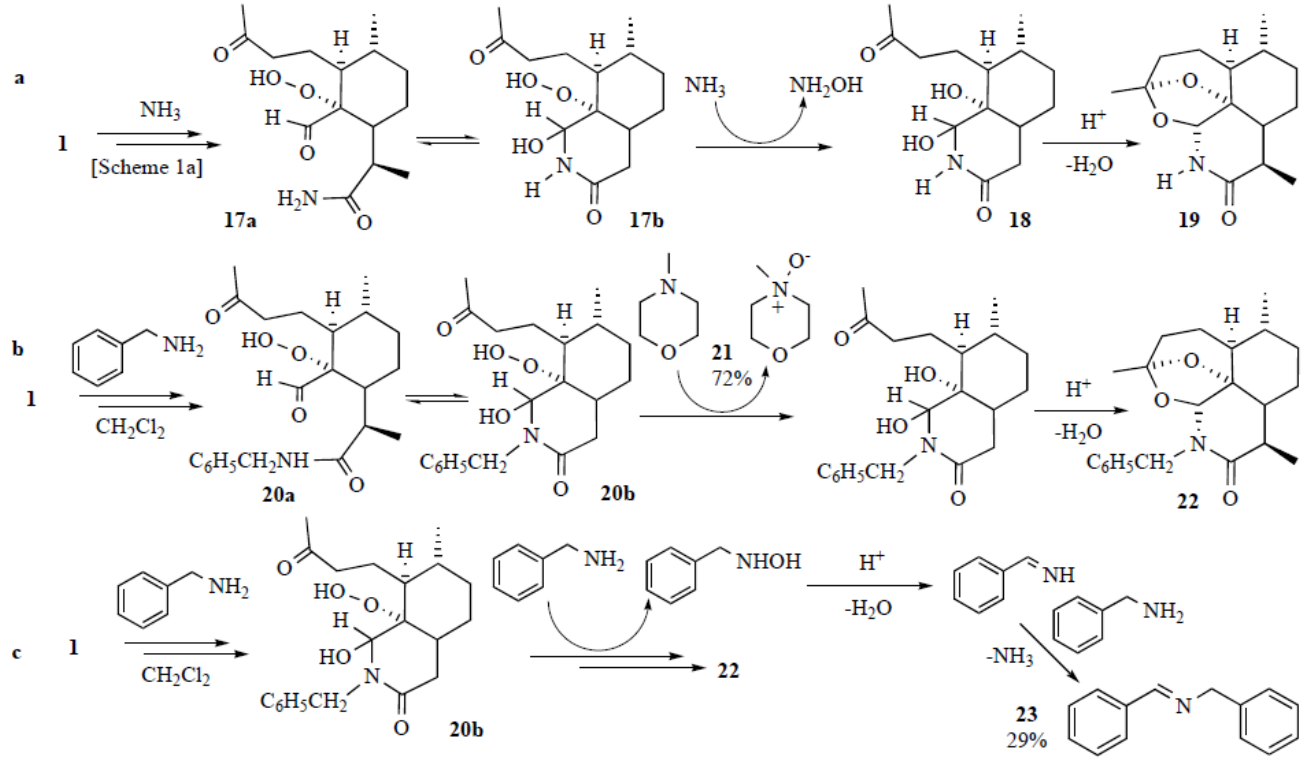


Fig. (3). a. Proposal for formation of 11-aza-deoxoartemisinin **19** from ammonia and artemisinin **1**: the open hydroperoxide **17** (*cf.* Scheme **3**) is reduced by ammonia to the alcohol **18** that undergoes acid-induced ring closure to **19** [39]; **b**. Outcome of experiment involving treatment of artemisinin **1** with benzylamine and excess of *N*-methylmorpholine in dichloromethane: the *N*-methylmorpholine *N*-oxide **21** is isolated in 72% yield [39]. **c**. Outcome of experiment involving treatment of artemisinin with excess of benzylamine in dichloromethane: the imine **23** is isolated in 29% yield [39].

with different artemisinin derivatives could not be demonstrated in the laboratory. It is now evident that the closed peroxide units of artemisinin and its derivatives and analogues have sufficient oxidizing power in their own right to interfere efficiently with intracellular reductants [10, 41].

3.3. Preparation of N-Alkyl-11-Azaartemisinins from Artemisinin 1 and Primary Amines

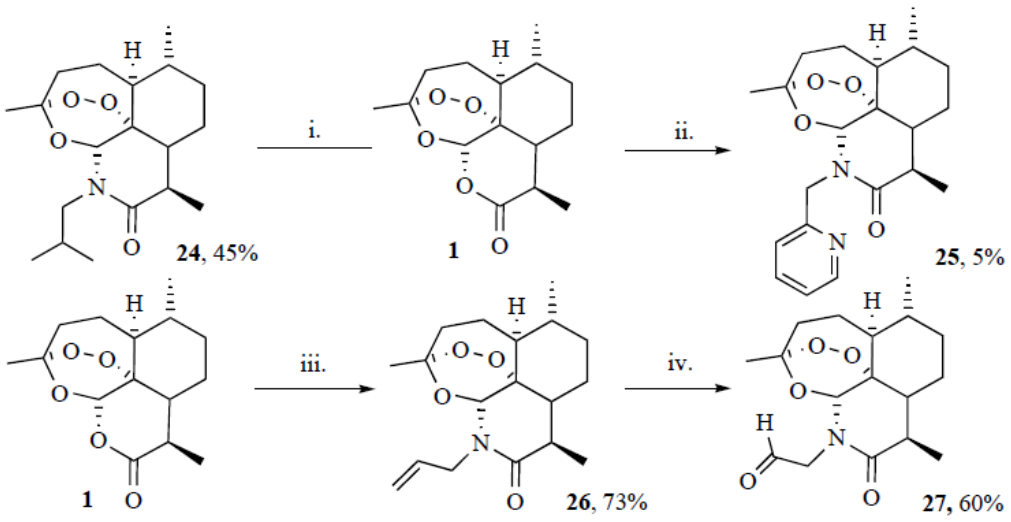
By extension of the above methodology to alkyl amines, *N*-alkyl-11-azaartemisinins were able to be prepared from artemisinin **1** (Scheme **4**) [31]. Here also, formation of the corresponding azadeoxy compounds interfered with these reactions when sulfuric acid was used at the cyclization step. When Amberlyst 15 was used, the deoxy products were not observed. In the case of allyl amine, the *N*-allyl-11-azaartemisinin **26** was functionalized by oxidative cleavage with ozone to generate the formylmethyl compound **27**.

The *N*-alkyl derivatives display good activities *in vitro* against the CQ-resistant strain FCR3 of *P. falciparum* (Table **2**). The data indicate that the parent 11-azaartemisinin is equipotent with artemisinin, and the *N*-alkyl derivatives are appreciably more potent. For compounds **25** and **27** that are relatively easily obtained from artemisinin, the data is impressive. *In vivo* efficacies were recorded by administering compounds, presumably *per os*, although this is not stated, to mice infected with *P. berghei*. End points were recorded based on the number of mice that survived post infection at given time points for specified amounts of each compound. Data for artemisinin **1**, arteether **4** and compound **27** indicate that compound **27** is at least 4 times more active than artemisinin **1** and is approximately equipotent with arteether **4** (Table **3**) [31]. Although the lactams are expected to be more stable in an acid medium such as the stomach as compared to the lactone in artemisinin or the acetal of artemether or arteether, no pharmacokinetic data is available for the foregoing azaartemisinins. Likewise nothing is known of their relative toxicity or metabolism.

Table 2. ***In vitro*** **activities of selected** ***N*****-alkyl-11-azaartemisinins (Scheme 3) against** ***P. falciparum*** **relative to artemisinin 1 [30].**[a]

Compound	*Pf* FCR3
7 Azaartemisinin	1.0
24	9.0
25	22.0
27	26.0

[a]Relative activity = IC_{50}artemisinin/IC_{50}derivative



Scheme 4. Conversion of artemisinin **1** into *N*-alkyl-11-azaartemisinins according to the method of Ziffer [31]. i. **1**, Isobutylamine, CH_3OH, room temperature, 1.5 h then H_2SO_4 (15%)-silica gel or Amberlyst-15, 2,6-di-*tert*-butyl-4-methylphenol, CH_2Cl_2, overnight: **24** 45%. ii. **1**, 2-(aminomethyl)pyridine, CH_3OH, room temperature, 1.5 h. then H_2SO_4 (15%)-silica gel, 2,6-di-*tert*-butyl-4-methylphenol, CH_2Cl_2, overnight: **25** 5%. iii. **1**, allylamine, CH_3OH, room temperature, 1.5 h then H_2SO_4 (15%)-silica gel, 2,6-di-*tert*-butyl-4-methylphenol, CH_2Cl_2, overnight: **26** 15% ; iv. O_3, CH_2Cl_2, -78 °C to room temperature: **27** 60%.

Table 3. ***In vivo*** **activities against** ***P. berghei*** **in mice [31].**

Cpd	Dose (mg/kg/day)	No. of Dead Mice/ Days Post-Infection	No. of Mice Alive 60 Days After Infection
1 Artemisinin	0	3/7, 11/10, 13/9	0/13
	8	1/13, 3/14, 6/16	0/5
	32	1/12, 1/18, 1/19, 1/20, 1/27	2/7
	128	1/17	6/7
4 Arteether	0	3/7, 5/8, 6/9, 7/10	0/7
	4	1/8, 2/9, 3/11, 4/12, 5/16	0/7
	16	2/18	7/7
	64	1/8	6/7
	256	-	7/7
27	8	1/15, 2/16, 3/19, 4/20, 5/26	0/5
	32	-	5/5
	128	-	5/5

3.4. Preparation of N-Functionalized-11-Azaartemisinins from Artemisinin 1 and Functionalized Primary Amines

As indicated above, the preparation of azaartemisinin and substituted derivatives by reaction of ammonia or primary amines with artemisinin is marred by reduction of the intermediate hydroperoxide by the amine. This becomes particularly evident when functionalized primary amines are used. Treatment of artemisinin in methanol with an excess of ethanolamine followed by acid treatment according to the standard conditions resulted in formation of the alcohol **28** and the azadeoxyartemisinin derivative **29** (Fig. **4**) [42]. The actual isolation of **28** confirms the idea that such alcohols (*cf.* **18**, Fig. **3**) are precursors of the azadeoxyartemisinins. With ethylenediamine, the deoxy dimer **30** was obtained. Interestingly, whilst compounds **28** and **29** had no antimalarial activity (as may have been anticipated because of the lack of the peroxide pharmacophore), the deoxy dimer **30** was active (Fig. **4**), and this respect appears unique among artemisinin derivatives in eliciting antimalarial activity in the absence of the peroxide pharmacophore. However, like other artemisinin deoxy-dimers [43] compound **30** is cytotoxic towards tumour cell lines - against human breast ductal carcinoma cells it displays an IC_{50} of 2.7 μg/mL (8.2 μM) [42].

The problem of reduction of the intermediate hydroperoxide by the ethanolamine was reported to be overcome by Singh and co-workers [8]; artemisinin **1** was converted into the desired *N*-(2'-hydroxyethyl)-11-azaartemisinin **31** (Scheme **5**, *cf.* Fig. **4**). Through the device of firstly running the reaction in a chloroform-methanol mixture, and extracting the reaction mixture with chloroform prior to mediating the acid induced ring closure employing the chloroform solution, the excess ethanolamine was removed into the aqueous layer before the cyclization step and competing reduction of the intermediate hydroperoxide apparently did not occur. Artemisinin **1** was also converted into the structurally interesting hydrazide, *N*-amino-11-azaartemisinin **31** by reaction with hydrazine hydrate on a multigram scale [8, 44]. The facility of the reaction itself is noteworthy, given that hydrazine should be susceptible to oxidation by the intermediate ring opened hydroperoxide as it is generated in the

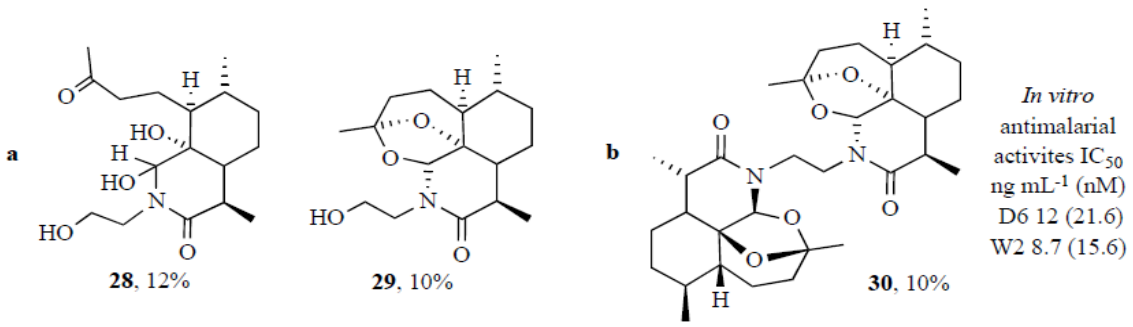
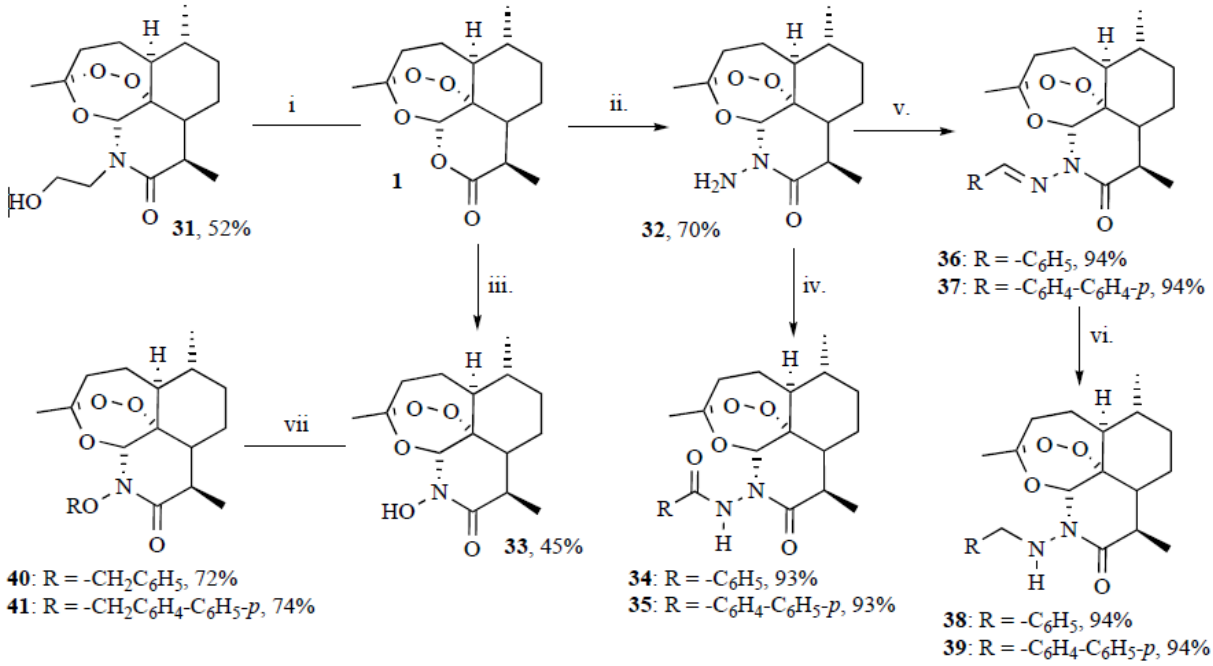


Fig. (4). a. Products **28** and **29** obtained from artemisinin **1** and excess of ethanolamine in methanol, room temperature, 3 h followed by treatment with H_2SO_4 (15%)-silica gel, 2,6-di-*tert*-butyl-4-methylphenol, CH_2Cl_2, overnight: **29** 10%, or **28** 12% after crystallization of the crude product mixture from hexane-ether. **b.** Product **30** obtained from artemisinin **1** and excess of ethylenediamine in methanol, room temperature, 3 h followed by treatment with H_2SO_4 (15%)-silica gel, 2,6-di-*tert*-butyl-4-methylphenol, CH_2Cl_2, overnight: **30** 10% [42]. Antimalarial activities *in vitro* for compound **30** are from [42].



Scheme 5. Amino- and hydroxyl- functionalized azaartemisinins prepared by Singh and co-workers [8,44]. i. $HOCH_2CH_2NH_2$, CH_3OH-$CHCl_3$ (7:3), 0 °C, 1 h; extraction with $CHCl_3$ then H_2SO_4 (20%)-silica gel, 2,6-di-*tert*-butyl-4-methylphenol, 12 h, room temperature: **31**, 52%; ii. $N_2H_4.H_2O$, CH_3OH-$CHCl_3$ (7:3), 0 °C , 1 h, extraction with $CHCl_3$ and then as for i. **32**, 70% ; iii. NH_2OH, MeOH-$CHCl_3$, room temperature, 1 h, extraction with $CHCl_3$ and then as for i. **33**, 45% ; iv. RCOCl, Et_3N, C_6H_6, 0 °C, 2 h; v. RCHO, Amberlyst-15, C_6H_6, room temperature, 2 h; vi. $NaBH_4$, C_6H_6, 0 °C, 4 h; vii. NaH, RBr, THF, 0 °C to room temperature, 12 h.

reaction mixture. Evidently this does not occur (Scheme **5**). Similarly, with hydroxylamine the *N*-hydroxy-11-azaartemisinin **32** was obtained from artemisinin, albeit in lower yield (Scheme **5**).

These functionalized azaartemisinins **32** and **33** were easily converted through application of relatively straightforward reactions into other derivatives. Treatment of **32** with acyl halides provided the acyl hydrazides **34** and **35**, and others in high yields. The acyl hydrazones **36** and **37** and others were prepared from the corresponding aldehyde under acid catalysis. Reduction of the latter with sodium borohydride provided the substituted hydrazides **38** and **39**, and others, again in high yields. The ethers **40** and **41**, and others were prepared from *N*-hydroxy-11-azaartemisinin **33** under standard conditions (Scheme **5**) [8,44]. The functionalized azaartemisinins and their derivatives were evaluated for antimalarial activity against *P. yoelii* in Swiss mice by the oral route [45]. Regrettably, activities *in vitro* were not ascertained, as data would allow for a meaningful comparison with activities of other azaartemisinins. In the *in vivo* model, arteether **4** provided 100% protection at a dose of 48 mg/kg/day for 4 days and 20% protection at 24 mg/kg/day for 4 days. Since the objective of this study was to discover compounds with a better activity profile than that of arteether, all the compounds were initially screened at a dose of 24 mg/kg for 4 days. Compounds that provided 100% protection at 24 mg/kg/day for 4 days were further screened at 12 mg/kg and 6 mg/kg/day for 4 days (Table **4**) [8, 44] The results of the compounds with the best activity profile are given in Table **4**. The parent functionalized azaartemisinins **32** and **33** were the least active, whilst hydrazone **37** was the most active. In general, with the exception of the ethers **40** and **41**, the most active compounds (among others not displayed here) were the biphenyl derivatives that were considered to display some 4-fold greater potency than arteether **4** [44]. However, the nature of the cytotoxicity evaluation coupled with the restricted nature of the sampling process prevents proper assessment of structure-activity relationships. The authors note that Log*P* values of "the six most active compounds of the series lie in the range 5.40−6.65" [44] although it is clear also from the data that other equally or more lipophilic compounds are less active. Although the trend to increased lipophilicity correlating with enhancement in activity for *in vitro* data is

Table 4. Activity of compounds administered orally against *P. yoelii* in mice [8,44].

Compound	Dose mg/kg/day x 4 Days	% Suppression on Day 4[a,b]	Mice Alive on Day 28
4 Arteether	48	100	5/5
	24	100	1/5
32	24	100	2/5
33	24	54	0/5
34	24	100	5/5
	12	62	0/5
35	24	100	5/5
	12	100	5/5
	6	94	0/5
36	24	100	2/5
37	24	100	5/5
	12	100	5/5
	6	100	4/5
38	24	100	5/5
	12	100	0/5
39	24	100	5/5
	12	100	5/5
	6	99	1/5
40	24	100	5/5
	12	100	4/5
41	24	100	5/5
	12	100	3/5

[a]Percent suppression = [(C - T)/C] x 100; where C is parasitaemia in control group and T is parasitaemia in treated group. [b]100% suppression of parasitaemia: parasites not detected in 50 oil immersion fields during microscopic observation [46].

well-established, and has been fully expressed by others on earlier occasions [47], for these *in vivo* assays a correlation simply cannot be drawn - it is clear that distribution, metabolism, and permeation, *etc.* must play a role in determining activities [32]. Irrespective of this, the relatively facile and economic preparation of these functionalized azaartemisinins represents an important advance.

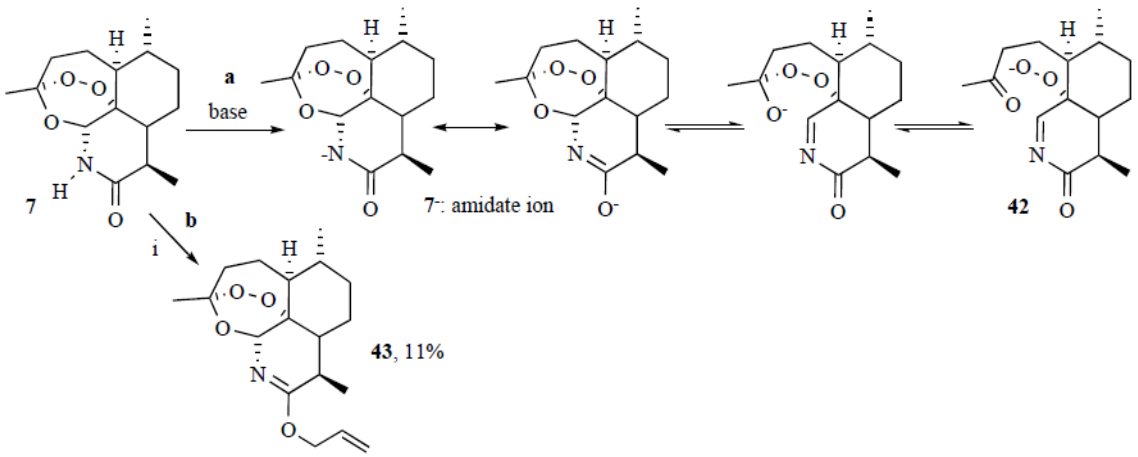
4. DIRECT CONVERSION OF 11-AZAARTEMISININ INTO FUNCTIONALIZED N-SUBSTITUTED DERIVATIVES

11-Azaartemisinin **7** incorporates a six-membered lactam ring, whose pK_a would approximate to that of δ-valerolactam, namely 26.6 [48]. In principle, the NH group may be deprotonated under equilibrating conditions (thermodynamic control) with moderately strong bases such as hydroxide ion, amidine bases such as 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) or conjugated amine bases such as 4-*N*,*N*-dimethyl-aminopyridine (DMAP) [49], or under kinetic control using aprotic conditions with strong bases such as lithium dialkylamides or sodium hydride to generate the amidate anion **7**- (Scheme **6**). However, there is the regiochemical issue involving reactions of ambident anions from secondary amides especially as generated under thermodynamic conditions which may interfere via formation of *O*-alkylated products. An additional potentially complicating issue is that the anion may undergo ring opening by virtue of connectivity with the electronegative oxygen atoms of the trioxane unit to deliver ultimately the peroxide anion **42** that must be less basic that the amidate (Scheme **6a**). Additionally, it is important that the intact peroxide itself does not oxidize the dialkylamide base, or undergo nucleophile induced cleavage of the peroxide triggered by hydride [10].

Nevertheless, artemisinin **1** is efficiently deprotonated at C-9 with lithium *N*,*N*-diisopropylamide under aprotic conditions at low temperature and the resulting enolate is of sufficient stability to react readily with electrophiles [50]. Thus, depending upon its stability with respect to ring-opening, alkylation of the amidate anion **7**- may enable access to a variety of *N*-substituted azaartemisinin derivatives. However, with respect to the regiochemical issue, it is noted that treatment of **7** with allyl bromide-silver oxide in *N*,*N*-dimethyl formamide (DMF) yields not the *N*-alkylated product, but rather the *O*-alkylated product, the imino-ether **43**, albeit in low yield, although such a reaction does not necessarily involve formation of a discrete anion [31]. Reactions of 11-azaartemisinin **7** with other alkylating agents, in particular of the amidate anion under aprotic conditions, have apparently not been recorded. Providing the anion is stable under the latter conditions, one expects formation of *N*-alkylated products to take place according to established chemistry [51]. As is now discussed, it turns out that the anion is indeed of sufficient stability to react easily with a variety of other electrophiles under widely differing conditions.

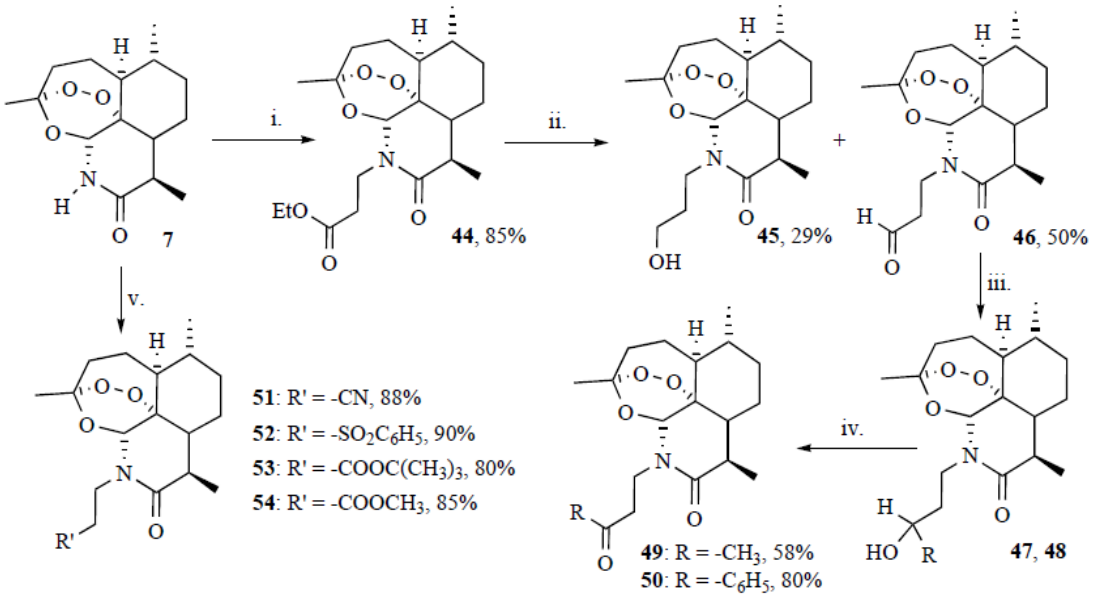
4.1. Addition of 11-Azaartemisinin 7 to Electron-Deficient Alkenes

Ziffer and co-workers identified straightforward conditions for converting 11-azaartemisinin **7** into functionalized *N*-substituted derivatives via addition of the amidate anion **7**- to electron deficient alkenes [52-54].

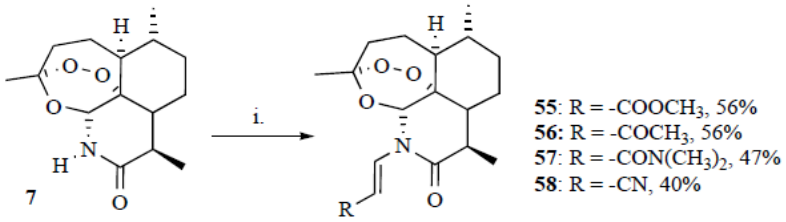


Scheme 6. a. Deprotonation of azaartemisinin with base to generate the amidate ion 7^- that in principle may undergo ring opening to generate ultimately the peroxide anion **42** (see text); **b**. Conversion of 11-azaartemisinin **7** into the imino-ether **43** [31]: i. Ag_2O, allyl bromide, DMF, room temperature, 20 h: **43** 11%.

Azaartemisinin in anhydrous tetrahydrofuran (THF) containing solid sodium hydroxide and ethyl acrylate at room temperature gave in good yield the *N*-(ethoxycarbonyl)ethyl adduct **44** (Scheme **7**). Further, adduct **44** was able to be reduced with diisobutylaluminium hydride (DIBAL-H) to the aldehyde **46**. Carbonyl addition of each of methyl-lithium and phenyllithium generated alcohols **47** and **48** that were oxidized to the carbonyl compounds **49** and **50**. The reactions are noteworthy given the evident compatibility of all reagents with the peroxide in the azaartemisinin. The addition reaction itself is notable for its simplicity. It also worked well for other electron deficient alkenes. Propynoate electrophiles in conjunction with acetonitrile as solvent and with 4-*N*,*N*-dimethylaminopyridine as base gave adducts in acceptable to good yields (Scheme **8**) [54] in accord with reactions involving nucleic acid bases with these electrophiles wherein the *E*-alkene is obtained as the predominant product [55].



Scheme 7. Conversion of 11-azaartemisinin **7** into *N*-ethyl substituted derivatives via addition to electron-deficient alkenes [52-54]. i. **7**, ethyl acrylate, NaOH -anhydrous THF, 1-2 h, room temperature; **44** 85%; ii. **44**, DIBAL-H, toluene, N_2, -78 °C, 1.5 h: **45** 29%, **46** 50%; iii. **47**, Et_2O, N_2, -78 °C, CH_3Li or C_6H_5Li then -40 °C, 2 h, alcohols **47**, **48** not isolated; iv. **47** or **48**, acetone, Jones reagent 0 °C then isopropanol quench: **49**, 58% or **50**, 80%; v. **7**, CH_2=CHR' [R' = -CN, -SO_2Ph, -$COOC(CH_3)_3$, -$COOCH_3$], NaOH-anhydrous THF, 1-2 h, room temperature: **51-54**, 85-90% [54].



Scheme 8. Conversion of 11-azaartemisinin **7** into *N*-vinyl substituted derivatives via addition to electron-deficient alkynes [54] **7**, HC≡CR [R = -$COOCH_3$, -$COCH_3$, -$CON(CH_3)_2$, -CN], acetonitrile, DMAP, room temperature, several minutes: **55-58** 40-56%.

Although the conversions are easily carried out, the antimalarial activities *in vitro* as recorded against *P. falciparum* for the most active adducts were not appreciably greater than that of artemisinin **1** itself or indeed of azaartemisinin **7** (Table **5**, *cf.* Table **2**). The most active compound was the ketone **49** derived via a multistep sequence from the adduct **44**. Therefore, the study overall does not identify potently active new artemisinin derivatives.

Table 5. ***In vitro*** **antimalarial activities of selected *N*-substituted 11-azaartemisinins against *P. falciparum* relative to artemisinin 1 [54].**[a]

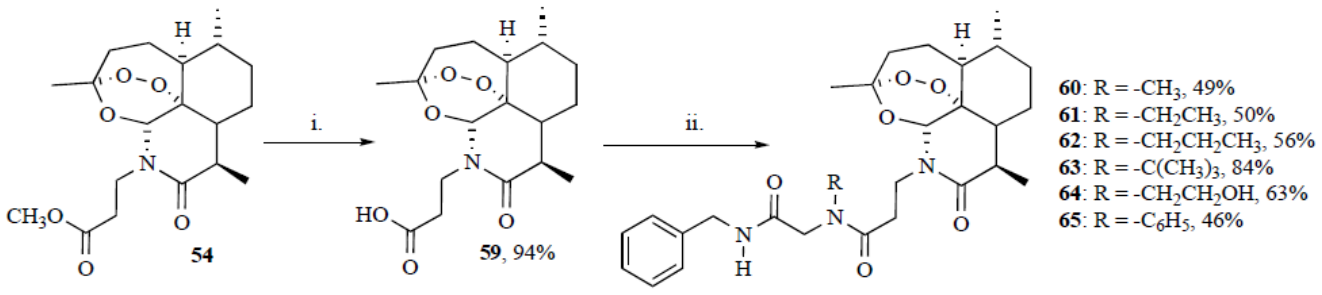
Compound	*Pf* W2	*Pf* D6
44 R = -$CO_2CH_2CH_3$	0.41	1.5
49 R = -$COCH_3$	3.0	3.2
50 R = -COPh	1.8	1.8
54 R = -CO_2CH_3	1.0	1.77
55 R = -CO_2CH_3	1.2	2.0
56 R = -$COCH_3$	1.25	2.54
58 R = -CN	1.7	2.7

[a]Relative activity = IC_{50}artemisinin/IC_{50}derivative

4.2. Functionalization of the Acrylate Adduct 54 of 11-Azaartemisinin 7 by the Ugi Reaction

Significantly, modification of the adduct **54** by hydrolysis to the free carboxylic acid **59** (Scheme **9**) followed by attachment of polar groups markedly enhances antimalarial activities. The carboxylic acid **59** was treated with a mixture of formaldehyde, benzyl isocyanide and selected alkyl and aromatic amines leading to compounds **60-65** among others in acceptable to good yields (Scheme **9**) [56]. This represents a clever application of the Ugi reaction, a multicomponent condensation of a ketone or aldehyde, amine, and alkyl isocyanide with a carboxylic acid to form dipeptide products [57].

The antimalarial activities of these polar functionalized azaartemisinins were evaluated against the CQ-resistant FcB1 strain of *P. falciparum* (Table **6**) [56]. Trends in activities are not readily apparent: an increase length and bulk of the alkyl chain R attached to the amide nitrogen abruptly results in enhanced activities (*cf.* Table **1**). Whilst this in part may be ascribed to increasing lipophilicity, it is noted that attachment of lipophilic aromatic substituents does not bestow enhanced activities. Nevertheless, overall activities of the alkyl amide derivatives **61-64** are significantly greater than that of artemether **3**, and the



Scheme 9. Functionalization of adduct **54** by way of the Ugi reaction [56]. i. NaOH-THF/H_2O; **59**, 94%. ii. $(CH_2O)_n$, RNH_2 [R = -CH_3, -CH_2CH_3, -$CH_2CH_2CH_3$, -$C(CH_3)_3$, -CH_2CH_2OH, -C_6H_5], CH_3OH then **59**, $C_6H_5CH_2N{\equiv}C$, room temperature, 1 - 2 d, **60 - 65**: 46-84%.

compounds thereby represent an attractive addition to the azaartemisinin library.

Table 6. ***In vitro*** **activities of polar functionalized azaartemisinins against** ***P. falciparum*** **[56].**

Compound	*Pf* FcB1 IC_{50} (nM)
60 R = -CH_3	140
61 R = -CH_2CH_3	15
62 R = -$CH_2CH_2CH_3$	0.3
63 R = -$C(CH_3)_3$	0.7
64 R = -CH_2CH_2OH	1.5
65 R = -C_6H_5	16
3 Artemether	19

4.3. Conversion of 11-Azaartemisinin into N-Sulfonyl- and N-Carbonyl-11-Azaartemisinins

Mindful of the problems with the current clinically-used used artemisinins, especially in so far that they essentially act as prodrugs for dihydroartemisinin (DHA) **2**, or comprise DHA itself, and are thermally unstable [12], Haynes and co-workers focussed on azaartemisinin with the view to attach an electron-withdrawing group directly at the nitrogen atom in order to provide new derivatives that are thermally stable and cannot provide DHA on hydrolysis or metabolism [38]. In general, attachment of electron-withdrawing groups to an ensemble containing the peroxide group tends to *enhance* stabilities by raising the homolytic bond dissociation energies of the peroxides. Thus, electron-withdrawing groups raise the activation energy for homolysis of diacyl peroxides [58] or, of greater relevance to the present case, of peroxy esters [59]. Comparative stabilities here are most easily gauged by comparing decomposition thresholds for each compound through use of thermogravimetric analysis (TGA). This technique is normally applied to determine the thermal stability of a material by monitoring the temperature increase (X axis) as a function of %change in weight (Y axis). The onset of loss of volatiles such as solvent of recrystallization, or of decomposition associated with loss of volatile products is thereby recorded. According to this technique, artemisinin **1** with the electronegative lactone oxygen atom is more stable than is azaartemisinin **7** with the less-electronegative lactam nitrogen atom. Artemisinin commences decomposition at 149 °C whereas 11-azaartemisinin commences decomposition at 132 °C (Fig. **5**). When the electron withdrawing lactone carbonyl group of artemisinin **1** is replaced by the electron-releasing hydroxyl group [60] of the lactol of DHA **2** or the electron releasing lactol ether group of artemether **3**, the decomposition thresholds become 110 °C and 116 °C respectively [12] In notable contrast, when the hydroxyl group of DHA becomes attached to the electron withdrawing carbonyl group within the ester group of artesunate, the decomposition threshold rises to 152 °C.

11-Azaartemisinin **7** was deprotonated under aprotic conditions using lithium *N,N*-diisopropylamide in THF at -78 °C to generate the amidate anion $\mathbf{7}^-$ that was treated with methanesulfonyl chloride to give the *N*-methanesulfonylazaartemisinin **66** in 62% yield (Scheme **10**) [38]. Although the sulfonyl electrophile may be classified as 'hard', only the *N*-sulfonylated derivative was obtained under these conditions. Similarly the sulfonamides **67-71** among others were also prepared. Sodium hydride was also used for deprotonation although generally yields were lower than in the case of LDA. Acid chlorides reacted with the amidate ion to give *N*-carbonyl derivatives **72-76**, and urea derivatives such as compound **77** were also obtained by reaction with isocyanates, albeit in low yields (Scheme **10**).

Antimalarial activities *in vitro* were assessed against the drug sensitive 3D7 clone of the NF54 isolate and the CQ resistant K1 strain with artesunate as the comparator drug (Table **6**) [38]. Generally, both classes of compounds with longer alkyl chains attached tended to be approximately equipotent with artesunate, conforming to the idea mentioned above that more lipophilic artemisinin derivatives of a particular class are more active. The most potent compounds were the carbonyl compounds bearing nitro-aryl groups, and the urea derivative **77**, the latter which is approximately equipotent with artemisone (IC_{50} 3D7 1.0 nM and IC_{50} K1 1.0 nM) [38]. Given that lipophilic artemisinins tend to have higher cytotoxicity [27, 61], it was important to establish if cytotoxicity was an issue in the current class. It is noted elsewhere that attachment of long alkyl chains (C10-16) to artemisinins at C10 via an amide linker results in drastic enhancement of cytotoxicity, as gauged by the effects of Hep G2 cancer cell lines [62] and the compounds are so cytotoxic that they cannot be screened *in vivo*. Here, preliminary screens were conducted on the sulfonyl azaartemisinins against Hep G2 cell lines at a concentration of 10 μM during a 6-day treatment.

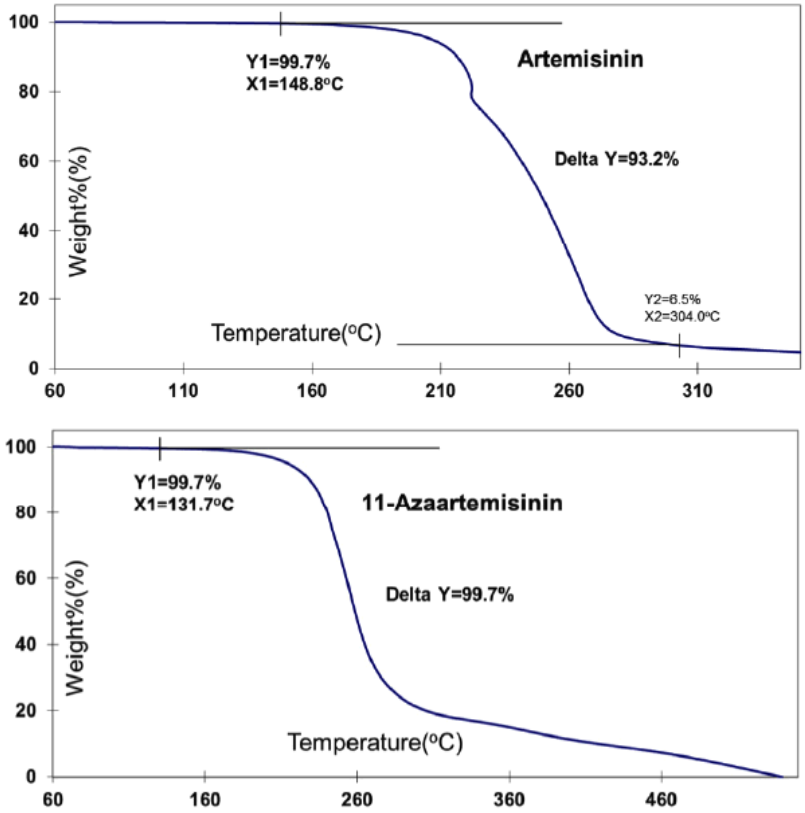
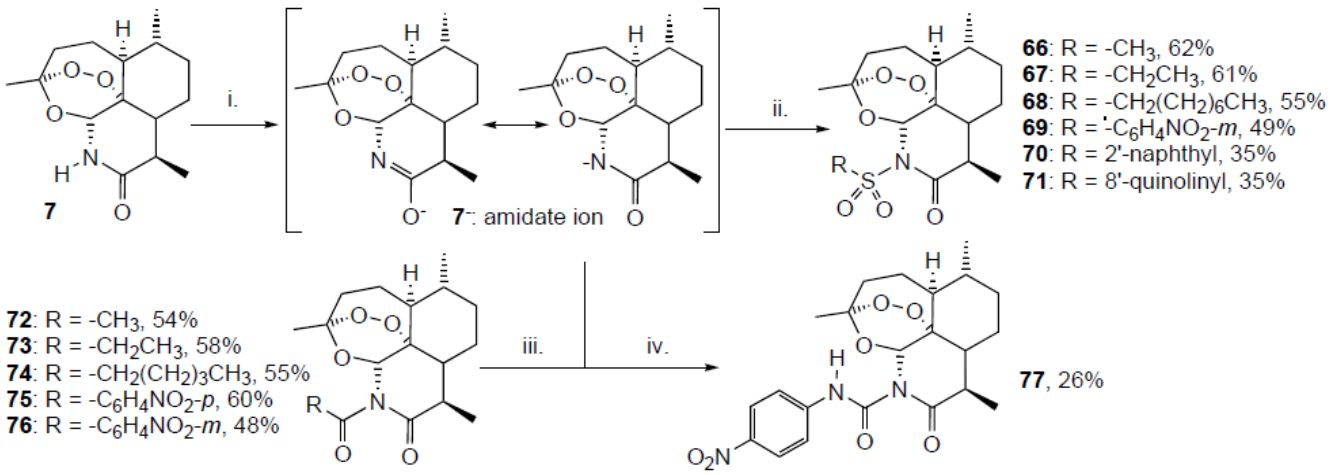


Fig. (5). Thermogravimetric analysis (TGA) of artemisinin **1** and 11-azaartemisinin **7** heated at a rate of 108 °C min^{-1} under N2. X1 and Y1 respectively refer to temperature and weight of the sample at the incipient decomposition event, and Delta Y represents the percent weight loss of sample between the designated temperatures.



Scheme 10. Preparation of *N*-sulfonyl, *N*-carbonyl and acyl urea derivatives of 11-azaartemisinin [38]. i. LDA (1.5 equiv), THF, N_2, -78 °C, 3 h, ii. RSO_2Cl (1.8 equiv) (R = $-CH_3$, $-CH_2CH_3$, $-CH_2(CH_2)_6CH_3$, $-C_6H_4F$-*p*, $-C_6H_4NO_2$-*m*, 2-naphthyl, 8-quinolinyl), -78 °C, 3 h, **66-71** 35-62%; iii. RCOCl (R = $-CH_3$, $-CH_2CH_3$, $-CH_2(CH_2)_4CH_3$, $-C_6H_4NO_2$-*p*), -78 °C, 3 h; **72-76** 48-60%; iv. O=C=N-C_6H_4-NO_2-*p*, -78 °C, 3 h, **77**, 26%.

Only compound **68** and other long chain derivatives not discussed here depressed cell viability to 75, 50 and 50% of the initial values, respectively. The other compounds depressed cell viability to 20% or less and were therefore considered relatively non-cytotoxic. So although compound **68** displays good antimalarial activity, it cannot be considered for further development due to its cytotoxicity [38].

Table 7. ***In vitro*** **activities of selected *N*-sulfonyl- and *N*-carbonyl-11-azaartemisinins against *P. falciparum* [38]**

Compound	*Pf* 3D7 IC_{50} (nM)	*Pf* K1 IC_{50} (nM)
Sulfonyl Derivatives		
66 R = $-CH_3$	11.1	8.9
67 R = $-CH_2CH_3$	4.6	9.9
68 R = $-CH_2(CH_2)_6CH_3$	2.4	4.6
69 R = $-C_6H_4NO_2$-*m*	4.5	4.7
70 R = 2'-naphthyl	5.9	0.42
71 R = 8'-quinolinyl	4.0	4.2
Carbonyl Derivatives		
72 R = $-CH_3$	9.3	13.9
73 R = $-CH_2CH_3$	5.9	3.0
74 R = $-CH_2(CH_2)_3CH_3$	2.6	5.3
75 R = $-C_6H_4NO_2$-*p*	1.0	1.4
76 R = $-C_6H_4NO_2$-*m*	1.1	1.4
77 R = $-NHC_6H_4NO_2$-*p*	1.3	0.9
5 Artesunate	3.9	5.7

With the general exception of compound **68** and others containing longer alkyl chains not shown here, the sulfonamides are highly crystalline compounds with melting points above 200 °C. The methanesulfonamide **66** melts at 245 °C, which appears to be the highest melting point recorded for any artemisinin derivative. As assessed by TGA, compound **66** shows a decomposition threshold of 185 °C indicating a greater thermal stability than other artemisinins (*cf.* Fig. **5**). Thus, although the nitrogen atom in azaartemisinin is remote to the peroxide, through-bond inductive effects serve to stabilize the peroxide once the electron-withdrawing sulfonyl group is attached. The carbonyl compounds tended to be lower melting, showed lower propensity to crystallize and were thermally less stable than the sulfonyl counterparts. Thus the methanecarbonyl compound **72** melts at 128 °C, and has a decomposition threshold of 152 °C. A direct comparison of the thermogravimetric analyses appears in Fig. (**6**) [38]. However, the methanesulfonamide has poor aqueous solubility (<1 mg/L), although the measured Log*P* of 2.32 places it in a range regarded as appropriate for development of new artemisinins [27].

CONCLUSION

11-Azaartemisinin **7** is readily and economically obtained from artemisinin **1** by means of treatment with aqueous ammonia, and in this respect is more easily and economically obtained than is dihydroartemisinin **2** from artemisinin **1** through reduction of the latter with sodium borohydride [2]. Azaartemisinin must thereby be less expensive than the DHA derivatives artemether **3** and artesunate **5**. The conversion of the lactone of artemisinin into the lactam of azaartemisinin **7** does not reduce the antimalarial activity *in vitro*, although clearly this is some fivefold less than that of DHA and its derivatives. 11-Azaartemisinin **7** is highly crystalline, relatively stable, and has an aqueous solubility at pH 7.2 exceeding that of artesunate. This is of potential importance for development of intramuscular or intravenous formulations for treatment of severe malaria. The measured Log*P* value (2.32) marks this as a relatively polar artemisinin derivative, and one which satisfies requirements as previously delineated for development of new artemisinins [26]. Against this, nothing appears known of its toxicity relative to that of the current derivatives, or indeed of its metabolism and pharmacokinetics. Clearly however, DHA cannot be formed. It is further not known if 11-azaartemisinin **7** retains activity against artemisinin-resistant parasite phenotypes. Thus, in order to better appreciate the suitability of taking this compound forward, these gaps in our knowledge of this compound need to be filled as soon as possible.

It is unfortunate that coherent data sets of *in vitro* activities for other azaartemisinins are not at hand, and thus a comparison of activities of the derivatives cannot readily be made. However, the *in vitro* results indicate that the compounds generally do possess better anti-malarial activities than artemisinin **1**, and hence of their immediate parent 11-azaartemisinin **7**. Of the derivatives, the most accessible appear to be those prepared from artemisinin and primary alkyl amines (Scheme **4**, Table **2**), functionalized primary amines such as hydrazine and hydroxylamine (compounds **31-33**, Scheme **5**), and the adducts obtained from azaartemisinin itself and electron deficient alkenes (Schemes **7** and **8**). However, with the notable exception of compounds **24** and **25** (Table **2**), these primary derivatives are not especially active. Whilst the compounds **31-33** may be readily converted into other derivatives dependent upon the type of functionality

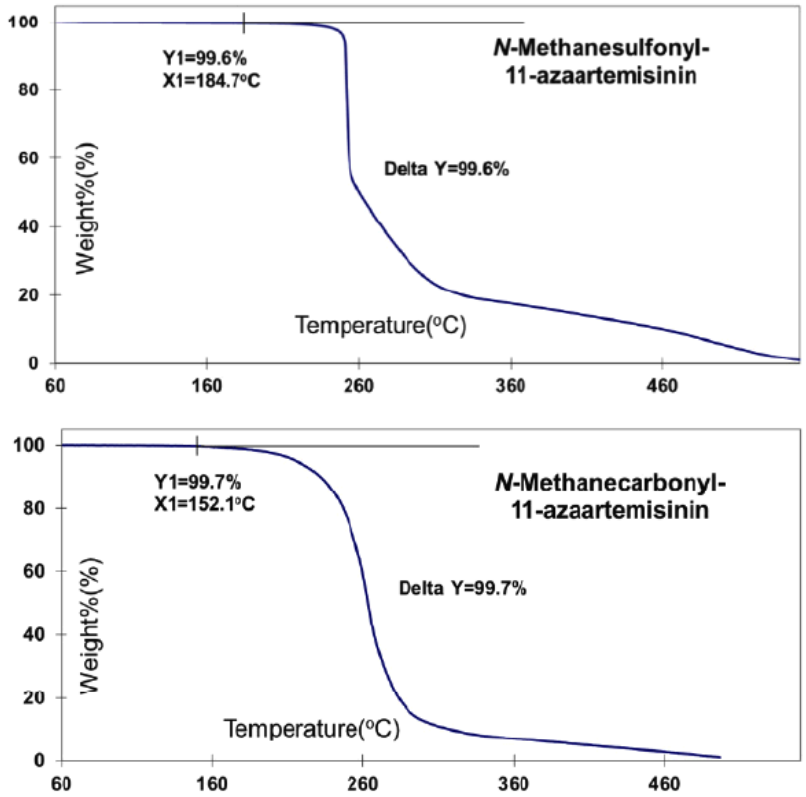


Fig. (6). Thermogravimetric analyses (TGA) of methanesulfonylazaartemisinin **65** and methanecarbonylazaartemisinin **72** heated at a rate of 108 °C min^{-1} under N2. X1 and Y1 respectively refer to temperature and weight of the sample at the incipient decomposition event, and Delta Y represents the percent weight loss of sample between the designated temperatures.

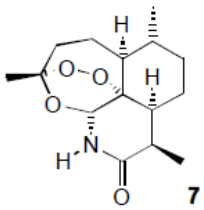
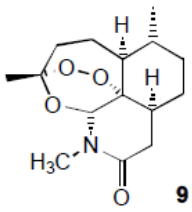
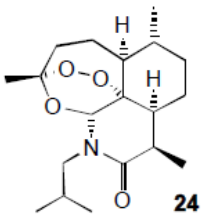
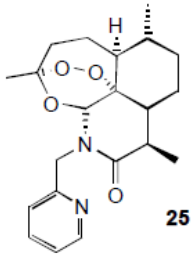
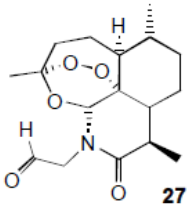
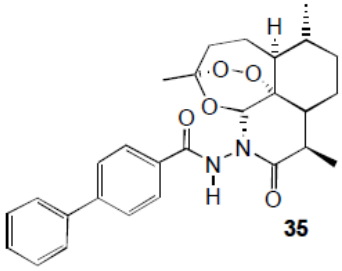
present (Scheme **5**), it is not possible to gauge how active these compounds are, although the claim is made that on the basis of *in vivo* activities, several compounds may be more active than arteether **4** (Table **5**). However, the Ugi adducts that are readily obtained from the acrylate adduct **54** (Table **6**, Scheme **9**) display notable antimalarial activities, and the data does support their further development. It would be important to establish how thermally stable these compounds are, and if indeed they have acceptable toxicity profiles. Whilst several of the *N*-sulfonyl- and *N*-carbonyl derivatives derived from azaartemisinin via deprotonation with strong bases (Table **7**, Scheme **10**) are equipotent or more active than artesunate **5**, the relative expense of preparing these compounds (use of aprotic conditions, low temperatures, and extractive work-up) is a drawback. However, against this, it is the notable thermal stabilities of the sulfonyl compounds in particular that renders these compounds attractive as future prospects for new drug candidates for treatment of malaria. The immediate challenge therefore is to evolve new chemistry to gain facile access to these compounds. This applies in particular to the highly active urea **77** (Table **7**, Scheme **10**) that is not readily obtained under the conditions of Scheme **6**. In this class also, it is shown that rendering the compounds more lipophilic through attachment of longer alkyl chains to the *N*-sulfonyl or carbonyl groups tends to enhance cytotoxicities, as gauged by effects on Hep G2 cell lines. Thus the original paradigm of constraining Log*P* to be less than that of arteether (Log*P* 3.89-3.99) would appear to be valid [27]. It is important not to lose sight of the fact that more lipophilic artemisinins tend to be more neurotoxic [27], a trend that becomes especially important now given the likely need of having to increase dose regimens of artemisinins against resistant parasites as discussed in the Introduction. For the compound class in general, it is necessary to conduct drug metabolism and pharmacokinetic studies on azaartemisinin itself, and on selected hit compounds.

A summary of the antimalarial activities of the more active azaartemisinin derivatives discussed in this article is given in Table **8** below.

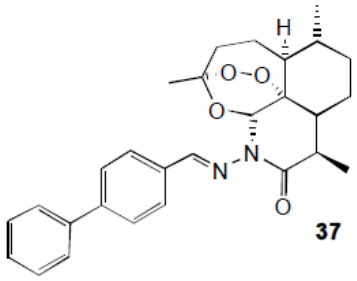
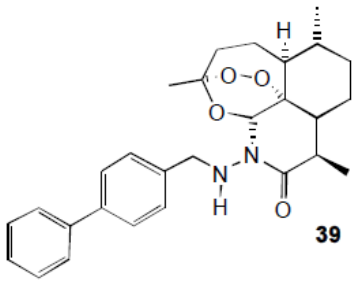
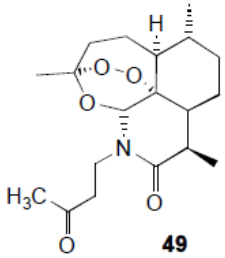
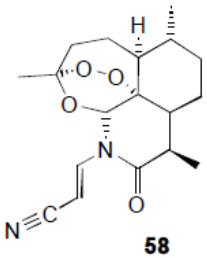
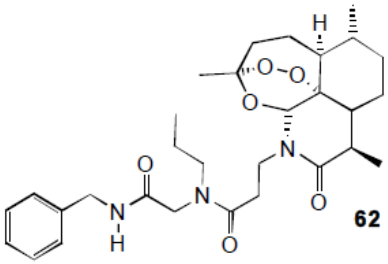
CONFLICT OF INTEREST

Each of the authors Rozanne Harmse, Ho Ning Wong, Frans Smit, David D. N'Da and Richard K. Haynes declare that they have no conflicts of interest.

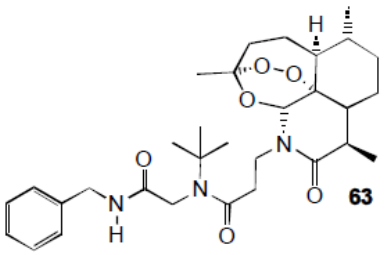
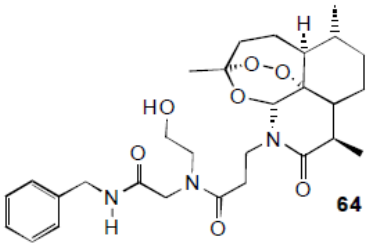
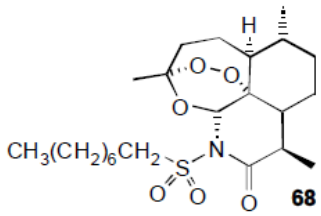
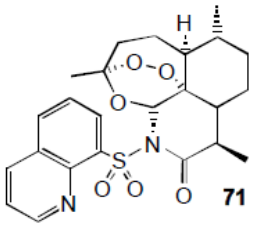
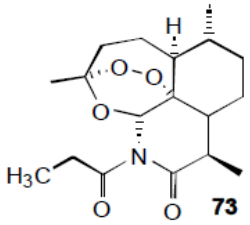
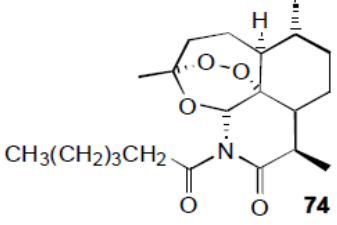
Table 8. Summary of antimalarial activities of selected azaartemisinins

Compound/Log*P*	*In vitro/in vivo*	Reference
 **7** LogP: 2.32	IC_{50} nM *Pf* W2: 6.1 *Pf* D6: 9.2	[38]
 **9** LogP: 2.76	*In vitro* activity relative to artemisinin **1** *Pf* W2: 5 *Pf* D6: 2.13	[32]
 **24** ClogP: 4.05 [a]	*In vitro* activity relative to artemisinin **1** *Pf* FCR3: 9	[31]
 **25** ClogP: 2.94	*In vitro* activity relative to artemisinin **1** *Pf* FCR3: 22	[31]
 **27** ClogP: 2.87	*In vitro* activity relative to artemisinin **1** *Pf* FCR3: 26	[31]
 **35** LogP: 5.53	*P. yoelii* in mice, oral 12 mg/kg for 4 days: 100% protection	[8, 44]

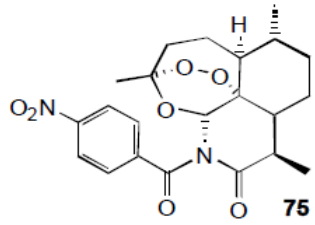
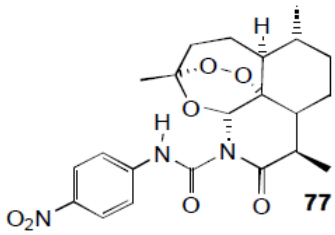
(Table 8) contd….

Compound/Log*P*	*In vitro/in vivo*	Reference
 **37** LogP: 6.59	*P. yoelii* in mice, oral 6 mg/kg for 4 days: 80% protection 12 mg/kg for 4 days: 100% protection	[8, 44]
 **39** LogP: 5.89	*P. yoelii* in mice, oral 12 mg/kg for 4 days: 100% protection.	[8, 44]
 **49** ClogP: 2.74	*In vitro* activity relative to artemisinin **1** *Pf* W2: 3.0 *Pf* D6: 3.2	[53]
 **58** ClogP: 3.19	*In vitro* activity relative to artemisinin **1** *Pf* W2: 1.7 *Pf* D6: 2.6	[53]
 **62** ClogP: 3.97	IC_{50} nM *Pf* FcB1: 0.3	[55]

(Table 8) contd….

Compound/Log*P*	*In vitro/in vivo*	Reference
 **63** ClogP: 4.14	IC_{50} nM *Pf* FcB1: 0.7	[55]
 **64** ClogP: 2.90	IC_{50} nM *Pf* FcB1: 1.5	[55]
 **68** ClogP: 4.88	IC_{50} nM *Pf* 3D7: 2.4 *Pf* K1: 4.6	[38]
 **71** ClogP: 4.04	IC_{50} nM *Pf* 3D7: 4.0 *Pf* K1: 4.2	[38]
 **73** ClogP: 3.62	IC_{50} nM *Pf* 3D7: 5.9 *Pf* K1: 3.0	[38]
 **74** ClogP: 5.21	IC_{50} Nm *Pf* 3D7: 2.6 *Pf* K1: 5.3	[38]

(Table 8) contd….

Compound/Log*P*	*In vitro/in vivo*	Reference
 **75** Clog*P*: 3.10	IC_{50} Nm *Pf* 3D7: 1.0 *Pf* K1: 1.4	[38]
 **77** Clog*P*: 3.42	IC_{50} nM *Pf* 3D7: 1.3 *Pf* K1: 0.9	[38]

[a] Clog*P* = Log*P* values calculated with ACD Labs ChemSketch 2015

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REFERENCES

[1] World Health Organisation Global Malaria Programme, World Malaria Report 2014. http://www.who.int/malaria/publications/world_malaria_report_2014 (Accessed April 23, 2015).

[2] (a) Qinghaosu Antimalarial Coordinating Research Group. Antimalarial studies on qinghaosu. *Chinese Medical Journal*, **1979**, *92*, 811-816; (b) Klayman, D.L. Qinghaosu (artemisinin) - an antimalarial drug from China. *Science*, **1985**, *228*, 1049-1055; (c) Ying, L.; Wu, Y.-L. An over four millennium story behind qinghaosu (artemisinin) - a fantastic antimalarial drug from a traditional Chinese herb. *Curr. Med. Chem.*, **2003**, *10*, 2197-2230; (d) Zhang, J.-F. *A detailed chronological record of project 523 and the discovery and development of qinghaosu (Artemisinin)*. Yang Cheng Evening News Publishing Company: Yang Cheng, China, **2005**; (e) Woodrow, C.J.; Haynes, R.K.; Krishna, S. Artemisinins. *Postgrad. Med. J.*, **2005**, *81*, 71-78.

[3] Cui, L.W.; Su, X.Z. Discovery, mechanisms of action and combination therapy of artemisinin. *Expert Rev. Anti-infective Ther.*, **2009**, *7*, 999-101.

[4] (a) Li, Y.; Yu, P.L.; Chen, Y.X.; Li, L.Q.; Gai, Y.Z.; Wang, D.S.; Zheng, Y.P. Synthesis of some derivatives of artemisinin. *Ke Xue Tong Bao*, **1979**, *24*, 667-669; (b) Li, Y.; Yu, P.-L.; Chen, Y.-X.; Li, L.Q.; Gai, Y.Z.; Wang, D.S.; Zheng, Y.-P. Studies on analogs of artemisinin. I. The synthesis of ethers, carboxylic esters and carbonates of dihydroartemisinin. *Acta Pharmaceutica Sinica*, **1981**, *16*, 429-439; (c) China Co-operative Research Group on qinghaosu and its derivatives as antimalarials. The chemistry and synthesis of qinghaosu derivatives. *J. Traditional Chinese Medicine*, **1982**, *2*, 9-16.

[5] Brossi, A.; Venugopalan, B.; Dominguez Gerpe, L.; Yeh, H.J.C.; Flippen-Anderson, J.L.; Buchs, P.; Luo, X.D.; Milhous, W.; Peters, W. Arteether, a new antimalarial drug: synthesis and antimalarial properties. *J. Med. Chem.*, **1988**, *31*, 645-650.

[6] Jiang, J.B.; Li, G.Q.; Guo, X.B.; Kong, Y.C.; Arnold, K. Antimalarial activity of mefloquine and qinghaosu. *Lancet*, **1982**, *8293*, 285-288.

[7] (a) Krishna, S.; Planche, T.; Agbenyega, T.; Woodrow, C.; Agranoff, D.; Bedu-Addo, G.A.; Owusu-Ofori, A.K.; Appiah, J.A.; Ramanathan, S.; Mansor, S.M.; Navaratnam, V. Bioavailability and preliminary clinical efficacy of intrarectal artesunate in Ghanaian children with moderate malaria. *Antimicrobial Agents Chemother.*, **2001**, *45*, 509-516; (b) Binh, T.Q.; Ilett, K.F.; Batty, K.T.; Davis, T.M.; Hung, N.C.; Powell, S.M.; Thu, L.T.; Thien, H.V.; Phuöng, H.L.; Phuong, V.D. Oral bioavailability of dihydroartemisinin in Vietnamese volunteers and in patients with falciparum malaria. *Brit. J. Clin. Pharmacol.*, **2001**, *51*, 541-546.

[8] Singh, A.S.; Verma, V.P.; Hassam, M.; Krishna, N.N.; Puri, S.K.; Singh, C. Amino- and hydroxy-functionalized 11-azaartemisinins and their derivatives. *Org. Lett.*, **2008**, *10*, 5461-5464.

[9] van Agtmael, M.A.; Gupta, V.; van der Wösten, T.H.; Rutten, J.-P. B.; van Boxtel, C.J. Grapefruit juice increases the bioavailability of artemether. *Eur. J. Clin. Pharmacol.* **1999**, *55*, 405-410.

[10] Haynes, R.K.; Chan, W.-C.; Wong, H.-N.; Li, K.-Y.; Wu, W.-K.; Fan, K.-M.; Sung, H.H.Y.; Williams, I.D.; Prosperi, D.; Melato, S.; Coghi, P.; Monti, D. Facile oxidation of leucomethylene blue and dihydroflavins by artemisinins: relationship with flavoenzyme function and

antimalarial mechanism of action. *ChemMedChem*, **2010**, *5*, 1282-1299.

[11] Baker, J.K.; McChesney, J.D.; Tao Chi, HT.. Decomposition of arteether in simulated stomach acid yielding compounds retaining antimalarial activity. *Pharmaceut. Research*, **1993**, *10*, 662-666.

[12] Haynes, R.K.; Chan, H.-W.; Lung, C.-M.; Ng, N.-C.; Wong, H.-N.; Shek, L.-Y.; Williams, I.D.; Gomes, M.F.; Cartwright, A. Artesunate and dihydroartemisinin (DHA): unusual decomposition products formed under mild conditions and comments on the fitness of DHA as an antimalarial drug. *ChemMedChem*, **2007**, *2*, 1448-1463.

[13] Olliaro, P.L.; Nair, N.K.; Haynes, R.K.; Wong, H.-N.; Tang, M.M.-K.; Decosterd, L A.; Navaratnam, V. [unpublished work].

[14] Parapini, S.; Olliaro, P.; Navaratnam, V.; Taramelli, D.; Basilico, N. Stability of the antimalarial drug dihydroartemisinin under physiologically-relevant conditions: implications for clinical treatment and pharmacokinetic and *in vitro* assays. *Antimicrob. Agents Chemother.* **2015**, *59*, 4046-4052.

[15] (a) Brewer, T.G.; Peggins, J.O.; Grate, S.J.; Petras, J.M.; Levine, B.S.; Weina, P.J.; Swearengen,J.; Heiffer, M.H.; Schuster, B.G. Neurotoxicity in animals due to arteether and artemether. *Trans. Roy. Soc. Trop. Med. Hygiene*, **1994**, *88*, 33-36; (b) Brewer, T.G.; Grate, S.J.; Peggins, J.O.; Weina, P.J.; Petras, J.M.; Levine, B.S.; Heiffer, M.H.; Schuster, B.G. Fatal neurotoxicity of arteether and artemether. *Am. J. Trop. Med. Hyg.*, **1994**, *51*, 251-259; (c) Nontprasert, A.; Nosten-Bertrand, M.; Pukrittayakamee, S.; Vanijanonta, S.; Angus, B.J.; White, N.J. Assessment of the neurotoxicity of parenteral artemisinin derivatives in mice. *Am. J. Trop. Med. Hyg.,* **1998**, *59*, 519-522; (d) Genovese, R.F.; Newman, D.B.; Brewer, T.G.. Behavioral and neural toxicity of the artemisinin antimalarial, arteether, but not artesunate and artelinate, in rats. *Pharmacol. Biochem. Behavior*, **2000**, *67*, 37-44; (e) Schmuck, G.; Haynes, R.K. Establishment of an *in vitro* screening model for neurodegeneration induced by antimalarial drugs of the artemisinin-type. *Neurotox. Res.*, **2000**, 2, 37-49; (f) Schmuck, G.; Roehrdanz, E.; Haynes, R.K.; Kahl, R. Neurotoxic mode of action of artemisinin. *Antimicrob. Agents Chemother.*, **2002**, *46*, 821-827.

[16] Genovese, R.F.; Newman, D.B. Understanding artemisinin-induced brainstem neurotoxicity. *Arch. Toxicol.,* **2008**, *82*, 379-385.

[17] Li, G.Q.; Arnold, K.; Guo, X.B.; Jian, H.X.; Fu, L.C. Randomised comparative study of mefloquine, qinghaosu, and pyrimethamine-sulfadoxine in patients with *falciparum* malaria. *Lancet,* **1984**, *2*(8416), 1360-1361.

[18] (a) White, N.J.; Olliaro, P. Artemisinin and derivatives in the treatment of uncomplicated malaria. *Medecine Tropicale (Marseilles),* **1998**, *58*, 54−56; (b) Nosten, F.; White, N.J.. Artemisinin-based combination treatment of falciparum malaria. *Am. J. Trop. Med. Hyg.*, **2007**, *77*, 181-192; (c) Aweeka, F.T.; German, P.I. Clinical pharmacology of artemisinin-based combination therapies. *Clin. Pharmacokinetics,* **2008**, *47*, 91-102; (d) Eastman, R.T.; Fidock, D.A.H. Artemisinin-based combination therapies: a vital tool in efforts to eliminate malaria. *Nat. Rev. Microbiol.* **2009**, *7*, 864-874.

[19] (a) Jambou, R.; Legrand, E.; Niang, M.; Khim, N.; Lim, P.; Volney, B.; Therese-Ekala, M.; Bouchier, C.; Esterre, P.; Fandeur, T.; Mercereau-Puijalon, O.. Resistance of *Plasmodium falciparum* field isolates to *in vitro* artemether and point mutations of the SERCA-type PfATPase6. *Lancet*, **2005**, *3*, 1960-1963; (b) Li, Q.-P.; Weina, P. Artesunate: the best drug in the treatment of severe and complicated malaria. *Pharmaceuticals*, **2010**, *3*, 2322-2332; (c) Hendriksen, I.C.E.; Mtove, G.; Kent, A.; Gesase, S.; Reyburn, H.; Lemnge, M.M.; Lindegardh, N.; Day, N.P.J.; von Seidlein, L.; White, N.J.; Dondorp, A.M.; Tarning, J. Population pharmacokinetics of intramuscular artesunate in African children with severe malaria: implications for a practical dosing regimen. *Clin. Pharmacol. Therapeutics*, **2013**, *93*, 443-450.

[20] Rolling, T.; Agbenyega, T.; Krishna, S.; Kremsner, P.G.; Kramer, J.P. Delayed haemolysis after artesunate treatment of severe malaria - Review of the literature and perspective. *Travel Med. Infect. Disease*. **2015**, *13*, 143-149.

[21] (a) Dondorp, A.M.; Phyo, A.P.; Nkhoma, S.; Stepniewska, K.; Ashley, E.A.; Nair, S.; McGready, R.; Ler, M.C.; Al-Saai, S.; Lwin, K.M.; Singhasivanon, P.; White, N.J.; Anderson, J.C.; Nosten, F.. Emergence of artemisinin-resistant malaria on the Western border of Thailand: a longitudinal study. *Lancet*, **2012**, *379*, 1960-1966; (b) Lubell, Y.; Dondorp, A.; Guérin, P.J.; Drake, T.; Meek, S.; Ashley, E.; Day, N.P.J.; White, N.J.; White, L.J. Artemisinin resistance - modelling the potential human and economic costs. *Malaria J.*, **2014**, *13*, 452.

[22] Ariey, F.; Witkowski, B.; Amaratunga C.; Beghain, J.; Langlois, A.C.; Khim, N.; Kim, S.; Duru, V.; Bouchier, C.; Ma, L.; Lim.; Leang, R.; Duong, S.; Sreng, S.; Suon, S.; Chuor, C.M.; Bout, D.M.; Ménard, S. Rogers, W.O.; Genton, B.; Fandeur, T.; Miotto, O.; Ringwald, P.; Le, B. J.; Berry, A.; Barale, J.C.; Fairhurst, R.M.; Benoit-Vical, F.; Mercereau-Puijalon, O.; Ménard, D.A. molecular marker of artemisinin resistant Plasmodium falciparum malaria. *Nature*, **2014** *505*, 50-55.

[23] Ashley, E.A.; Dhorda, M.; Fairhurst, R.M.; Amaratunga, C.; Lim, P.; Suon, S.; Sreng, S.; Anderson, J.M.; Mao, S.; Sam, B.; Sopha, C.; Chuor, C.M.; Nguon, C.; Sovannaroth, S.; Pukrittayakamee, S.; Jittamala, P.; Chotivanich, K.; Chutasmit, K.; Suchatsoonthorn, C.; Runcharoen, R.; Hien, T.T.; Thuy-Nhien, N.T.; Thanh, N.V.; Phu, N.H.; Htut Y.; Han, K.T.; Aye, K.H.; Mokuolu, O.A.; Olaosebikan, R.R.; Folaranmi, O.O.; Mayxay, M.; Khanthavong, M.; Hongvanthong, B.; Newton, P.N.; Onyamboko, M.A.; Fanello, C.I.; Tshefu, A.K.; Mishra, N.; Valecha, N.; Phyo, A.P.; Nosten, F.; Yi, P.; Tripura, R.; Borrmann, S.; Bashraheil, M.; Peshu, J.; Faiz, M.A.; Ghose, A.; Hossain, M.A.; Samad, R.; Rahman, M.R.; Hasan, M.M.; Islam, A.; Miotto, O.; Amato, R.; MacInnis, B.; Stalker, J.; Kwiatkowski, D.P.; Bozdech, Z.; Jeeyapant, A.; Cheah, P.Y.; Sakulthaew, T.; Chalk, J.; Intharabut, B.; Silamut, K.; Lee, S.J.; Vihokhern, B.; Kunasol, C.; Imwong, M.; Tarning, J.; Taylor, W.J.; Yeung, S.; Woodrow, C.J.; Flegg, J.A.; Das, D.; Smith, J.; Venkatesan, M.; Plowe, C.V.; Stepniewska, K.; Guerin, P.J.; Dondorp, A.M.; Day, N.P.; White, N.J. Spread of artemisinin resistance in Plasmodium falciparum malaria. *New Eng. J. Med.*, **2014**, *371*, 411-423.

[24] (a) Jansen, F.H.; Soomro, S.A. Chemical instability determines the biological action of the artemisinins. *Curr. Med. Chem.*, **2007**, *14*, 3243-3259; (b) Jansen, F.H. The pharmaceutical death-ride of dihydroartemisinin. *Malaria J.*, **2010**, *9*, 212.

[25] Gitua, J.; Beck, A.; Rovers, J. Quality and stability of artemether-lumefantrine stored under ambient conditions in rural Mali. *Malaria J.*, **2014**, *13*, 474.

[26] Kopp, S. Stability testing of pharmaceutical products in a global environment. *RAJ Pharma*, **2006**, *5*, 291-294.

[27] Haynes, R.K.; Fugmann, B.; Stetter, J.; Rieckmann, K.; Heilmann, H.-D.; Chan, H.-W.; Cheung, M.-K.; Lam, W.-L.; Wong, H.-N.; Croft, S.L.; Vivas, L.; Rattray, L.; Stewart, L.; Peters, W.; Robinson, B. L.; Edstein, M.D.; Kotecka, B.; Kyle, D.E.; Beckermann, B.; Gerisch, M.;

Radtke, M.; Schmuck, G.; Steinke, W.; Wollborn, U.; Schmeer, K.; Römer, A. Artemisone - A highly active antimalarial drug of the artemisinin class. *Angew. Chem. Internet. Ed.*, **2006**, *46*, 1989-2148.

[28] Nagelschmitz, J.; Voith, B.; Wensing, G.; Roemer, A.; Fugmann, B.; Haynes, R.K.; Kotecka, B.M.; Rieckmann, K.H.; Edstein, M.D. First assessment in humans of the safety, tolerability, pharmacokinetics, and ex vivo pharmacodynamic antimalarial activity of the new artemisinin derivative artemisone. *Antimicrob. Agents Chemother.*, **2008**, *52*, 3085-3091.

[29] Schmuck, G.; Temerowski, M.; Haynes, R.K.; Fugmann. B. Identification of a non-neurotoxic artemisinin derivative *in vivo* and *in vitro*. *Research Advan. Antimicrob. Agents Chemother.,* **2003**, *3*, 35-47.

[30] Bach, R.D.; Dmitrenko, O. The effect of carbonyl substitution on the strain energy of small ring compounds and their six-member ring reference compounds. *J. Am. Chem. Soc*., **2006**, *128*, 4598-4611.

[31] Torok, D.S.; Ziffer, H.; Meshnick, S.R.; Pan, X.-Q.; Ager, A. Syntheses and antimalarial activities of N-substituted 11-azaartemisinins. *J. Med. Chem.*, **1995**, *38*, 5045-5050.

[32] Avery, M.A.; Wesley, J.D.B.; Chong, K.M.; Mehrotra, S.; Miller, R.; Milhous, W.; Goins, D.K.; Venkatesan, S.; Wyandt, C.; Khan, I.; Avery, B.A. Structure-activity relationships of the antimalarial agent artemisinin. 2. Effect of heteroatom substitution at 0-11: Synthesis and bioassay of *N*-Alkyl-11-aza-9-desmethylartemisinins. *J. Med. Chem.,* **1996**, *38*, 5038-5044.

[33] Avery, M.A.; Chong, W.K.M.; Jennings-White, C. Stereoselective total synthesis of (+) artemisinin, the antimalarial constituent of *Artemisia Annua*. *J. Am. Chem. Soc.,* **1992**, *14*, 974-979.

[34] Avery, M.A.; Chong, W.K.M.; Jennings-White, C. The total synthesis of (+)-artemisinin and (+)-9-desmethylartemisinin. *Tetrahedron Lett.,* **1987**, *28*, 4629-4632.

[35] Avery, M.A.; Gao, F.; Chong, W.K.M.; Mehotra, S.; Milhous, W.K. Structure-activity relationships of the antimalarial agent artemisinin. 1. Synthesis and comparative molecular field analysis of C-9 analogs of artemisinin and 10-deoxoartemisinin. *J. Med. Chem.,* **1993**, *36*, 4264-4275.

[36] Avery, M.A.; Gao, F.; Chong, W.K.M.; Mehrotra, S.; Jennings-White, C. The organic and medicinal chemistry of artemisinin and analogs. *Trends Org. Chem*., **1993**, *4*, 413-468.

[37] Matsumoto, K.; Hashimoto, S.; Okamoto, T.; Otani, S.; Hayami, J. High pressure ammonolysis of lactones to hydroxyamides. *Chem. Lett.* **1987**, 803-804.

[38] Haynes, R.K.; Wong, H.-N.; Lee, K.-W.; Lung, C.-M.; Shek, L.Y.; Williams, I.D.; Croft, S.L.; Vivas, L.; Rattray, L.; Stewart, L.; Wong, V.K.W.; Ko, B.C.B. Preparation of N-sulfonyl- and N-carbonyl-11-azaartemisinins with greatly enhanced thermal stabilities: *in vitro* antimalarial activities. *Chem. Med. Chem.*, **2007**, *2*, 1464-1479.

[39] Haynes, R.K.; Pai, H.-O.; Voerste, A. Ring opening of artemisinin (qinghaosu) and dihydroartemisinin and interception of the open hydroperoxides with formation of *N*-oxides - a chemical model for antimalarial mode of action. *Tetrahedron Lett.*, **1999**, *40*, 4715-4718.

[40] Olliaro, P.L.; Haynes, R.K.; Meunier, B.; Yuthavong, Y. Possible modes of action of the artemisinin-type compounds. *Trends Parasitol.,* **2001**, *17*, 121-126.

[41] (a) Haynes, R.K.; Cheu, K.-W.; Tang, M.M.-K.; Chen, M.-J.; Guo, Z.-F.; Guo, Z.-H.; Coghi, P.; Monti, D. Reactions of antimalarial peroxides with each of leucomethylene blue and dihydroflavins: flavin reductase and the co-factor model exemplified. *Chem. Med. Chem.,* **2011** *6*, 279-291; (b) Haynes, R.K.; Cheu, K.-W.; Chan, H.-W.; Wong, H.-N.; Li, K.-Y.; Tang M.M.-K.; Chen, M.-J.; Guo, Z.-F.; Guo, Z.-H.; Sinniah, K.; Witte, A.B.; Coghi, P.; Monti, D. Interactions between artemisinins and other antimalarial drugs in relation to the co-factor model - a unifying proposal for drug action. *ChemMedChem* **2012**, *7*, 2204-2226.

[42] Al-Oqail, M.; Galal, A.M.; Ahmad, M.S.; Al-Fishawi, A.M.; El-Feraly, F.S. New bioactive azaartemisinin derivatives. *Molecules*, **2003**, *8*, 901-909.

[43] Beekman, A.C.; Barentsen, A.R.W.; Woerdenbag, H.J.; Van Uden, W.; Pras, N.; Konings, A.W.T; El-Feraly, F.S.; Galal, A.M.; Wikström, H.V. Stereochemistry-dependent cytotoxicity of some artemisinin derivatives. *J. Nat. Prod.* **1997**, *60*, 325-330.

[44] Singh, C.; Verma, V.P.; Hassam, M.; Singh, A.S.; Naikade, N.K.; Puri, S.K.. New orally active amino- and hydroxy-functionalized 11-azaartemisinins and their derivatives with high order of antimalarial activity against multidrug-resistant *Plasmodium yoelii* in Swiss mice. *J. Med. Chem.*, **2014**, *57*, 2489-2497.

[45] Peters, W. Techniques for the Study of Drug Response in Experimental Malaria. In: *Chemotherapy and Drug Resistance in Malaria*. Academic Press: London, **1970**, pp. 64-136.

[46] 100% Suppression of parasitemia means no parasites were detected in 50 oil immersion microscopic fields; parasites if present were below the detection limit. The parasites present below the detection limit could multiply and eventually could be detected during observation on subsequent days. In such cases though the drug was providing near 100% suppression of the parasitemia on day 4, it would not provide full protection to the treated mice in the 28-day survival assay. Multidrug-resistant *Plasmodium yoelii nigeriensis* used in this study is resistant to chloroquine, mefloquine, and halofantrine; 100% protection means that all the treated mice survived until day 28. Similarly 60% protection means three out of five treated mice survived until day 28.

[47] Ramu, K.; Baker, J.K. Synthesis, characterization, and antimalarial activity of the glucuronides of the hydroxylated metabolites of arteether. *J. Med. Chem.,* **1995**, *38*, 1911-1921.

[48] Bordwell, F.G.; Fried, H.E. Heterocyclic aromatic anions with 4n + 2 π-electrons. *J. Org. Chem.* **1991**, *56*, 4218-4223. http://www.chem.wisc.edu/areas/reich/pkatable (accessed 31 March 2015);

[49] Kaljurand, I.; Kütt, A.; Sooväli, L.; Rodima, T.; Mäemets, V.; Leito, I.; Koppel, I.A. Extension of the self-consistent spectrophotometric basicity scale in acetonitrile to a full span of 28 pKa units: unification of different basicity scales. *J. Org. Chem*. **2005**, *70*, 1019-1028.

[50] Paitayatat, S.; Tarnchompoo, B.; Thebtaranonth, Y.; Yuthavong, Y. Correlation of antimalarial activity of artemisinin derivatives with binding affinity with ferroprotoporphyrin IX. *J. Med. Chem.* **1997**, *40*, 634-638.

[51] Carey, F.A.; Sundberg, R.J. *Advanced Organic Chemistry*. Part A *Structure and Mechanisms*, Springer Verlag: US, **2007**; Ch. 3, pp 366-368.

[52] Mekonnen, B.; Ziffer, H. A new route to N-substituted 11-azaartemisinins. *Tet. Lett,* **1997**, *38,* 731-734.

[53] Katz, E.; Ma, J.; Kyle, D.; Ziffer, H. Structure and antimalarial activity of adducts of 11-azaartemisinin with conjugated terminal acetylenes. *Bioorg. Med. Chem. Lett.*, **1999**, *9*, 2969-2972.

[54] Mekonnen, B.; Weiss, E.; Katz, E.; Ma, J.; Ziffer, H.; Kyle, D.E. Synthesis and antimalarial activities of base-catalyzed adducts of 11-azaartemisinin. *Bioorg. Med. Chemistry*, **2000**, *8*, 1111-1116.

[55] Faja, M.; Ariza, X.; Galvez, C.; Vilarrasa, S. Reaction of uridines and thymidines with methyl propynoate. A new N-3 protecting group. *Tetrahedron Lett.*, **1995**, *36*, 3261-3264.

[56] Le, T.N.; De Borggraeve, W.M.; Grellier, P.; Pham, V.C.; Dehaen, W.; Nguyen, V.H. Synthesis of 11-azaartemisinin derivatives using the Ugi reaction and an evaluation of their antimalarial activity. *Tetrahedron Lett.*, **2014**, *55*, 4892-4894.

[57] (a) Domling, A.; Ugi, I. Multicomponent reactions with isocyanides. *Angew. Chem. Internat. Ed.*, **2000**, *39*, 3168-3210; (b) Domling, A. Recent developments in isocyanide based multicomponent reactions in applied chemistry. *Chem. Rev.*, **2006**, *106*, 17-89.

[58] Swain, C.G.; Stockmayer, W.H.; Clarke, J.T. Effect of structure on the rate of spontaneous thermal decomposition of substituted benzoyl peroxides. *J. Am. Chem. Soc.* **1950**, *72*, 5426-5434.

[59] Sawaki, Y. "Peroxy Acids and Peroxy Esters". In *Organic Peroxides* Ando, W. Ed., Wiley, New York, **1992**, pp. 456-457.

[60] Carey, F.A.; Sundberg R.J. Advanced Organic Chemistry. Part A: Structure and Mechanisms, *Springer Verlag US*, **2007**, *Vol. 3*, pp. 297-299.

[61] Haynes, R.K. From artemisinin to new artemisinin antimalarials: biosynthesis, extraction, old and new derivatives, stereochemistry and medicinal chemistry requirements. *Curr. Topics Med. Chem.*, **2006**, *6*, 509-537.

[62] Liu, Y.; Wong, V.K.-W.; Ko, B.C.-B.; Wong, M.-K.; Che, C.-M. Synthesis and cytotoxicity studies of artemisinin derivatives containing lipophilic alkyl carbon chains. *Org. Lett.*, **2005**, *7*, 1561-1564.

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CHAPTER 4
Antimalarial activities and cytotoxicities of *N*-sulfonyl-11-azaartemisinin derivatives – Article 2

This chapter contains the manuscript entitled “Antimalarial activities and cytotoxicities of *N*-sulfonyl-11-azaartemisinin derivatives” that is yet to be submitted to the European Journal of Medicinal Chemistry. This manuscript contains the aim, background, experimental details and results of the compounds that were synthesized in this study. The manuscript was also prepared according to the standards set out in the author information pack available in the author’s guidelines at: https://www.elsevier.com/journals/european-journal-of-medicinal-chemistry/0223-5234/guide-for-authors

Antimalarial Activities and Cytotoxicities of *N*-Sulfonyl-11-Azaartemisinin Derivatives

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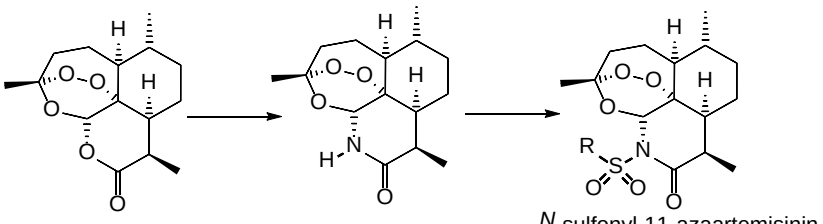
Abstract

Given the current thesis that the antimalarial drug dihydroartemisinin (DHA) either used in its own right, or as the active drug generated *in vivo* from the other current clinically-used artemisinins artemether and artesunate, induces quiescence in ring-stage parasites of *Plasmodium falciparum* (*Pf*) as the trigger of artemisinin resistance, we have turned our attention to the examination of structurally disparate artemisinins that are incapable of providing DHA on metabolism. Accordingly, *N*-sulfonyl-11-azaartemisinin derivatives were prepared from 11-azaartemisinin and screened against intraerythrocytic stages of chloroquine (CQ) sensitive *Pf* NF54 and CQ resistant K1 and W2 parasites. Cytotoxicities were determined against human fetal lung fibroblasts WI-38 cell line (HFLF). Most compounds displayed appreciable activities against all three strains with IC_{50} values <10 nM. The *p*-trifluoromethylbenzene-sulfonyl-11-azaartemisinin derivative **11** was the most active with IC_{50} values between 2 – 3 nM. All compounds were relatively non-toxic to HFLF. The compounds were also screened against *Pf* NF54 blood-stage gametocytes using the luciferase and pLDH assays. The 2'-thienylsulfonyl derivative **16** was the most active against late stage (IV-V) gametocytes with an IC_{50} value of 8.7 nM. Compounds **11** and **16** are potential candidates for further development.

Keywords: *Malaria, artemisinin, azaartemisinin, gametocytes*

Graphical abstract



Antimalarial activity (IC_{50}):
NF54: 2 - 42 nM
K1: 3 - 61 nM
W2: 2 - 65 nM

Gametocytocidal activity (IC_{50}):
Early stage Luciferase: 165 - 465 nM
Late stage Luciferase: 12 - 167 nM
Late stage $_{pLDH}$: 8 - 656 nM

Introduction

Malaria is caused by the protozoan parasite of the genus *Plasmodium* and is transmitted through the bite of an infected female *Anopheles* mosquito. Globally, an estimated 3.3 billion people in 97 countries are at risk of being infected with malaria, and 1.2 billion people are at high risk.[1] According to World Health Organization (WHO) estimates, 212 million cases of malaria occurred globally in 2015 resulting in 429 000 deaths. The malaria burden is heaviest in Africa and is ranked amongst the top five causes of death in Africa. Drugs once used in standard chemotherapeutic regimens for treatment of malaria have become ineffective through development of resistance. The most notable example is of chloroquine (CQ), for which resistance was first reported in the late 1950s on the Thai-Cambodian border. The resistant phenotype was transmitted to Africa and other parts of the world, thus essentially nullifying efforts to eradicate the disease.[2-5]

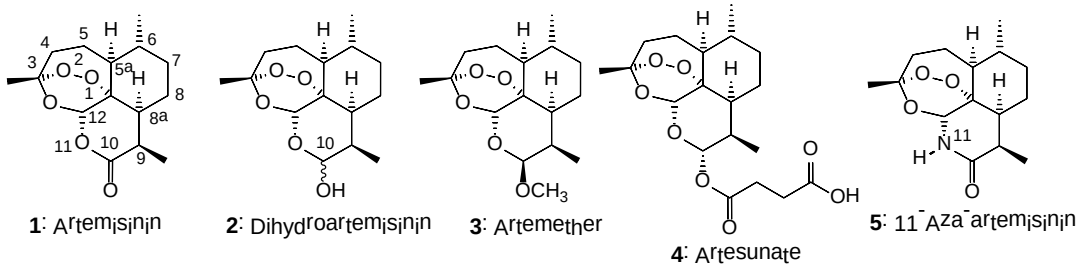


Figure 1: Structures of artemisinin **1** and its current clinical derivatives, the hemiacetal dihydroartemisinin (DHA) **2**, the lactol ether artemether **3** and hemiester artesunate **4**. The latter two drugs essentially act as prodrugs for DHA via facile metabolism or hydrolysis, respectively. Azaartemisinin **5** with the lactam replacing the lactone of artemisinin is expected to be more stable at physiological pH, and is chemically incapable of providing DHA by hydrolysis or metabolism.

The introduction of artemisinin **1** and its derivatives dihydroartemisinin (DHA) **2**, the oil-soluble artemether **3** and water-soluble artesunate **4** (Fig. 1), all first developed in China as antimalarial drugs,[6-11,12-14] appeared in large measure to redress the ineffectiveness of CQ in providing a new drug class that was notably active against CQ-resistant parasites, and in particular, induced rapid killing of blood stage parasites. In light of earlier experience with antimalarial drugs vis-à-vis emergence of resistance, the WHO proscribed the use of the clinically used artemisinins **2** – **4** in monotherapy, and recommended artemisinin combination therapies (ACTs) employing usually a fixed-dose combination of the artemisinin with a longer half-life drug.[15-18] However, in spite of this measure, reports on greatly increased parasite clearance times in patients from the Thai-Cambodian border treated with ACTs began to appear.[19,20] Resistance is now known to be associated with induction of dormancy by intraerythrocytic ring-stage parasites, and genetic markers of the resistant phenotype have been identified.[21] Further, it is claimed that DHA **2**, used as a drug in its own

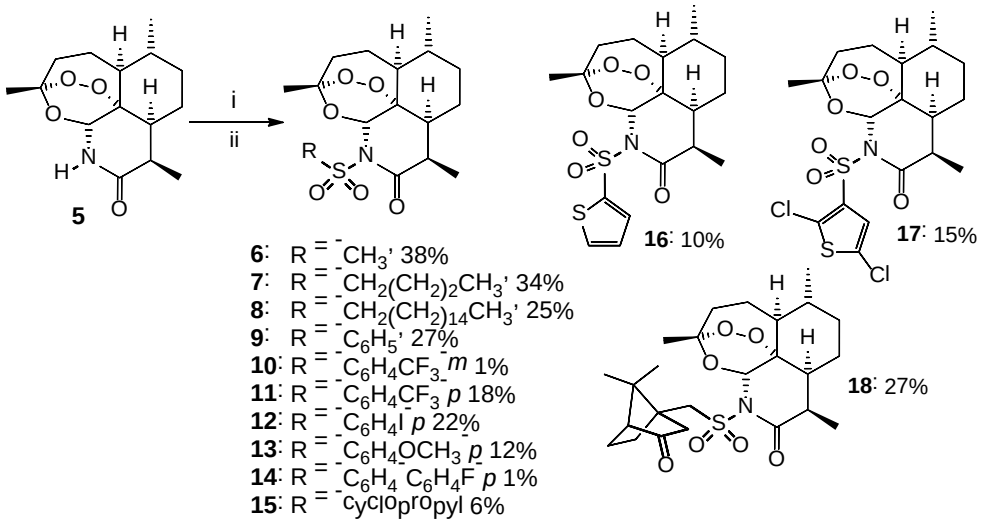
right or that is active as the metabolite common to the most widely used clinical artemisinins artemether **3** and artesunate **4**, is key to inducing ring stage dormancy through binding to the kinase phosphatidylinositol-3-kinase *Pf*PI3K to prevent its ubiquitinylation.[22-24] Interestingly, *in silico* modelling presents an optimum pose for DHA in the kinase binding site interacting with the key residues D1889 and Y1915. Structurally distinct artemisinins such as artelinate or artemisone are unable to adopt the DHA pose, and do not interact with these residues. It is notable that DHA is labile under physiological conditions, yet it is modelled intact into the *Pf*PKI3 target. Further, it is claimed that when the drug is 'washed out', activity of *Pf*PKI3 is restored.[22] Yet it was not established if indeed the drug was 'washed out'; although the normal procedure of 'washing out' was followed, no analyses for drug residues in the washings were carried out. Given the structural diversity of artemisinins and synthetic peroxides that elicit potent antimalarial activities, the presentation of a model invoking classical inhibition by binding into an endogenous receptor site is not convincing. According to a precedent for this type of target involving redox-sensitive signal transduction pathways, the nature of the inhibition must equate with generation of reactive oxygen species (ROS) by the artemisinin and evidently, inhibition of PI3P by ROS.[25-27] Overall, irrespective of the exact molecular mechanism that DHA does play in induction of resistance, an examination of the efficacies of artemisinins that are unable to provide DHA on metabolism is mandated. Further, as emphasis has to be placed on blocking transmission of resistant phenotypes,[28] those stages of intraerythrocytic parasites transmitted from host to vector, namely late-stage gametocytes, ideally should also be susceptible to the new drug. However, the current clinical artemisinins display moderate transmission blocking activities: they are at best moderately active against late stage mature gametocytes,[29] and ACTs such as artemether-lumefantrine and sulfadoxine-pyrimethamine-artesunate decrease gametocyte densities and clearance times.[30,31] Thus, our aim here is to develop newer artemisinins that do not provide DHA on metabolism or hydrolysis, and are active against asexual blood-stage parasites and late stage gametocytes.

11-Azaartemisinin **5** (Fig. 1) is readily obtained from artemisinin and incorporates a lactam unit which typically is more stable under acidic and basic conditions than is the lactone of artemisinin.[32] It is therefore expected that azaartemisinin and its derivatives will be more stable than artemisinin either at pH 7.4 or under the more acidic conditions of the stomach.[33] Further, azaartemisinin cannot provide DHA either by hydrolysis or metabolism as it is at a higher oxidation level than is DHA.[34] Therefore, derivatives of 11-azaartemisinin fulfil the criterion of not providing DHA and are potentially attractive as follow up drugs to the current clinical artemisinins. We have already demonstrated that certain azaartemisinin derivatives possess greatly enhanced thermal stability[35] and several have good antimalarial activities.[33,36]

Results and Discussion

Preparation of N-sulfonylalkyl and –aryl azaartemisinins

The parent compound, 11-azaartemisinin **5** was obtained in good yield (73%) from artemisinin and aqueous ammonia in a tetrahydrofuran-methanol mixture below 0 °C according to the literature method.[35] It was deprotonated under aprotic conditions with lithium *N*,*N*-diisopropylamide (LDA) as previously described and then treated with a sulfonyl chloride to yield the corresponding *N*-sulfonyl-11-azaartemisinin derivatives **6** – **18** (Scheme 1) in low to moderate yields (1 – 38%).



Scheme 1: Preparation of *N*-sulfonyl-azaartemisinin derivatives: i. LDA (1.5 equiv.), THF, N_2, -78 °C, 3 h. ii. RSO_2Cl (1.5 equiv.), -78 °C, 3h, silica gel chromatography, ethyl acetate-hexane (4:6), **6** – **18** (1 – 38%).

As this method follows that as previously reported by us[35] and provides the products in low yields, an examination of other potentially more economic methods was carried out in order to improve access to the products. Previous work of Ziffer and co-workers identified simplified conditions for converting 11-azaartemisinin into functionalized derivatives using electron deficient alkenes.[37-39] Thus, azaartemisinin in anhydrous THF containing solid sodium hydroxide and ethyl acrylate at room temperature gave in good yield (85%) the *N*-(ethoxycarbonyl)ethyl adduct.[37,38] Likewise, propynoate electrophiles in acetonitrile as solvent and with 4-*N*,*N*-dimethylaminopyridine (DMAP) as base gave adducts in acceptable to good yields (40-56%).[38] However, use of sulfonyl halides 3-(trifluoromethyl)benzene, 4-(trifluoromethyl)benzene, 4-methoxybenzene, 4-fluorobiphenyl, cyclopropyl, thiophene and 2,5-dichlorothiophene in acetonitrile in the presence of DMAP either alone or under

microwave irradiation did give the desired products, these were not obtained in acceptable yields, and were not easily separated from other components of the reaction mixtures.

Antimalarial activities and cytotoxicities

All compounds, with the exception of the *N*-alkylsulfonyl azaartemisinin **8** that was poorly soluble in the culture medium, were screened *in vitro* against CQ sensitive NF54 and CQ resistant K1 and W2 strains of intraerythrocytic *P. falciparum* parasites. The IC_{50} values are summarized in Table 1. Overall the compounds were active against all three strains with the most active displaying IC_{50} values of <10 nM. The *p*-trifluorobenzenesulfonyl derivative **11** was the most active against all three strains with IC_{50} values ranging between 2.04 – 3.05 nM. This compound displays comparable activity to artemether **3** and artesunate **4** against all three strains. The *m*-triflouromethylbenzenesulfonyl derivative **10** was the least active against all three strains with IC_{50} values ranging between 41.3 – 64.5 nM. Although the *p*-iodobenzenesulfonyl derivative **12** also exhibited poor activities against all three strains (IC_{50} 27.30 – 58.14 nM), it was some twofold less active against the drug resistant K1 and W2 strains, as expressed in the relatively high resistance indices (RI, Table 1).

All compounds were screened against human fetal lung fibroblasts WI-38 cell line (HFLF) to gauge the relative cytotoxicities; emetine, known for its high toxicity,[40] was used as a standard (Table 1). Selectivity indices (SI) of the azaartemisinin derivatives indicated several thousand-fold lower activities towards mammalian cells with respect to asexual stages of the malaria parasite as indicative of the high selectivity indices.

Table 1: *In vitro* biological data for standard artemisinins, azaartemisinin **6** and *N*-sulfonyl derivatives. Results are representative of three independent biological replicates, each performed as technical triplicates.

		Antimalarial activities IC_{50} (nM) ± SEM			Resistance Index		Cytotoxicities IC_{50} (µM)	Selectivity Index
Cpd	ClogP[a]	NF54	K1	W2	RI[b]	RI[c]	WI-38 HFLF[d]	SI[e]
2	2.71	0.57 ± 0.01	0.8 ± 0.5	0.37 ± 0.11	1.40	0.65	ND	ND
3	3.05	1.86 ± 0.17	2.64 ± 0.18	6.7 ± 0.6	1.41	3.60	ND	ND
4	2.93	3 ± 0.29	3.26 ± 0.2	2.4 ± 0.2	1.09	0.8	ND	ND
5	2.63	10.48 ± 1.36	9.44 ± 2.93	6.02 ± 1.81	0.90	0.57	>100	>9 542
6	2.77	14.51 ± 1.64	11.78 ± 1.17	8.27 ± 2.41	0.81	0.57	>100	>6 892
7	4.36	4.39 ± 0.83	5.29 ± 1.52	4.36 ± 0.77	1.20	0.99	>100	>22779
8	10.70	ND	ND	ND	ND	ND	>100	ND
9	4.73	5.17 ± 0.44	5.26 ± 1.77	4.18 ± 0.65	1.02	0.81	>100	>19342
10	5.61	41.34 ± 13.93	60.74 ± 7.10	64.50 ± 7.65	1.47	1.56	>100	>2 419
11	5.61	2.04 ± 0.59	3.15 ± 1.26	2.39 ± 0.50	1.55	1.17	>100	>49020
12	5.85	27.30 ± 4.89	51.89 ± 5.26	58.41 ± 12.06	1.90	2.14	>100	>3663
13	4.97	27.57 ± 8.04	40.51 ± 12.13	30.31 ± 2.50	1.47	1.10	>100	>3627
14	6.76	26.37 ± 2.23	19.93 ± 9.27	21.30 ± 3.56	0.76	0.81	>100	>3792
15	3.35	10.15 ± 1.05	6.19 ± 2.40	8.89 ± 2.80	0.61	0.88	>100	>9852
16	4.47	5.68 ± 0.53	6.27 ± 2.31	4.26 ± 0.76	1.10	0.75	>100	>17606
17	5.92	3.74 ± 0.95	5.11 ± 2.11	2.82 ± 0.37	1.37	0.75	43.20	11551
18	5.07	13.23 ± 2.93	9.01 ± 5.14	4.45 ± 0.39	0.68	0.34	>100	>7559
EM[f]	4.36	ND	ND	ND	ND	ND	0.05	ND

[a]CLogP = calculated LogP values using ChemBioDraw 2014. [b]Resistance index (RI) = IC_{50} K1/IC_{50} NF54. [c]Resistance index (RI) = IC_{50} W2/IC_{50} NF54. [d]Cytotoxicity data. [e]Selectivity index (SI) = IC_{50} WI-38/IC_{50} NF45. [f]EM = Emetine. ND = not determined

The *in vitro* gametocytocidal activity was determined using two different assay methods. The luciferase assay was used to determine effect of the compounds on early (>90% stages I-III) and late-stage (>90% stage IV-V) *Pf* NF54 gametocytes. The pLDH assay was used to determine effect of compounds on late stages NF54 gametocytes. Two transgenic parasite lines are employed in the luciferase assays: NF54-PfS16-GFP-Luc and NF54-Mal8p1.16-GFP-Luc. The most active derivatives **5** – **7**,**9**, **11**, **15** – **18** against asexual int***raerythrocytic*** parasites were selected for assessment of gametocytocidal activity with the luciferase assay. In those cases, where the assay did not provide meaningful results, their IC_{50} values were determined using the pLDH assay (Table 2).

The compounds showed good activities (IC_{50} <1 μM) against early- and late-stage gametocytes. Compounds **16**, **17** and **18** showed preference towards late-stage gametocytes as determined with the pLDH assay. All the other compounds had preference for early-stage gametocytes. Compound **11**, which demonstrated the best activity against asexual NF54 (IC_{50} 2.04 nM, Table 1) was also active against early- and late-stage gametocytes. Although compound **18** was specific for late-stage gametocytes (IC_{50} 25.3 nM), it was less active against asexual NF54 (IC_{50} 13.23 nM, Table 1). The standout compounds were **16** and **17** with activities against asexual NF54 (IC_{50} 5.68 and 3.74 nM, respectively, Table 1) and specific towards late-stage gametocytes (IC_{50} 1.53 and 3.18 nM, respectively, Table 2). The comparator compounds artemether **3** and artesunate **4** were active against early-stage gametocytes, but do not display the stage specificity of compound **16**.

Table 2: *In vitro* gametocytocidal data for artemisinins, azaartemisinin **6** and *N*-sulfonyl derivatives on early stage (I-III) and late stage (IV-V) gametocytes.[a]

	Early stage	Late stage		Stage specificity[b]
Cpd	IC_{50} (nM) ± SEM (n = 3) (Luciferase 48 h)	IC_{50} (nM) (n = 1) (Luciferase 72 h)	IC_{50} (nM) (n = 1) (pLDH 72 + 72 h)	[pLDH IC_{50} (nM)/ NF54 IC_{50} (nM)]
3	37.74 ± 2.08	223.9	ND	122.35[b]
4	62.83 ± 3.14	171.0	ND	57.0[b]
5	170.4 ± 19.3	166.1	622.2	59.37
6	233.1 ± 18.0	22.0	655.9	45.2
7	169.3 ± 27.8	ND	564.2	128.52
9	186.1 ± 49.5	ND	175.1	33.87
11	356.9 ± 78.6	ND	85.1	41.72
15	296.6 ± 60.5	12.0	32000	3152.71
16	165.7 ± 25.9	ND	8.7	1.53
17	421.6 ± 148.2	ND	11.9	3.18
18	464.8 ± 111.2	ND	25.3	1.91

[a]Results against the early stages are from three independent biological replicates, performed as technical triplicates (± SEM), and for the late stages as a single independent biological replicate, performed as technical triplicates;.[b] Stage specificity for artemether and artesunate: luciferase 72 h IC_{50} (nM)/NF54 IC_{50} (nM); ND = not determined

Ideally, an antimalarial drug or drug combinations should target both the asexual blood stages and the transmissible stages such as the gametocytes so as to hinder transmission of resistant parasites from an infected individual. Most antimalarial drugs target the asexual blood stage, that may cure a malaria patient but also allowing further transmission weeks after the clearance of the asexual parasites.[41] There is also evidence of enhanced rates of transmission of drug resistant parasites from drug-treated individuals.[42] Although it has been ascertained that the current clinical artemisinins can reduce gametocyte carriage,[43,44] in general these are not regarded as transmission blocking drugs. Thus, compounds in the current series such as the *p*-trifluoromethylbenzenesulfonyl derivative **11** and the 2-thienylsulfonyl derivative **16** are relatively active against both asexual- and late stage gametocytes represents an important development in the development of transmission blocking antimalarial drugs.

Conclusion

A series of *N*-sulfonylazaartemisinin derivatives have been prepared, and most are shown to be significantly active against blood stage asexual parasites, and against early and late stage gametocytes. Overall the compounds are relatively non-toxic and displayed good selectivity values towards the malaria parasite vis-a-vis normal mammalian cells. Compounds **11** and **16** represent hit compounds that have been taken forward for pharmacokinetic studies and further screening studies *in vivo*.

Experimental Section

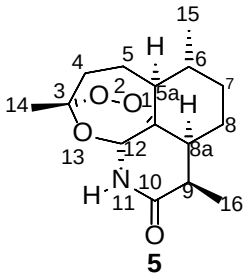
Materials and methods

All reagents were of analytical grade, and were obtained from Sigma-Aldrich (South Africa) and used as supplied. Solvents were purchased from Associated Chemical Enterprises (ACE, South Africa). Tetrahydrofuran (THF) (Sigma-Aldrich) was dried by storing over sodium and benzophenone under a nitrogen atmosphere and distilled prior to use. Lithium diisopropylamide (LDA) was generated *in situ* under a nitrogen atmosphere using *n*-butyllithium, *N*,*N*-diisopropylamide (DIPA) and 2,2'-bipyridine as endpoint indicator. Artemisinin was purchased from Changzhou Kaixuan Chemical Co (Chunjiang, China).

^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance^{TM} III spectrometer as solutions in chloroform-*d* ($CDCl_3$). Chemical shifts (δ) are reported in parts per million (ppm) and the ^{1}H 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). Spectra were analysed with MestReNova Software, version 5.3.2-4936. Infrared (IR) spectra were recorded on a Bruker Alpha-P FTIR instrument in conjunction with the attenuated total reflectance (ATR)

sampling technique. 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 nebulizer set at 0.4 Bar, r, and a collision cell RF voltage of 100 Vpp. 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 ($60F_{254}$) from Merck.

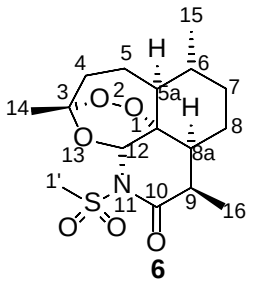
Syntheses



5

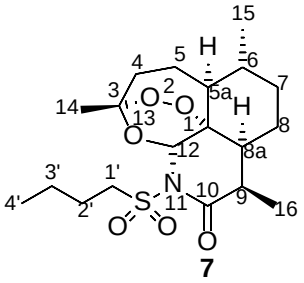
*11-Azaartemisinin **5***

A solution of artemisinin **1** (10.0 g, 35.42 mmol) in THF (200 mL) and methanol (60 mL) was cooled down to -15 ºC and treated with NH_4OH (33% aqueous, 100 mL). The resulting mixture was left to stir for 10 h at this temperature, during which time the color changed to a very pale yellow. The solution was evaporated under reduced pressure, without heating, to leave a pale yellow foam. This was dissolved in CH_2Cl_2 (250 mL) and treated with *p*-tolenesulfonic acid monohydrate (6.8 g, 35.84 mmol) at room temperature. The resulting mixture was left to stir for 12 h after which it was washed with 5% aqueous sodium bicarbonate (400 mL) and water (500 mL). The organic layer was separated and dried over anhydrous $MgSO_4$. The $MgSO_4$ was removed *via* filtration and the filtrate was evaporated under reduced pressure to leave the residue as a pale foam. The foam was crystallized using ethyl acetate: hexane (4:6) to yield 11-azaartemisinin **5** (7.2 g, 73%) (Scheme 1) as colourless needles; yield: 7.2 g (73%); mp: 143 – 144.5 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 6.25 (s, 1H, H-11), 5.39 (s, 1H, H-12), 3.27 – 3.19 (m, 1H, H-9), 2.44 - 2.35 (m, 1H, H-4α), 2.04 – 1.96 (m, 2H), 1.84 – 1.67 (m, 3H), 1.49 – 1.30 (m, 6H), 1.13 (d, *J* = 7.4 Hz, 3H, H-16), 1.04 – 0.95 (m, 5H); ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 173.48 (C-10), 104.96 (C-3), 79.97 (C-12a), 75.70 (C-12), 51.14 (C-5a), 46.03 (C-8a), 37.75 (C-9), 36.66 (C-25), 33.89 (C-7), 32.88 (C-9), 25.71 (C-14), 25.25 (C-14), 23.16 (C-5), 19.91 (C-15), 12.25 (C-16); IR: (ATR) ν_{max}/cm^{-1} 3298, 2964, 2884, 1656, 1444, 1133, 830, 730, 690; HRMS (APCI) *m/z* $[M + H]^+$ 282.1700 (Calcd for $C_{15}H_{24}NO_4$: 282.1705).



6

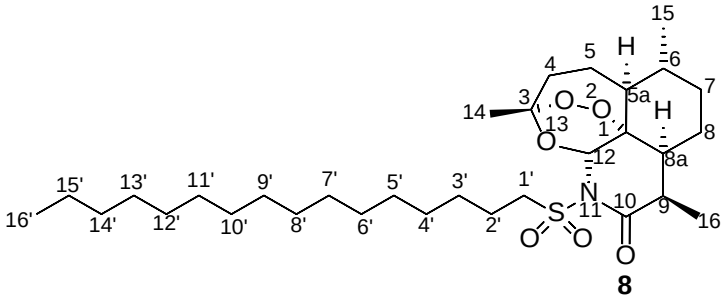
N-Methanesulfonylazaartemisinin **6**: 11-azaartemisinin **5** (1 g, 3.55 mmol) in dry THF (10 mL) was added to a stirred solution of LDA (5.35 mmol, 1.5 equiv.) in dry THF (15 mL) at -78 ºC. The resulting mixture was left to stir for 3 h under a nitrogen atmosphere at -78 ºC after which methanesulfonyl chloride (0.5 mL, 6.46 mmol, 1.5 equiv.) was added to the reaction mixture. The solution was left to stir for another 3h at -78 ºC and then for another 30 min at room temperature. The mixture was quenched with saturated aqueous ammonium chloride (20 mL), diluted with water (10 mL) and extracted with ethyl acetate (3 x 30 mL). The extracts were washed with brine (30 mL), dried ($MgSO_4$) and then filtered. The filtrate was evaporated under reduced pressure to leave a white solid, which was submitted to flash column chromatography with ethyl acetate-hexane (4:6). The eluent was evaporated to dryness to leave **6** as a pale yellow powder (481 mg, 38%), mp: 245 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 6.04 (s, 1H, H-12), 3.43 – 3.29 (m, 4H, H-1' and H-9), 2.44 – 2.34 (m, 1H, H-4α), 2.03 – 1.95 (m, 2H), 1.84 – 1.67 (m, 3H), 1.50 - 1.30 (m, 2H), 1.43 – 1.33 (m, 4H), 1.26 – 1.20 (m, 4H), 1.06 – 0.98 (m, 4H); ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 174.09 (C-10), 105.37 (C-3), 80.37 (C-12a), 78.83 (C-12), 51.45 (C-5a), 44.43 (C-8a), 44.23 (C-1'), 37.34 (C-1'), 36.51 (C-9), 36.15 (C-4), 33.71 (CH_2), 25.45 (C-14), 24.88 (CH_2), 22.40 (CH_2), 19.66 (C-15), 13.79 (C-16); IR: (ATR) ν_{max}/cm^{-1} 2978, 1709, 1446, 1349, 1165, 963, 519, 485; HRMS (APCI) *m/z* $[M + H]^+$ 360.1439 (Calcd for $C_{16}H_{26}NO_6S$: 360.1480). The data is in agreement with that reported in the literature.[35]



7

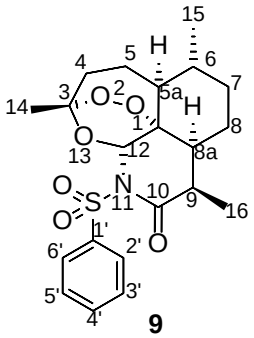
Butanesulfonyl-azaartemisinin **7**: Compound **7** was obtained from **5** (1.0 g, 3.55 mmol), LDA and 1-butanesulfonyl chloride (0.7 mL, 5.33 mmol, 1.5 equiv.), and chromatography (ethyl acetate: hexane, 4:6) as pale yellow powder (484 mg, 34%), mp 120 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 6.03 (s, 1H, H-12), 3.71 – 3.61 (m, 1H, H-1'α), 3.52 – 3.44 (m, 1H, H-1'β), 3.40 – 3.32 (m, 1H, H-9), 2.43 – 2.35 (m, 1H, H-4α), 2.08 – 1.99 (m, 2H), 1.96 – 1.85 (m, 2H, CH_2), 1.81 – 1.66 (m, 3H), 1.55 – 1.45 (m, 4H), 1.44 - 1.30 (m, 4H, H-14 and H-5a), 1.23 – 1.17 (m,

4H), 1.06 – 0.99 (m, 4H), 0.95 (t, *J* = 7.4 Hz, 3H, H-4'); ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 174.19 (C-10), 105.39 (C-3), 80.38 (C-12a), 78.34 (C-12), 56.76 (C-1'), 51.54 (C-5a), 44.82 (C-8a), 37.29 (CH), 36.59 (CH_2), 35.90 (C-9), 33.66 (CH_2), 25.41 (C-14), 24.85 (CH_2), 24.59 (CH_2), 22.33 (CH_2), 21.67 (CH_2), 19.65 (C-15), 13.67 (C-16), 13.52 (C-4'); IR: (ATR) ν_{max}/cm^{-1} 2931, 2879, 1700, 1344, 1201, 1146, 1023, 946, 648, 534, 487; HRMS (APCI) *m/z* $[M + H]^+$ 402.1930 (Calcd for $C_{19}H_{32}NO_6S$: 402.1950).



8

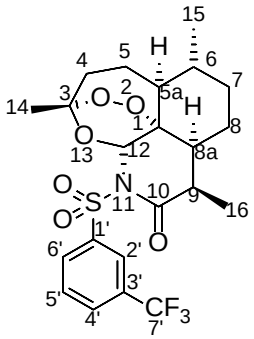
1-Hexadecanesulfonyl-azaartemisinin **8**: Compound **8** was obtained from **5** (1.0 g, 3.55 mmol), LDA and 1-hexadecanesulfonyl chloride (1.73 g, 5.33 mmol, 1.5 equiv.), and chromatography (ethyl acetate:hexane, 4:6) as a bright yellow oil (498 mg, 25%); ^{1}H NMR (600 MHz, DMSO) δ (ppm): 5.88 (s, 1H, H-12), 3.61 (ddd, *J* = 14.1, 10.0, 5.6 Hz, 1H, H-1'α), 3.45 (ddd, *J* = 14.0, 10.1, 5.7 Hz, 1H, H-1'β), 3.30 – 3.25 (m, 1H, H-2'α), 3.18 – 3.13 (m, 1H, H-9), 2.28 – 2.20 (m, 1H, H-4α), 2.08 – 2.02 (m, 1H), 2.00 – 1.95 (m, 1H), 1.80 – 1.76 (m, 1H), 1.75 – 1.61 (m, 6H), 1.38 – 1.33 (m, 9H), 1.28 – 1.24 (m, 14H), 1.08 (d, *J* = 7.5 Hz, 3H, H-16), 1.01 (t, *J* = 8.6 Hz, 2H), 0.95 (d, *J* = 6.2 Hz, 3H, H-15), 0.90 – 0.81 (m, 9H); ^{13}C NMR (151 MHz, DMSO) δ (ppm): 173.30 (C-10), 104.74 (C-3), 80.08 (C-12a), 77.72 (C-12), 55.59 (C-1'), 50.55 (C-5a), 43.30 (CH), 36.73 (CH), 35.97 (CH_2), 35.43 (C-9), 32.85 (CH_2), 31.31 (CH_2), 29.11 – 28.21 (C-13' – 4'), 27.28 (C-3'), 24.84 (C-14), 22.28 (CH_2), 22.11 (CH_2), 19.30 (C-15), 13.97 (C-16), 13.33 (C-16'); IR: (ATR) ν_{max}/cm^{-1} 2921, 2852, 1707, 1571, 1377, 1366, 1306, 1040, 645, 487; HRMS (APCI) *m/z* $[M + H]^+$ 570.3819 (Calcd for $C_{19}H_{32}NO_6S$: 570.3828).



9

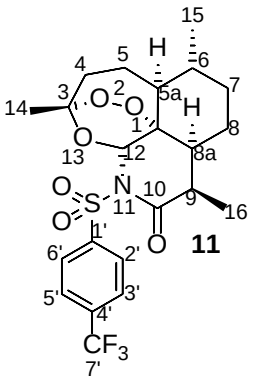
Benzenesulfonyl-azaartemisinin **9**: Compound **9** was obtained from **5** (1.0 g, 3.55 mmol), LDA and benzenesulfonyl chloride (0.7 mL, 5.33 mmol, 1.5 equiv.), and chromatography (ethyl acetate:hexane, 4:6) to give **9** as off-white crystals; yield: 400 mg (27%); mp: 147 °C; ^{1}H NMR

(600 MHz, $CDCl_3$) δ (ppm): 8.19 (d, *J* = 7.5 Hz, 2H, H-2' and H-6'), 7.59 (t, *J* = 7.4 Hz, 1H, H-4'), 7.50 (t, *J* = 7.8 Hz, 2H, H-3' and H-5'), 6.21 (s, 1H, H-12), 3.33 – 3.26 (m, 1H, H-9), 2.35 (td, *J* = 14.5, 3.9 Hz, 1H, H-4α), 2.06 – 1.99 (m, 2H), 1.84-1.76 (m, 2H), 1.72 – 1.64 (m, 1H, H-8a), 1.61 – 1.52 (m, 2H), 1.36 (dt, *J* = 11.1, 7.3 Hz, 1H, H-5a), 1.28 – 1.14 (m, 7H), 1.08 – 0.96 (m, 4H); ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 172.98 (C-10), 140.22 (C-1'), 133.68 (C-4'), 129.21 (C-2' and C-6'), 128.53 (C-3' and C-5'), 105.34 (C-3), 80.42 (C-12a), 78.70 (C-12), 51.54 (C-5a), 44.93 (C-8a), 37.36 (CH), 36.62 (CH_2), 35.92 (C-9), 33.70 (CH_2), 24.88 (CH_2), 22.42 (CH_2), 19.67 (C-15), 13.46 (C-16); IR: (ATR) ν_{max}/cm^{-1} 2921, 1702, 1413, 1199, 881, 828, 560; HRMS (APCI) *m/z* $[M + H]^+$ 422.1612 (Calcd for $C_{21}H_{28}NO_6S$: 422.1637).

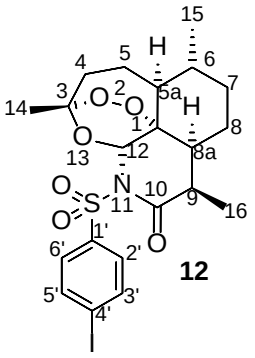


10

3-(Trifluoromethyl)benzenesulfonyl-azaartemisinin **10**: Compound **10** was obtained from **5** (1.0 g, 3.55 mmol), LDA and 3-(trifluoromethyl)benzenesulfonyl chloride (0.85 mL, 5.33 mmol, 1.5 equiv.), and chromatography (ethyl acetate:hexane, 4:6) to give **10** as white crystals; yield: 16 mg (1%); mp: 167 °C; ^1H NMR (600 MHz, $CDCl_3$) δ (ppm): 8.47 (d, *J* = 8.0 Hz, 1H, H-6'), 8.42 – 8.34 (s, 1H, H-2'), 7.86 (d, *J* = 7.8 Hz, 1H, H-4'), 7.68 (t, *J* = 7.9 Hz, 1H, H-5'), 6.21 (s, 1H, H-12), 3.33 – 3.26 (m, 1H, H-9), 2.37 – 2.30 (m, 1H, H-4α), 2.07 – 2.00 (m, 2H), 1.85 – 1.78 (m, 2H), 1.72 – 1.65 (m, 1H, H-8a), 1.59 – 1.50 (m, 2H), 1.38 – 1.33 (m, 1H), 1.26 – 1.22 (m, 1H), 1.20 (d, *J* = 7.5 Hz, 3H, H-16), 1.13 (s, 3H, H-14), 1.10 – 1.01 (m, 4H); ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 173.08 (C-10), 141.07 (C-1'), 132.78 (C-6'), 130.97 (C-7'), 130.20 (C-4'), 129.26 (C-5'), 126.13 (C-2'), 105.29 (C-3), 80.13 (C-12a), 78.67 (C-12), 51.33 (C-5a), 44.73 (C-8a), 37.21 (CH), 36.38 (CH_2), 35.84 (C-9), 33.47 (CH_2), 24.70 (C-14), 24.59 (CH_2), 22.22 (CH_2), 19.48 (C-15), 13.29 (C-16); IR: (ATR) ν_{max}/cm^{-1} 2972, 2831, 2718, 2488, 1702, 1467, 1397, 1152, 1103, 509; HRMS (APCI) *m/z* $[M + H]^+$ 490.1499 (Calcd for $C_{22}H_{27}F_3NO_6S$: 490.1511).

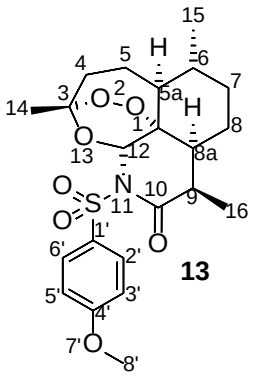


4-(Trifluoromethyl)benzenesulfonyl-azaartemisinin **11**: Compound **11** was obtained from **5** (1.0 g, 3.55 mmol), LDA and 4-(trifluoromethyl)benzenesulfonyl chloride (1.3 g, 5.33 mmol, 1.5 equiv.), and chromatography (ethyl acetate:hexane, 4:6) to give **11** as an off-white powder; yield: 306 mg (18%); mp: 168.1 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 8.32 (d, *J* = 8.3 Hz, 2H, H-2' and H-6'), 7.77 (d, *J* = 8.4 Hz, 2H, H-3' and H-5'), 6.21 (s, 1H, H-12), 3.34 – 3.26 (m, 1H, H-9), 2.41 – 2.33 (m, 1H, H-4α), 2.08 – 2.00 (m, 2H), 1.86 – 1.79 (m, 2H), 1.73 – 1.67 (m, 1H, H-8a), 1.60 – 1.51 (m, 2H), 1.40 – 1.34 (m, 1H, H-5a), 1.27 – 1.21 (m, 1H), 1.20 – 1.13 (m, 6H, H-16 and H-14), 1.11 – 0.99 (m, 4H); ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 173.19 (C-10), 143.62 (C-1'), 135.35 (C-4'), 129.83 (C-2'), 129.79 (C-6'), 125.72 (C-3'), 125.69 (C-5'), 124.21 (C-7'), 105.44 (C-3), 80.35 (C-12a), 78.89 (C-12), 51.50 (C-5a), 44.89 (C-8a), 37.39 (CH), 36.57 (CH_2), 35.99 (C-9), 33.65 (CH_2), 24.88 (C-14), 24.87 (CH_2), 22.43 (CH_2), 19.65 (C-15), 13.44 (C-16); IR: (ATR) ν_{max}/cm^{-1} 2984, 2855, 1705, 1366, 1166, 711, 554, 429; HRMS (APCI) *m/z* [M + H]$^{+}$ 490.1497 (Calcd for $C_{22}H_{27}F_3NO_6S$: 490.1511).

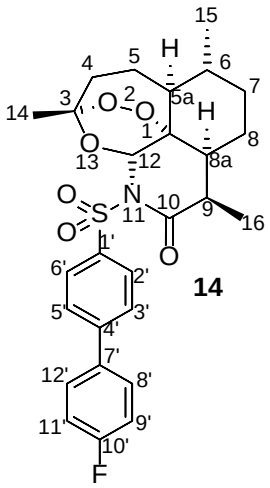


4-Iodobenzenesulfonyl-azaartemisinin **12**: Compound **12** was obtained from **5** (1.0 g, 3.55 mmol), LDA and 4-iodobenzenesulfonyl chloride (1.61 g, 5.33 mmol, 1.5 equiv.), and chromatography (ethyl acetate:hexane, 4:6) to give **12** as a pale pink powder; yield: 431 mg (22%); mp: 182.8 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 7.90 – 7.85 (m, 4H, H-2', H-3, H-5' and H-6'), 6.18 (s, 1H, H-12), 3.32 – 3.27 (m, 1H, H-9), 2.36 (dt, *J* = 14.5, 3.8 Hz, 1H, H-4α), 2.07 – 2.00 (m, 2H), 1.84 – 1.78 (m, 2H), 1.70 – 1.66 (m, 1H, H-8a), 1.58 – 1.50 (m, 2H), 1.36 (td, *J* = 11.0, 6.4 Hz, 1H, H-5a), 1.25 – 1.16 (m, 7H), 1.06 – 0.96 (m, 4H); ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 172.93 (C-10), 139.69 (C-1'), 137.66 (C-2' and C-6'), 130.50 (C-3' and C-5'),

105.23 (C-3), 101.49 (C-4'), 80.18 (C-12a), 78.59 (C-12), 51.36 (C-5a), 44.78 (C-8a), 37.20 (CH), 36.44 (CH_2), 35.75 (C-9), 33.49 (CH_2), 24.82 (C-14), 24.71 (CH_2), 22.24 (CH_2), 19.49 (C-15), 13.26 (C-16); IR: (ATR) ν_{max}/cm^{-1} 2934, 2854, 1704, 1567, 1385, 1235, 1054, 1018, 895, 601, 561; HRMS (APCI) *m/z* $[M + H]^+$ 548.0586 (Calcd for $C_{21}H_{27}INO_6S$: 548.0603).

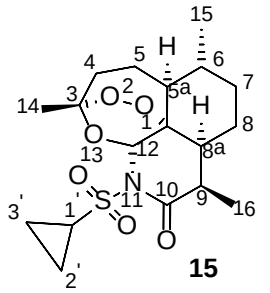


4-Methoxybenzenesulfonyl-azaartemisinin **13**: Compound **13** was obtained from **5** (1.0 g, 3.55 mmol), LDA and 4-methoxybenzenesulfonyl chloride (1.1 g, 5.33 mmol, 1.5 equiv.), and chromatography (ethyl acetate:hexane, 4:6) to give **13** as off-white crystals; yield: 19 mg (12%); mp 159 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 8.11 (d, *J* = 9.0 Hz, 2H, H-2' and H-6'), 6.95 (d, *J* = 9.0 Hz, 2H, H-3' and H-5'), 6.19 (s, 1H, H-12), 3.86 (s, 2H, H-8'), 3.32 – 3.25 (m, 1H, H-9), 2.38 – 2.29 (m, 1H, H-4α), 2.07 – 2.00 (m, 2H), 1.83 – 1.73 (m, 2H), 1.70 – 1.55 (m, 3H), 1.36 (dt, *J* = 11.0, 7.5 Hz, 1H, H-5a), 1.25 – 1.20 (m, 4H), 1.17 (d, *J* = 7.4 Hz, 3H, H-16), 1.05 – 0.95 (m, 4H); ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 172.79 (C-10), 163.59 (C-4'), 131.54 (C-1'), 131.45 (C-2' and C-6'), 113.48 (C-3' and C-5'), 105.12 (C-3), 80.23 (C-12a), 78.40 (C-12), 55.64 (C-8'), 51.43 (C-5a), 44.87 (C-8a), 37.18 (CH), 36.50 (CH_2), 35.66 (C-9), 33.54 (CH_2), 24.94 (C-14), 24.73 (CH_2), 22.23 (CH_2), 19.51 (C-15), 13.26 (C-16); IR: (ATR) ν_{max}/cm^{-1} 2965, 2828, 2717, 2487, 1702, 1397, 1313, 1152, 1026, 695, 508; HRMS (APCI) *m/z* $[M + H]^+$ 452.1714 (Calcd for $C_{22}H_{30}NO_7S$: 452.1742).



14

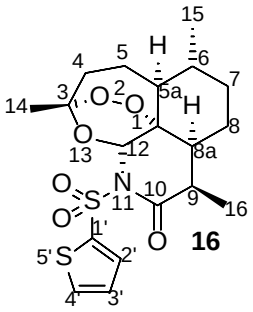
4-(4-fluorophenyl)benzene-1-sulfonyl-azaartemisinin **14**: Compound **14** was obtained from **5** (1.0 g, 3.55 mmol), LDA and 4-(4-fluorophenyl)benzene-1-sulfonyl chloride (1.16 g, 5.33 mmol, 1.5 equiv.), and chromatography (ethyl acetate:hexane, 4:6) to give **14** as pale yellow crystals; yield: 21 mg (1%); mp: 142 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 8.23 (d, *J* = 8.5 Hz, 2H, H-2' and H-6'), 7.65 (d, *J* = 8.6 Hz, 2H, H-3' and H-5'), 7.56 (dd, *J* = 8.8, 5.2 Hz, 2H, H-8' and H-12'), 7.18 – 7.13 (m, 2H, H-9' and H-11'), 6.23 (s, 1H, H-12), 3.34 – 3.28 (m, 1H, H-9), 2.36 (dt, *J* = 14.6, 3.8 Hz, 1H, H-4α), 2.06 – 2.02 (m, 2H), 1.84 – 1.79 (m, 2H), 1.71 – 1.67 (m, 1H, H-8a), 1.61 – 1.53 (m, 2H), 1.38 (dd, *J* = 11.4, 5.9 Hz, 1H, H-5a), 1.26 – 1.22 (m, 4H), 1.19 (d, *J* = 7.4 Hz, 3H, H-16), 1.08 – 1.02 (m, 4H); ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 172.97 (C-10), 162.30 (C-10'), 145.41 (C-1'), 138.56 (C-4'), 135.44 (C-7'), 129.71 (C-2' and C-6'), 129.13 (C-8' and C-12'), 126.84 (C-3' and C-5'), 116.06 (C-9' and C-11'), 105.21 (C-3), 80.25 (C-12a), 78.52 (C-12), 50.86 (C-5a), 44.83 (C-8a), 37.19 (CH), 36.47 (CH_2), 35.73 (C-9), 33.51 (CH_2), 24.84 (C-14), 24.71 (CH_2), 22.25 (CH_2), 19.51 (C-15), 13.26 (C-16); IR: (ATR) ν_{max}/cm^{-1} 3014, 2936, 2748, 2518, 1703, 1603, 1472, 1244, 1145, 1084, 680, 550, 508; HRMS (APCI) *m/z* $[M + H]^+$ 516.1827 (Calcd for $C_{27}H_{31}FNO_6S$: 516.1856).



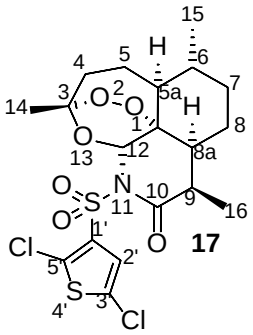
15

Cyclopropanesulfonyl-azaartemisinin **15**: Compound **15** was obtained from **5** (1.0 g, 3.55 mmol), LDA and cyclopropanesulfonyl chloride (0.55 mL, 5.33 mmol, 1.5 equiv.), and chromatography (ethyl acetate:hexane, 4:6) to give **15** as a white powder; yield: 76 mg (6%); mp: 133.1 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 5.95 (s, 1H, H-12), 3.43 – 3.35 (m, 1H, H-9), 3.22 – 3.16 (m, 1H, H-1'), 2.42 – 2.30 (m, 1H, H-4α), 2.07 – 1.98 (m, 2H), 1.82 – 1.74 (m, 2H), 1.72 – 1.66 (m,

1H, H-8a), 1.58 – 1.46 (m, 3H), 1.44 – 1.35 (m, 5H), 1.25 (d, *J* = 7.5 Hz, 3H, H-16), 1.18 – 0.99 (m, 7H); ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 173.52 (C-10), 105.18 (C-3), 80.12 (C-12a), 77.94 (C-12), 51.38 (C-5a), 44.89 (C-8a), 37.12 (CH), 36.42 (CH_2), 35.57 (C-9), 33.56 (C-1'), 33.49 (CH_2), 25.41 (C-14), 24.69 (CH_2), 22.17 (CH_2), 19.49 (C-15), 13.27 (C-16), 6.73 (C-3'), 6.42 (C-2'); IR: (ATR) ν_{max}/cm^{-1} 2966, 2940, 1710, 1350, 1273, 1204, 1129, 975, 927, 707, 594, 492; HRMS (APCI) *m/z* $[M + H]^+$ 386.1619 (Calcd for $C_{18}H_{28}NO_6S$: 386.1637).

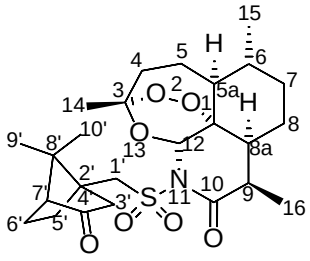


2'-Thiophenesulfonylazaartemisinin **16**: Compound **16** was obtained from **5** (1.0 g, 3.55 mmol), LDA and 2-thiophenesulfonyl chloride (0.97 g, 5.33 mmol, 1.5 equiv.), and chromatography (ethyl acetate:hexane, 4:6) to give **16** as a brown powder; yield: 156 mg (10%); mp: 150.1 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 7.94 (d, *J* = 3.7 Hz, 1H, H-2'), 7.66 (d, *J* = 4.9 Hz, 1H, H-4'), 7.09 – 7.05 (m, 1H, H-3'), 6.18 (s, 1H, H-12), 3.38 – 3.27 (m, 1H, H-9), 2.35 (td, *J* = 14.6, 3.9 Hz, 1H, H-4α), 2.07 – 1.96 (m, 2H), 1.83 – 1.75 (m, 2H), 1.72 – 1.65 (m, 1H, H-8a), 1.59 – 1.53 (m, 2H), 1.40 – 1.32 (m, 1H, H-5a), 1.26 – 1.19 (m, 4H), 1.15 (s, 3H, H-14), 1.07 – 0.99 (m, 4H); ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 173.08 (C-10), 140.47 (C-1'), 135.33 (C-2'), 133.68 (C-4'), 126.61 (C-3'), 105.21 (C-3), 80.29 (C-12a), 79.09 (C-12), 51.32 (C-5a), 44.53 (C-8a), 37.22 (CH), 36.41 (CH_2), 35.94 (C-9), 33.57 (CH_2), 24.74 (C-14), 24.71 (CH_2), 22.27 (CH_2), 19.51 (C-15), 13.47 (C-16); IR: (ATR) ν_{max}/cm^{-1} 2976, 2879, 1706, 1365, 1142, 1128, 1013, 696, 670, 596, 551, 466; HRMS (APCI) *m/z* $[M + H]^+$ 428.1178 (Calcd for $C_{19}H_{26}NO_6S_2$: 428.1201).



2,5-Dichlorothiophenesulfonyl-azaartemisinin **17**: Compound **17** was obtained from **5** (1.0 g, 3.55 mmol), LDA and 2,5-dichlorothiophenesulfonyl chloride (1.3 g, 5.33 mmol, 1.5 equiv.), and chromatography (ethyl acetate:hexane, 4:6) to give **17** as a white powder; yield: 271 mg (15%);

mp: 168.3 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 7.31 (s, 1H, H-2'), 6.06 (s, 1H, H-12), 3.33 – 3.24 (m, 1H, H-9), 2.36 – 2.27 (m, 1H, H-4α), 2.00 – 1.91 (m, 2H), 1.75 – 1.66 (m, 2H), 1.65 – 1.60 (m, 1H, H-8a), 1.53 – 1.45 (m, 2H), 1.33 – 1.26 (m, 4H), 1.10 – 1.03 (m, 4H), 1.00 – 0.90 (m, 4H); ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 172.68 (C-10), 135.08 (C-1'), 132.11 (C-5'), 128.01 (C-2'), 126.68 (C-3'), 105.44 (C-3), 80.32 (C-12a), 79.26 (C-12), 51.42 (C-5a), 44.60 (C-8a), 37.18 (CH), 36.37 (CH_2), 33.55 (C-9), 24.91 (C-14), 24.66 (CH_2), 22.24 (CH_2), 19.51 (C-15), 13.25 (C-16); IR: (ATR) ν_{max}/cm^{-1} 2976, 2879, 1642, 1515, 1425, 1212, 1155, 1090, 1065, 646, 523, 483; HRMS (APCI) *m/z* $[M + H]^+$ 496.0360 (Calcd for $C_{19}H_{24}Cl_2NO_6S_2$: 496.0422).



18

10-Camphoryl-azaartemisinin **18**: Compound **18** was obtained from **5** (1.0 g, 3.55 mmol), LDA and (1S)-(+)-10-camphorsulfonyl chloride (1.3 g, 5.33 mmol, 1.5 equiv.), and chromatography (ethyl acetate:hexane, 4:6) to give **18** as a white powder; yield: 484 mg (27%); mp: 186 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 6.06 (s, 1H, H-12), 4.27 (d, *J* = 14.7 Hz, 1H, H-1'α), 3.40 – 3.33 (m, 2H), 2.60 – 2.53 (m, 1H, H-6'α), 2.43 – 2.36 (m, 2H, H-4α), 2.10 (t, *J* = 4.5 Hz, 1H, H-7'), 2.07 – 1.98 (m, 3H), 1.94 (d, *J* = 18.4 Hz, 1H, H-3'β), 1.81 – 1.75 (m, 2H), 1.74 – 1.66 (m, 2H), 1.57 – 1.48 (m, 2H), 1.44 (s, 3H, H-14), 1.43 – 1.34 (m, 2H), 1.26 – 1.20 (m, 7H), 1.04 – 0.98 (m, 4H), 0.94 (s, 3H, H-9'); ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 214.15 (C-4'), 173.60 (C-10), 105.22 (C-3), 80.31 (C-12a), 78.62 (C-12), 59.26 (C-2'), 54.27 (C-1'), 51.37 (C-5a), 47.44 (C-8'), 44.35 (C-8a), 43.24 (C-7'), 42.46 (C-3'), 37.18 (CH), 36.39 (CH_2), 35.94 (C-9), 33.61 (CH_2), 26.81 (C-5'), 25.86 (C-6'), 25.37 (C-14), 24.65 (CH_2), 22.23 (CH_2), 20.19 (C-9'), 19.76 (C-10'), 19.50 (C-15), 13.59 (C-16); IR: (ATR) ν_{max}/cm^{-1} 2935, 1743, 1705, 1571, 1405, 1356, 1014, 855, 645, 621; HRMS (APCI) *m/z* $[M + H]^+$ 496.2383 (Calcd for $C_{25}H_{38}NO_7S$: 496.2369).

Biological screening

Malaria parasite proliferation can be directly monitored in their intra-erythrocytic environment through detecting and monitoring DNA replication (without background forthcoming from erythrocytes, which lack DNA). SYBR Green I is a fluorescent dye that interacts with DNA, therefore a correlation between DNA content (SYBR Green I signal) and parasitaemia can be used to monitor decrease in parasitaemia as a measurement of inhibition of parasite proliferation.

P. falciparum parasites were maintained at 37 °C in human erythrocytes (O^+) suspended in complete culture medium (RPMI 1640 medium supplemented with 25 mM HEPES, 20 mM D-glucose, 200 µM hypoxanthine, 0.2% sodium bicarbonate, 24 µg/ml gentamycin and 0.5% AlbuMAX II) in a gaseous environment of 90% N_2, 5% O_2, and 5% CO_2 according to the method established by Verlinden and co-workers.[45] *In vitro* ring-stage intra-erythrocytic *P. falciparum* parasite cultures (200 µL at 1% haematocrit, 1% parasitaemia) were treated with the synthesized compounds. The controls for this assay included CQ disulphate (1 µM, as positive control) and complete RPMI media (as negative control), grown for 96 h at 37 °C under the 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 µL/mL 10 000 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 CQ-treated red blood cell 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.[46,47] Data obtained were analyzed in Excel 2007 and sigmoidal dose-response curves were plotted using GraphPad 5.0. Experiments are always performed in triplicate, and repeated mainly 3 times.

The cytotoxic effects of the compounds were tested by Sulforhodamine B (SRB) assay on the WI-38 cell line. The SRB assay was developed by Skehan and colleagues[48] to measure drug-induced cytotoxicity and cell proliferation. Its principle is based on the ability of the protein dye sulforhodamine B (Acid Red 52) to bind electrostatically in a pH-dependent manner to protein basic amino acid residues of trichloroacetic acid-fixed cells. Under mild acidic conditions it binds to the fixed cellular protein, while under mild basic conditions it can be extracted from cells and solubilized for measurement. The SRB assay is performed at the CSIR in accordance with the protocol of the Drug Evaluation Branch, NCI, and the assay has been adopted for this screen. The WI-38 cell line - normal Human Fetal Lung Fibroblast (HFLF) from ECACC was routinely maintained as a monolayer cell culture at 37 ºC, 5% CO_2, 95% air and 100% relative humidity in EMEM containing 10% foetal bovine serum, 2 mM L-glutamine and 50 µg/ml gentamicin. For the screening experiment, the cells (21 – 50 passages) were inoculated in a 96-well microtiter plates at plating densities of 10000 cells per well and were incubated for 24 h. After 24 h the cells were treated with the synthesized compounds which were previously dissolved in DMSO and diluted in medium to produce 5 concentrations. Cells without any drug addition served as control. The blank contains complete medium without cells. Emetine was used as a standard. The plates were incubated for 48 h after addition of the synthesized compounds. Viable cells

were fixed to the bottom of each well with cold 50% trichloroacetic acid, washed, dried and dyed by SRB. Unbound dye was removed and protein-bound dye was extracted with 10 mM Tris base for optical density determination at the wavelength 540 nm using a multiwell spectrophotometer. Data analysis was performed using GraphPad Prism software, where 50% of cell growth inhibition (IC_{50}) was determined by non-linear regression.

Gametocytes were produced as per Reader and co-workers.[49] The luciferase reporter assay was established to enable accurate, reliable and quantifiable investigations of the stage-specific action of gametocytocidal compounds for each of the early- and late-stage gametocyte marker cell lines; NF54-PfS16-GFP-Luc and NF54-Mal8p1.16-GFP-Luc. Drug assays were set up on day 5 and 10 (representing >90% of either early stage I/II/III or mature stage IV/V gametocytes, respectively). In each instance, assays were set up using a 2 – 3% gametocytaemia, 1.5% haematocrit culture and 48 h for early-stage gametocytes and 72 h for late-stage gametocytes drug pressure in a gas chamber (90% N_2, 5% O_2, and 5% CO_2) at 37ºC. Luciferase activity was determined in 20 µL parasite lysates by adding 50 µL luciferin substrate (Promega Luciferase Assay System) at room temperature and detection of resultant bioluminescence at an integration constant of 10 s with the GloMax®-Multi+ Detection System with Instinct® Software.
The pLDH assay was set up on day 10 late-stage gametocytes (>90% mature stage IV/V gametocytes). The assays were set up using a 1% gametocytaemia and 2% haematocrit culture and 72 h drug pressure in a gas chamber (90% N_2, 5% O_2, and 5% CO_2) at 37ºC, after which the media was replaced with fresh media and the culture incubated for another 72 h. Gametocyte viability was measured by lysing 20 µL of the parasite suspension with 100 µL of a Malstat reagent [0.21% v/v Triton-100; 222 mM L-(+)-lactic acid; 54.5 mM Tris; 0.166 mM 3-acetylpyridine adenine dinucleotide (APAD; Sigma-Aldrich); adjusted to pH 9 with 1 M NaOH] followed by addition of 25 µL PES/NBT [1.96 mM nitro blue tetrazoliumchloride NBT; 0.239 mM phenazine ethosulphate (PES)]. Absorbance was measured with a Multiskan Ascent 354 multiplate scanner (Thermo Labsystems, Finland) at 620 nm.

Disclaimer

Any opinion, findings and conclusions, or recommendations expressed in this article are those of the author(s) and therefore the NRF does not accept any liability in regard thereto.

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REFERENCES

(1) WHO; WHO: Geneva http://www.who.int/malaria/publications/world_malaria_report_2016, 2016.

(2) Wellems, T. E.; Plowe, C. V. *J. Infect. Dis.* **2001**, *184*, 770-776.

(3) Wootton, J. C.; Feng, X.; Ferdig, M. T.; Cooper, R. A.; Mu, J.; Baruch, D. I.; Magill, A. J.; Su, X.-z. *Nature* **2002**, *418*, 320-323.

(4) Wongsrichanalai, C.; Pickard, A. L.; Wernsdorfer, W. H.; Meshnick, S. R. *Lancet Infect. Dis.* **2002**, *2*, 209-218.

(5) Roper, C.; Pearce, R.; Nair, S.; Sharp, B.; Nosten, F. o.; Anderson, T. *Science* **2004**, *305*, 1124-1124.

(6) Qinghaosu Antimalaria Coordinating Research, G. *Chinese Medical Journal* **1979**, *92*, 811-6.

(7) Klayman, D. L. *Science* **1985**, *228*, 1049-1055.

(8) Li, Y.; Wu, Y.-L. *Curr. Med. Chem.* **2003**, *10*, 2197-2230.

(9) Zhang, J.-F. *A detailed chronological record of Project 523 and the discovery and development of qinghaosu (artemisinin)*; Strategic Book Publishing: China, 2005.

(10) Woodrow, C. J.; Haynes, R. K.; Krishna, S. *Postgrad. Med. J.* **2005**, *81*, 71-78.

(11) Cui, L.; Su, X.-z. *Expert Rev. Anti. Infect. Ther.* **2009**, *7*, 999-1013.

(12) Li, Y. *Reprinted from Kexue Tongbao.* **1979**, *24*, 667-669.

(13) Li, Y.; Yu, P.-L.; Chen, Y.-X.; Li, L.-Q.; Gai, Y.-Z.; Wang, D.-S.; Zheng, Y.-P. *Acta Pharmaceutica Sinica* **1981**, *16*, 429.

(14) China Cooperative Research, G. *J. Trad. Chin. Med.* **1982**, *2*, 1-16.

(15) White, N. J.; Olliaro, P. *Med. Trop. (Mars).* **1997**, *58*, 54-56.

(16) Nosten, F.; White, N. J. *Am. J. Trop. Med. Hyg.* **2007**, *77*, 181-192.

(17) Aweeka, F. T.; German, P. I. *Clin. Pharmacokinet.* **2008**, *47*, 91-102.

(18) Eastman, R. T.; Fidock, D. A. *Nature Rev. Microbiol.* **2009**, *7*, 864-874.

(19) Phyo, A. P.; Nkhoma, S.; Stepniewska, K.; Ashley, E. A.; Nair, S.; McGready, R.; ler Moo, C.; Al-Saai, S.; Dondorp, A. M.; Lwin, K. M. *The Lancet* **2012**, *379*, 1960-1966.

(20) Lubell, Y.; Dondorp, A.; Guèrin, P. J.; Drake, T.; Meek, S.; Ashley, E.; Day, N. P. J.; White, N. J.; White, L. J. *Malar. J.* **2014**, *13*, 1.

(21) Ariey, F.; Witkowski, B.; Amaratunga, C.; Beghain, J.; Langlois, A.-C.; Khim, N.; Kim, S.; Duru, V.; Bouchier, C.; Ma, L. *Nature* **2014**, *505*, 50-55.

(22) Mbengue, A.; Bhattacharjee, S.; Pandharkar, T.; Liu, H.; Estiu, G.; Stahelin, R. V.; Rizk, S. S.; Njimoh, D. L.; Ryan, Y.; Chotivanich, K. *Nature* **2015**, *520*, 683-687.

(23) VanHook, A. M. *Sci. Signal.* **2015**, *8*, ec118-ec118.

(24) Paloque, L.; Ramadani, A. P.; Mercereau-Puijalon, O.; Augereau, J.-M.; Benoit-Vical, F. o. *Malaria J.* **2016**, *15*, 1.

(25) Kim, J. H.; Chu, S. C.; Gramlich, J. L.; Pride, Y. B.; Babendreier, E.; Chauhan, D.; Salgia, R.; Podar, K.; Griffin, J. D.; Sattler, M. *Blood* **2005**, *105*, 1717-1723.

(26) Liu, J.; Zhou, J.; Xing, D. *PLoS One* **2012**, *7*, e33817.

(27) Okoh, V. O.; Felty, Q.; Parkash, J.; Poppiti, R.; Roy, D. *PLoS One* **2013**, *8*, e54206.

(28) Burrows, J. N.; van Huijsduijnen, R. H.; Möhrle, J. J.; Oeuvray, C.; Wells, T. N. C. *Malaria J.* **2013**, *12*, 1.

(29) Chotivanich, K.; Sattabongkot, J.; Udomsangpetch, R.; Looareesuwan, S.; Day, N. P. J.; Coleman, R. E.; White, N. J. *Antimicrob. Agents Chemother.* **2006**, *50*, 1927-1930.

(30) Bousema, J. T.; Schneider, P.; Gouagna, L. C.; Drakeley, C. J.; Tostmann, A.; Houben, R.; Githure, J. I.; Ord, R.; Sutherland, C. J.; Omar, S. A. *J. Infect. Dis.* **2006**, *193*, 1151-1159.

(31) Bousema, T.; Okell, L.; Shekalaghe, S.; Griffin, J. T.; Omar, S.; Sawa, P.; Sutherland, C.; Sauerwein, R.; Ghani, A. C.; Drakeley, C. *Malaria J.* **2010**, *9*, 1.

(32) Bach, R. D.; Dmitrenko, O. *J. Am. Chem. Soc.* **2006**, *128*, 4598-4611.

(33) Torok, D. S.; Ziffer, H.; Meshnick, S. R.; Pan, X.-Q.; Ager, A. *J. Med. Chem.* **1995**, *38*, 5045-5050.

(34) Haynes, R. K. *Curr. Opin. Infect. Dis.* **2001**, *14*, 719-726.

(35) Haynes, R. K.; Wong, H.-N.; Lee, K.-W.; Lung, C.-M.; Shek, L. Y.; Williams, I. D.; Croft, S. L.; Vivas, L.; Rattray, L.; Stewart, L. *ChemMedChem* **2007**, *2*, 1464-1479.

(36) Avery, M. A.; Bonk, J. D.; Chong, W. K. M.; Mehrotra, S.; Miller, R.; Milhous, W.; Goins, D. K.; Venkatesan, S.; Wyandt, C. *J. Med. Chem.* **1995**, *38*, 5038-5044.

(37) Mekonnen, B.; Ziffer, H. *Tetrahedron Lett.* **1997**, *38*, 731-734.

(38) Mekonnen, B.; Weiss, E.; Katz, E.; Ma, J.; Ziffer, H.; Kyle, D. E. *Bioorg. Med. Chem.* **2000**, *8*, 1111-1116.

(39) Katz, E.; Ma, J.; Kyle, D.; Ziffer, H. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 2969-2972.

(40) Akinboye, E., S.; Bakare, O. *J. Nat. Prod.* **2011**, *4*.

(41) Schneider, P.; Bousema, J. T.; Gouagna, L. C.; Otieno, S.; Van de Vegte-Bolmer, M.; Omar, S. A.; Sauerwein, R. W. *Am. J. Trop. Med. Hyg.* **2007**, *76*, 470-474.

(42) Hallett, R. L.; Sutherland, C. J.; Alexander, N.; Ord, R.; Jawara, M.; Drakeley, C. J.; Pinder, M.; Walraven, G.; Targett, G. A. T.; Alloueche, A. *Antimicrob. Agents Chemother.* **2004**, *48*, 3940-3943.

(43) Price, R. N.; Nosten, F.; Luxemburger, C.; Ter Kuile, F. O.; Paiphun, L.; Chongsuphajaisiddhi, T.; White, N. J. *The Lancet* **1996**, *347*, 1654-1658.

(44) Sutherland, C. J.; Ord, R.; Dunyo, S.; Jawara, M.; Drakeley, C. J.; Alexander, N.; Coleman, R.; Pinder, M.; Walraven, G.; Targett, G. A. T. *PLoS Med* **2005**, *2*, e92.

(45) Verlinden, B. K.; Niemand, J.; Snyman, J.; Sharma, S. K.; Beattie, R. J.; Woster, P. M.; Birkholtz, L.-M. *J. Med. Chem.* **2011**, *54*, 6624-6633.

(46) Smilkstein, M.; Sriwilaijaroen, N.; Kelly, J. X.; Wilairat, P.; Riscoe, M. *Antimicrob. Agents Chemother.* **2004**, *48*, 1803-1806.

(47) Johnson, J. D.; Dennull, R. A.; Gerena, L.; Lopez-Sanchez, M.; Roncal, N. E.; Waters, N. C. *Antimicrob. Agents Chemother.* **2007**, *51*, 1926-1933.

(48) Skehan, P.; Storeng, R.; Scudiero, D.; Monks, A.; McMahon, J.; Vistica, D.; Warren, J. T.; Bokesch, H.; Kenney, S.; Boyd, M. R. *J. Natl. Cancer Inst.* **1990**, *82*, 1107-1112.

(49) Reader, J.; Botha, M. t.; Theron, A.; Lauterbach, S. B.; Rossouw, C.; Engelbrecht, D.; Wepener, M.; Smit, A. l.; Leroy, D.; Mancama, D. *Malar. J.* **2015**, *14*, 213.

CHAPTER 5
Activities of *N*-sulfonyl-11-azaartemisinin derivatives against the apicomplexan parasite *Neospora caninum* and comparative cytotoxicities – Article 3

This chapter contains the manuscript entitled “Activities of N-sulfonyl-11-azaartemisinin derivatives against the apicomplexan parasite Neospora caninum and comparative cytotoxicities” that is yet to be submitted to Bioorganic & Medicinal Chemistry Letters. This manuscript contains the aim, background, experimental details and results of the compounds that were synthesized in this study. The manuscript was also prepared according to the standards set out in the author information pack available in the author’s guidelines at: https://www.elsevier.com/journals/bioorganic-and-medicinal-chemistry-letters/0960-894X/guide-for-authors

Activities of *N*-sulfonyl-11-azaartemisinin derivatives against the apicomplexan parasite *Neospora caninum* and comparative cytotoxicities.

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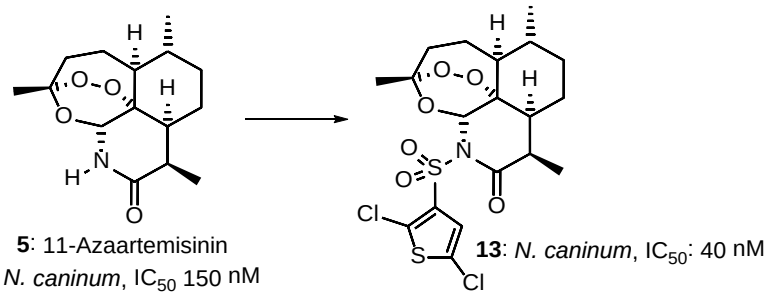
Previously synthesized *N*-sulfonyl-11-azaartemisinin derivatives were evaluated *in vitro* against *Neospora caninum*. The compounds were also screened *in vitro* against three cancer cell lines consisting of TK-10 (renal), UACC-62 (melanoma) and MCF-7 (breast).



5: 11-Azaartemisinin
N. caninum, IC_{50} 150 nM

13: *N. caninum*, IC_{50}: 40 nM

Abstract

Activities of *N*-sulfonyl-11-azaartemisinins were evaluated *in vitro* against *Neospora caninum,* a protozoan parasite afflicting dogs and cattle. As neosporosis induces abortion in dairy and beef cattle, it is of considerable economic importance. Although drug regimens are available for controlling or treating the disease, the number of drugs available is limited, and a major shortfall is the persistence of drug residues in milk and meat products of treated cattle. In an effort to enhance drug options for treatment, we have turned our attention to the naturally occurring peroxide artemisinin and its derivatives that are active against apicomplexan parasites, in particular *Plasmodia* species. 11-Azaartemisinin was converted into *N*-sulfonyl-11-azaartemisinins as previously described and screened. Compound **13** presented the best IC_{50} value of 40 nM. The compounds were also screened *in vitro* against three cancer cell lines consisting of TK-10 (renal), UACC-62 (melanoma) and MCF-7 (breast). Overall, most compounds in the series were found to be inactive against all cancer cell lines.

Keywords: *Neospora caninum, azaartemisinin, artemisinin, apicomplexan, antitumor*

Neospora caninum (*N*. *caninum*) is an obligate intracellular coccidian protozoan parasite of the phylum Apicomplexa[1] causing neosporosis which induces abortion in cattle. Losses to both the dairy and beef industries worldwide[2] is estimated to be 2 – 5% annually on most farms but can reach as high as 20%.[3, 4] The global economic impact of this disease is estimated to be approximately US$ 1.3 billion.[5]

The life cycle of *N*. *caninum* involves both sexual reproduction that takes place in a definitive host such as canids and asexual reproduction that takes place in an intermediate host such as cattle.[6] Infected canids shed the unsporulated oocysts in their faeces,[7] where the oocyst will sporulate to contain two sporocysts of which each contains four sporozoites.[8] These sporulated oocysts are ingested by cattle during feeding on contaminated pasture or water and thus become infected. Once the oocysts are ingested, sporozoites are released in the gut, invade the gut wall and transform into tachyzoites. The tachyzoites replicate rapidly through endodyogeny and infect different cells types in the host including fibroblast, neural, endothelial, liver, macrophage, vascular and muscle cells.[8, 9] Tachyzoites differentiate into bradyzoites which form cysts in tissues characteristically found in neural (spinal cord and brain) or skeletal muscle cells.[10] Once a tissue cyst has formed it can persist for life without causing any significant clinical manifestations.[11] The life cycle of *N*. *caninum* is completed when contaminated meat containing cysts are consumed by canids.[12]

Infection in cattle occur either by horizontal or vertical infection. Horizontal infection occurs when the definitive host excretes the sporulated oocysts that thereafter are consumed by the intermediate host, whereas vertical infection (congenital infection) occurs during pregnancy.[13, 14] Vertical transmission from dam to foetus is highly effective where up to 95% of calves born from infected dams are also born infected[13] which as a consequence sustains the level of infection in a herd.[15] The precise mechanism of transplacental transmission and foetal loss is poorly understood. However, acute infection in the intermediate host is caused by the tachyzoites which are distributed through the blood stream and lymphatic system and can trigger an immune response. Tachyzoites rupture the host cells through active replication which will lead to cell death.[16] Most *Neospora*-infected adult cattle do not exhibit clinical signs of the disease; the only physical sign in serologically positive adults is weight loss.[17] The majority of infected dams have normal pregnancies, but are more likely to abort than uninfected dams, especially if they are infected for the first time in early pregnancy. Infected dams can remain infected for their entire lifespan and can also infect their offspring in consecutive pregnancies.[18] Offspring born with neosporosis tend to be weak, have poor balance and are unable to stand, and also may have congenital neurological effects.[6] Diagnosis of *Neospora*-related abortion is usually made by pathology of aborted foetuses.[19]

There are a number of control options[20-23] available for neosporosis infection.[24] Although the test-and-cull approach is the most popular,[25] this is expensive[3] especially if a large part of the herd is infected.[26] Treatment relies on a limited number of drugs. Coccidiostatic drugs such as sulfonamides, antifolates and antibiotics are efficacious against different stages of the parasite *in vitro*,[27] but cost prohibits widespread use. Although ponazuril is effective against *N*. *caninum* in mice and cattle,[28, 29] it is toxic.[30] Other compounds that have been evaluated against neosporosis in animal models include sulfadiazine, amprolium,[31] miltefosine[32] and the pentamidines arylimidamide and nitazoxanide, all with differing results.[33, 34] A concern with the use of these compounds is the harmful effect they may have on milk and meat products,[35] especially if used in prophylaxis. The long-term treatment inherent in prophylaxis requires an extended withdrawal period prior to slaughter.[13, 23, 35] Clearly, the identification of more effective compounds that do not experience the foregoing problems is urgently required.

Artemisinin **1** and its derivatives (Figure 1), apart from their proven efficacy as antimalarial drugs, are active against other apicomplexan parasites including *Toxoplasma* and *Babesia* species.[5] Artemisinin is effective against *Eimeria tenella*, an apicomplexan parasite responsible for haemorrhagic cecal coccidiosis in young poultry[36] and *Cryptosporidium parvum*, also an apicomplexan parasite causing cryptosporidiosis in mammals.[37]

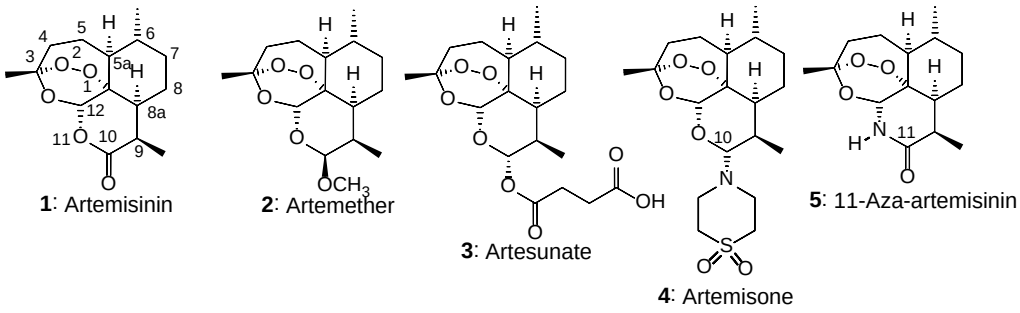
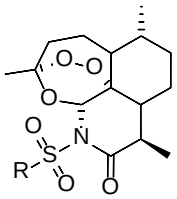


Figure 1: Artemisinin **1** and derivatives artemether **2**, artesunate **3**, artemisone **4** and 11-azaartemisinin **5**.

Artemisinin is able to reduce parasite burden of *N*. *caninum* and also slows down the intracellular multiplication of *N*. *caninum* tachyzoites.[38] Clearly, examination of newer derivatives of artemisinin already shown to be more efficacious than artemisinin against malaria is required. Artemisone **4** (Figure 1), is a newer artemisinin derivative that is highly active against *Plasmodium falciparum*[39, 40] and *Toxoplasma gondii*.[41] Artemisone exhibits inhibitory activity against *N*. *caninum* proliferation both *in vitro* and *in vivo*.[30] Totally synthetic trioxolane analogues of artemisinin possess *in vitro* activity against *N*. *caninum* of <100 nM.[42] These findings suggest that artemisinin derivatives have the potential to be used as drugs for the treatment of neosporosis. Therefore, in extending this line of research, we have screened a series of *N*-

sulfonyl-11-azaartemisinins (Figure 2). Although azaartemisinins have been synthesized since the early 1990's,[43] none have been evaluated against *N*. *caninum*. The azaartemisinins are relatively stable when compared with their first-generation artemisinin counterparts.[43] As they are unable to provide DHA as a product of metabolic breakdown characteristic of the current artemisinins, they are unlikely to possess the neurotoxicity of the current derivatives.[44-49]



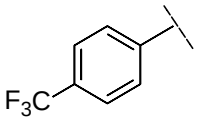
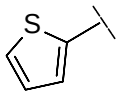
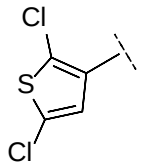
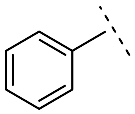
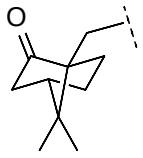
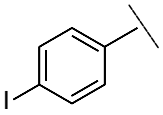
Cpd	R	Cpd	R
6	H_3C—	**11**	
7	$H_3C(H_2C)_2H_2C$—	**12**	
8	$H_3C(H_2C)_{14}H_2C$—	**13**	
9		**14**	
10			

Figure 2: *N*-Sulfonyl-11-azaartemisinins screened against *N*. *caninum* and three cancer cell lines TK-10 (renal), UACC-62 (melanoma) and MCF-7 (breast).

11-Azaartemisinin **5** and derivatives **6** – **14** were synthesized according to the literature method.[50] These were screened *in vitro* against *N. caninum* with artemisone **4** as control.[42] The IC_{50} values are summarized in Table 1 and shown in the histogram (Figure 3). Cytotoxicities of the compounds against the normal human fetal lung fibroblast WI-38 cell line are also included.

Table 1: *N*-sulfonyl-11-azaartemisinins against *N. caninum*

Compound	Neosporosis activity		Cytotoxicity IC_{50} (µM)	Selectivity Index
	IC_{50} (nM)	SE	WI-38 HFLF[a]	SI[b]
5	150	20	>100*	>667
6	230	40	>100*	>435
7	110	20	>100*	>909
8	430	100	>100*	>233
9	80	20	>100*	>1250
10	45	10	>100*	>2222
11	100	25	>100*	>1000
12	150	50	>100*	>667
13	40	10	43.20*	1080
14	90	30	>100*	>1111
Artemisone 4	20	10	ND	ND
EM[c]	ND[d]	ND	0.05	ND

[a] Cytotoxicity data. [b] Selectivity Index (SI) = IC_{50} WI-38/IC_{50} neosporosis. [c] EM = Emetine. [d] ND = not determined. * Cytotoxicities against the normal human fetal lung fibroblast WI-38 cell line as determined in one of our previous studies.

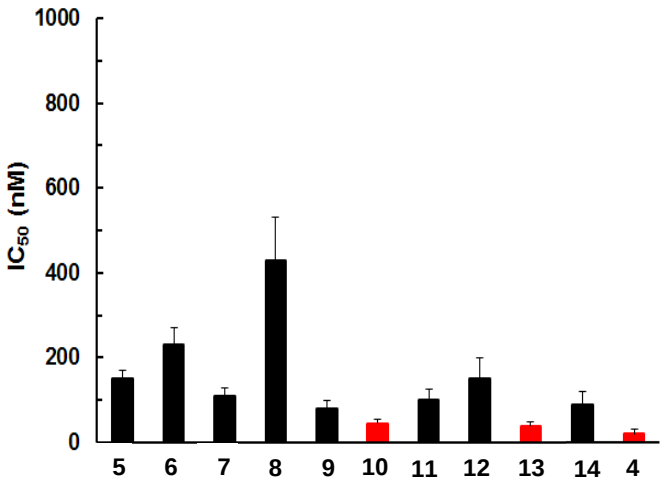


Figure 3: *In vitro* activities of the sulfonyl azaartemisinins as compared with artemisone **4**; the most active compounds are depicted in red.

The compounds are considered to be active if they present IC_{50} values below 100 nM.[42] Compounds **10** and **13** were the most active compounds of the series with IC_{50} values of 45 and 40 nM, respectively, making them some 3-4 times more active than the parent azaartemisinin **5** and some twofold less potent than artemisone **4** which had an IC_{50} value of 20 nM.[30, 42] The compounds were generally non-cytotoxic. The most active compound **13** with a cytotoxicity of 43.20 µM against the normal human fetal lung fibroblast cell line WI-38, but its selectivity index (SI) implies a very good therapeutic ratio (Table 1). Clearly, activity against *N. caninum* stems from intrinsic activity rather than systemic toxicity.

In order to assess parasitic versus intracellular activities at large, the derivatives were also screened *in vitro* against the tumour cell lines TK-10 (renal), MCF-7 (breast) and UACC-62 (melanoma) with parthenolide as control.[51] Results are depicted in Table 2. Generally, the compounds displayed poor antitumour activities. Standout activity was displayed by compound **7** against the MCF-7 cell line with an IC_{50} value of 4.21 µM, making it more active than the parthenolide standard (Table 2). Compounds **8** and **13** displayed the highest average SI values against all three cell lines. The second set of SI values demonstrates the anticancer selectivity of the compounds compared to *N. caninum*; overall, the compounds **5** – **14** show superior selectivity towards the parasite.

Table 2: Antitumor activities

Compound	Antitumor activity IC_{50} (µM)[a]			Selectivity Index Set 1[b]			Selectivity Index Set 2[c]		
	TK-10[d]	UACC-62[e]	MCF-7[f]	SI_1[g]	SI_2[h]	SI_3[i]	SI_1[j]	SI_2[k]	SI_3[l]
5	83.51	>100	>100	1.2	>1	>1	557	>667	>667
6	>100	99.95	57.83	>1	1	1.73	>435	435	251
7	60.27	70.24	37.05	1.66	1.42	2.7	548	639	337
8	32.48	22.82	4.21	3.07	4.38	23.75	76	53	10
9	69.73	>100	78.89	1.43	>1	1.27	872	>1250	986
10	95.08	>100	82.10	1.05	>1	1.22	2113	>2222	1824
11	>100	>100	77.52	>1	>1	1.29	>1000	>1000	775
12	>100	>100	>100	>1	>1	>1	>667	>667	>667
13	14.50	14.87	16	2.98	2.91	2.7	363	372	400
14	90.68	>100	>100	1.10	>1	>1	1008	>1111	>1111
PTD	6.40	14.96	5.81	ND[n]	ND	ND	ND	ND	ND

[a] Minimum concentration of compound inducing 50% cells growth inhibition. [b] Selectivity towards the cancer cell line. [c] Selectivity towards parasitic cells. [d] TK-10 - renal cancer cells. [e] UACC-62 - melanoma cancer cells. [f] MCF-7 - breast cancer cells. [g] Selectivity index (SI_1) = IC_{50} WI-38/IC_{50} TK-10. [h] Selectivity index (SI_2) = IC_{50} WI-38/IC_{50} UACC-62. [i] Selectivity index (SI_3) = IC_{50} WI-38/IC_{50} MCF-7. [j] Selectivity index (SI_1) = IC_{50} TK-10/IC_{50} neosporosis. [k] Selectivity index (SI_2) = IC_{50} UACC-62/ IC_{50} neosporosis. [l] Selectivity index (SI_3) = IC_{50} MCF-7/ IC_{50} neosporosis. [m] PTD = parthenolide. [n] ND = not determined.

In conclusion, compound **13** is the most active azaartemisinin derivative against *N*. *caninum* and also proved to be moderately active against the three tumour cell lines. It remains to be established if this compound can restrict the proliferation of *N*. *caninum* tachyzoites *in vitro*.

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REFERENCES

1. Dubey, J. P.; Carpenter, J. L.; Speer, C. A.; Topper, M. J.; Uggla, A. Newly recognized fatal protozoan disease of dogs. *JAVMA* 1988, 192, 1269-1285.
2. Larson, R. L.; Hardin, D. K.; Pierce, V. L. Economic considerations for diagnostic and control options for *Neospora caninum*-induced abortions in endemically infected herds of beef cattle. *JAVMA* 2004, 224, 1597-1604.
3. Reichel, M. P.; Ellis, J. T. If control of *Neospora caninum* infection is technically feasible does it make economic sense? *Vet. Parasitol.* 2006, 142, 23-34.
4. Reichel, M. P.; Ellis, J. T. Re-evaluating the economics of neosporosis control. *Vet. Parasitol.* 2008, 156, 361-362.
5. Reichel, M. P.; McAllister, M. M.; Pomroy, W. E.; Campero, C.; Ortega-Mora, L. M.; Ellis, J. T. Control options for *Neospora caninum* - is there anything new or are we going backwards? *Parasitol.* 2014, 141, 1455-1470.
6. Goodswen, S. J.; Kennedy, P. J.; Ellis, J. T. A review of the infection, genetics, and evolution of *Neospora caninum*: from the past to the present. *Infect. Genet. Evol.* 2013, 13, 133-150.
7. Reichel, M. P.; Ellis, J. T.; Dubey, J. P. Neosporosis and hammondiosis in dogs. *J. Small Anim. Pract.* 2007, 48, 308-312.
8. Dubey, J. P.; Barr, B. C.; Barta, J. R.; Bjerkås, I.; Björkman, C.; Blagburn, B. L.; Bowman, D. D.; Buxton, D.; Ellis, J. T.; Gottstein, B. Redescription of *Neospora caninum* and its differentiation from related coccidia. *Int. J. Parasitol.* 2002, 32, 929-946.
9. Barr, B. C.; Conrad, P. A.; Breitmeyer, R.; Sverlow, K.; Anderson, M. L.; Reynolds, J.; Chauvet, A. E.; Dubey, J. P.; Ardans, A. A. Congenital *Neospora* infection in calves born from cows that had previously aborted Neospora-infected fetuses: four cases (1990-1992). *JAVMA* 1993, 202, 113-117.
10. Dubey, J. P.; Koestner, A.; Piper, R. C. Repeated transplacental transmission of *Neospora caninum* in dogs. *JAVMA* 1990, 197, 857-860.
11. Dubey, J. P.; Lindsay, D. S. A review of *Neospora caninum* and neosporosis. *Vet. Parasitol.* 1996, 67, 1-59.
12. McAllister, M. M.; Dubey, J. P.; Lindsay, D. S.; Jolley, W. R.; Wills, R. A.; McGuire, A. M. Rapid communication: Dogs are definitive hosts of *Neospora caninum*. *Int. J. Parasitol.* 1998, 28, 1473-1479.
13. Dubey, J. P.; Schares, G.; Ortega-Mora, L. M. Epidemiology and control of neosporosis and *Neospora caninum*. *Clin. Microbiol. Rev.* 2007, 20, 323-367.
14. López-P□rez, I. C.; Collantes-Fern□ndez, E.; Rojo-Montejo, S.; Navarro-Lozano, V.; Risco-Castillo, V.; P□rez-P□rez, V.; Pereira-Bueno, J.; Ortega-Mora, L. M. Effects of *Neospora*

caninum infection at mid-gestation on placenta in a pregnant mouse model. *J. Parasitol.* 2010, 96, 1017-1020.

15. Williams, D. J. L.; Hartley, C. S.; Björkman, C.; Trees, A. J. Endogenous and exogenous transplacental transmission of *Neospora caninum* - how the route of transmission impacts on epidemiology and control of disease. *Parasitol.* 2009, 136, 1895-1900.

16. Dubey, J. P.; De Lahunta, A. Neosporosis associated congenital limb deformities in a calf. *Appl. Parasitol.* 1993, 34, 229-233.

17. Barling, K. S.; McNeill, J. W.; Thompson, J. A.; Paschal, J. C.; McCollum Iii, F. T.; Craig, T. M.; Adams, L. G. Association of serologic status for *Neospora caninum* with post-weaning weight gain and carcass measurements in beef calves. *JAVMA* 2000, 217, 1356-1360.

18. Fioretti, D. P.; Pasquali, P.; Diaferia, M.; Mangili, V.; Rosignoli, L. *Neospora caninum* infection and congenital transmission: serological and parasitological study of cows up to the fourth gestation. *J. Vet. Med. B.* 2003, 50, 399-404.

19. Buxton, D.; McAllister, M. M.; Dubey, J. P. The comparative pathogenesis of neosporosis. *Trends Parasitol.* 2002, 18, 546-552.

20. Bartels, C. J. M.; Wouda, W.; Schukken, Y. H. Risk factors for *Neospora caninum*-associated abortion storms in dairy herds in The Netherlands (1995 to 1997). *Theriogenology* 1999, 52, 247-257.

21. Quinn, H. E.; Windsor, P. A.; Kirkland, P. D.; Ellis, J. T. An outbreak of abortion in a dairy herd associated with *Neospora caninum* and bovine pestivirus infection. *Aus. Vet. J.* 2004, 82, 99-101.

22. Haddad, J. P. A.; Dohoo, I. R.; Van Leewen, J. A. A review of *Neospora caninum* in dairy and beef cattle - a Canadian perspective. *Can. Vet. J.* 2005, 46, 230-243.

23. Reichel, M. P.; Ellis, J. T. Control options for *Neospora caninum* infections in cattle - current state of knowledge. *N. Z. Vet. J.* 2002, 50, 86-92.

24. Weston, J. F.; Heuer, C.; Williamson, N. B. Efficacy of a *Neospora caninum* killed tachyzoite vaccine in preventing abortion and vertical transmission in dairy cattle. *Prev. Vet. Med.* 2012, 103, 136-144.

25. Hall, C. A.; Reichel, M. P.; Ellis, J. T. *Neospora* abortions in dairy cattle: diagnosis, mode of transmission and control. *Vet. Parasitol.* 2005, 128, 231-241.

26. Reichel, M. P.; Ellis, J. T. *Neospora caninum* - How close are we to development of an efficacious vaccine that prevents abortion in cattle? *Int. J. Parasitol.* 2009, 39, 1173-1187.

27. Lindsay, D. S.; Rippey, N. S.; Cole, R. A.; Parsons, L. C.; Dubey, J. P.; Tidwell, R. R.; Blagburn, B. L. Examination of the activities of 43 chemotherapeutic agents against *Neospora caninum* tachyzoites in cultured cells. *Am. J. Vet. Res.* 1994, 55, 976-981.

28. Kritzner, S.; Sager, H.; Blum, J. R.; Krebber, R.; Greif, G.; Gottstein, B. An explorative study to assess the efficacy of Toltrazuril-sulfone (Ponazuril) in calves experimentally infected with *Neospora caninum*. *Ann. Clin. Microbiol. Antimicrob.* 2002, 1, 1.

29. Strohbusch, M.; Müller, N.; Hemphill, A.; Krebber, R.; Greif, G.; Gottstein, B. Toltrazuril treatment of congenitally acquired *Neospora caninum* infection in newborn mice. *Parasitol. Res.* 2009, 104, 1335-1343.

30. Mazuz, M. L.; Haynes, R.; Shkap, V.; Fish, L.; Wollkomirsky, R.; Leibovich, B.; Molad, T.; Savitsky, I.; Golenser, J. *Neospora caninum*: *in vivo* and *in vitro* treatment with artemisone. *Vet. Parasitol.* 2012, 187, 99-104.

31. Lindsay, D. S.; Dubey, J. P. Effects of sulfadiazine and amprolium on *Neospora caninum* (Protozoa: Apicomplexa) infections in mice. *J. Parasitol.* 1990, 76, 177-179.

32. Debache, K.; Hemphill, A. Effects of miltefosine treatment in fibroblast cell cultures and in mice experimentally infected with *Neospora caninum* tachyzoites. *Parasitol.* 2012, 139, 934-944.

33. Schorer, M.; Debache, K.; Barna, F.; Monney, T.; Müller, J.; Boykin, D. W.; Stephens, C. E.; Hemphill, A. Di-cationic arylimidamides act against *Neospora caninum* tachyzoites by interference in membrane structure and nucleolar integrity and are active against challenge infection in mice. *Int. J. Parasitol. Drugs Drug Resist.* 2012, 2, 109-120.

34. Debache, K.; Guionaud, C.; Kropf, C.; Boykin, D.; Stephens, C. E.; Hemphill, A. Experimental treatment of *Neospora caninum*-infected mice with the arylimidamide DB750 and the thiazolide nitazoxanide. *Exp. Parasitol.* 2011, 129, 95-100.

35. Dubey, J. P.; Schares, G. Neosporosis in animals - the last five years. *Vet. Parasitol.* 2011, 180, 90-108.

36. Oh, H. G.; Youn, H. J.; Noh, J. W.; Jang, D. H.; Kang, Y. B. Anticoccidial effects of artemisinin on the *Eimeria tenella*. *Korean J. Vet. Res.* 1995, 35, 123-130.

37. Noh, J. W.; Youn, H. J.; Jang, D. H. The anticryptosporidial effects of sulfadimethoxine, toltrazuril and the extracts of herbs for *Cryptosporidium parvum* isolated from mouse in Korea. *Korean J. Lab. Anim. Sci.* 1997.

38. Kim, J.-T.; Park, J.-Y.; Seo, H.-S.; Oh, H.-G.; Noh, J.-W.; Kim, J.-H.; Kim, D.-Y.; Youn, H.-J. *In vitro* antiprotozoal effects of artemisinin on *Neospora caninum*. *Vet. Parasitol.* 2002, 103, 53-63.

39. Haynes, R. K.; Fugmann, B.; Stetter, J.; Rieckmann, K.; Heilmann, H. D.; Chan, H.-W.; Cheung, M.-K.; Lam, W.-L.; Wong, H.-N.; Croft, S. L. Artemisone - a highly active antimalarial drug of the artemisinin class. *Angew. Chem.* 2006, 118, 2136-2142.

40. Waknine-Grinberg, J. H.; Hunt, N.; Bentura-Marciano, A.; McQuillan, J. A.; Chan, H.-W.; Chan, W.-C.; Barenholz, Y.; Haynes, R. K.; Golenser, J. Artemisone effective against murine cerebral malaria. *Malar. J.* 2010, 9, 1.

41. Dunay, I. R.; Chan, W. C.; Haynes, R. K.; Sibley, L. D. Artemisone and artemiside control acute and reactivated toxoplasmosis in a murine model. *Antimicrob. Agents Chemother.* 2009, 53, 4450-4456.

42. Müller, J.; Balmer, V.; Winzer, P.; Rahman, M.; Manser, V.; Haynes, R. K.; Hemphill, A. *In vitro* effects of new artemisinin derivatives in *Neospora caninum*-infected human fibroblasts. *Int. J. Antimicrob. Agents* 2015, 46, 88-93.

43. Harmse, R.; Wong, H. N.; Smit, F.; K Haynes, R.; N'Da, D. D. The case for development of 11-aza-artemisinins for malaria. *Curr. Med. Chem.* 2015, 22, 3607-3630.

44. China Cooperative Research Group. The chemistry and synthesis of qinghaosu derivatives. *J. Trad. Chin. Med.* 1982, 2, 1-16.

45. Brewer, T. G.; Grate, S. J.; Peggins, J. O.; Weina, P. J.; Petras, J. M.; Levine, B. S.; Heiffer, M. H.; Schuster, B. G. Fatal neurotoxicity of arteether and artemether. *Am. J. Trop. Med. Hyg.* 1994, 51, 251-259.

46. Nontprasert, A.; Nosten-Bertrand, M.; Pukrittayakamee, S.; Vanijanonta, S.; Angus, B. J.; White, N. J. Assessment of the neurotoxicity of parenteral artemisinin derivatives in mice. *Am. J. Trop. Med. Hyg.* 1998, 59, 519-522.

47. Genovese, R. F.; Newman, D. B.; Brewer, T. G. Behavioral and neural toxicity of the artemisinin antimalarial, arteether, but not artesunate and artelinate, in rats. *Pharmacol. Biochem. Behav.* 2000, 67, 37-44.

48. Schmuck, G.; Haynes, R. K. Establishment of an *in vitro* screening model for neurodegeneration induced by antimalarial drugs of the artemisinin-type. *Neurotox. Res.* 2000, 2, 37-49.

49. Schmuck, G.; Roehrdanz, E.; Haynes, R. K.; Kahl, R. Neurotoxic mode of action of artemisinin. *Antimicrob. Agents Chemother.* 2002, 46, 821-827.

50. Haynes, R. K.; Wong, H. N.; Lee, K. W.; Lung, C. M.; Shek, L. Y.; Williams, I. D.; Croft, S. L.; Vivas, L.; Rattray, L.; Stewart, L. Preparation of *N*-sulfonyl- and *N*-carbonyl-11-azaartemisinins with greatly enhanced thermal stabilities: *in vitro* antimalarial activities. *ChemMedChem* 2007, 2, 1464-1479.

51. Skehan, P.; Storeng, R.; Scudiero, D.; Monks, A.; McMahon, J.; Vistica, D.; Warren, J. T.; Bokesch, H.; Kenney, S.; Boyd, M. R. New colorimetric cytotoxicity assay for anticancer-drug screening. *JNCI* 1990, 82, 1107-1112.

CHAPTER 6
Summary and conclusion

Malaria is a dreadful disease caused by the protozoan parasite of the genus *Plasmodium*, affecting quite a large number of the world population. Between 2000 and 2014 the malaria incidence rate fell by 37% globally and by 42% in the African region (WHO, 2015). During the same time period the mortality rate of malaria fell by 60% globally and by 66% in the African region (WHO, 2015). Although this is a quite an achievement, current statistics show that malaria still affects 97 countries worldwide with an estimated 214 million reported cases with 438 000 deaths in 2014 (WHO, 2015). Prevalence for malaria is highest in Africa and predominates in children under the age of 5 (WHO, 2015).

Malaria has been recorded for generations, and cases that may be attributed to malaria date as far back as 2700 BC (Cox, 2010) to its official discovery in 1880 (Bruce-Chwatt, 1981). Although there have been many scientific advances in treatment and prevention, the ability of the *Plasmodium* parasite to develop resistance has long been a thorn in the side of scientific advancement and often contributes to the degradation of treatment regimes. Multidrug resistance has rendered older generations of drugs (quinolones, antifolates, etc.) useless against the fight against malaria. In an effort to combat the spread of any further resistance, the World Health Organization (WHO) set out standard treatment guidelines involving artemisinin combination therapies (ACTs) comprising the short half-life artemisinin antimalarial drug combined with a longer half-life partner drug. The rationale is that the artemisinin within its short pharmacological half-life provides rapid reduction of the parasite burden, while the longer half-life partner drug continues the clearance when the artemisinin concentration falls to sub-therapeutic levels.(Wellems and Plowe, 2001) Unfortunately, current artemisinin derivatives are also prone to developing resistance. Even with the efforts of the WHO in banning artemisinin monotherapy and only prescribing the use of ACTs, greatly increased parasite clearance times in patients treated with ACTs have been reported (Phyo *et al*., 2012, Lubell *et al*., 2014) and genetic markers of the apparently resistant phenotype have been identified (Ariey *et al*., 2014). The poor thermal stabilities of artemisinins also raise some concern over decomposition of formulated material in countries with hot and humid weather (Haynes *et al*., 2007). Also, current derivatives like artemether, arteether and artesunate are metabolized to dihydroartemisinin (DHA) *in vivo* which gives rise to more challenges as DHA is known to display neurotoxicity in cell and animal assays (China Cooperative Research Group, 1982, Brewer *et al*., 1994, Avery *et al*., 1995, Nontprasert *et al*., 1998, Genovese *et al*., 2000, Schmuck and Haynes, 2000,

Schmuck *et al*., 2002). Hence, plasma concentrations of DHA can rapidly drop below therapeutic effective levels because it is an unstable drug that is erratically absorbed (Li, 1979, Li *et al*., 1981).

Faced with these limitations in the arsenal of viable malaria drugs, the aim of this study is to evaluate new artemisinin derivatives incapable of providing DHA on metabolism or hydrolysis *in vivo*. Therefore, the aim of this study was to prepare new *N*-sulfonyl-11-azaartemisinin derivatives, to evaluate their antimalarial activity against both CQ-sensitive and -resistant strains of *P. falciparum*, to assess their gametocytocidal activity against early- and late-stage gametocytes and to determine their toxicity against mammalian cells with the ultimate goal to finding new artemisinin derivatives for the treatment of malaria. In principle, the new compounds should also be active against other apicomplexan parasites, and accordingly the efficacy of the new derivatives was evaluated against *N*. *caninum*, with the subsequent goal of evolving new treatments for neosporosis.

Chapter 3: "The case for development of 11-azaartemisinins for malaria" is the first article to be incorporated into this thesis. This is a review article in which previously synthesized analogues of the parent compound azaartemisinin are reviewed. This review critically focusses on the synthetic accessibility and biological activities of 11-azaartemisinin derivatives in order to assess their actual suitability for the treatment of malaria. Highlights of the review include the work of Avery and co-workers who were the first to synthesize 9-desmethyl azaartemisinins and that paved the way forward for preparation of an array of various functionalized azaartemisinin derivatives (Avery *et al*., 1996). The simplified synthesis of 11-azaartemisinin by Haynes and co-workers and the preparation of thermally stable and chemically robust derivatives (Haynes *et al*., 2007) are also discussed. The remarkable yet economically modest Ugi reaction which was used by Le and co-workers to create an array of new 11-azaartemisinin derivatives (Le *et al*., 2014) is also reviewed.

It is evident from the material collated in this review that 11-azaartemisinin is more easily and economically obtained from artemisinin than is DHA. Also, the *in vitro* antimalarial activity is retained with the conversion of the lactone of artemisinin into the lactam of azaartemisinin, although activity is some five-fold less than that of DHA and its derivatives. Little is known about metabolism and pharmacokinetics of azaartemisinin, although it is clear that DHA cannot form from azaartemisinin *in vitro* or *in vivo*. Unfortunately, for this review there were no coherent data sets of *in vitro* activities and therefore comparisons of activities between the various azaartemisinin derivatives reported in the literature could not be made. Nevertheless, the *in vitro* activities of the compounds did indicate that they possess better antimalarial activities than the parent 11-azaartemisinin, and therefore of artemisinin itself. Thus, the azaartemisinins were

selected in this PhD project for evaluation for further research and development into potential drug candidates.

Chapter 4: "Antimalarial activities and cytotoxicities of *N*-sulfonyl-11-azaartemisinin derivatives" is the second article of the thesis. In this article is described the syntheses of 13 *N*-sulfonyl-azaartemisinins and their *in vitro* antimalarial activities against chloroquine (CQ) sensitive (NF54) and CQ resistant (K1 and W2) strains of intraerythrocytic *P. falciparum* (*Pf*) parasites and cytotoxicities as determined against the human fetal lung fibroblast (HFLF) WI-38 cell line. The aim of the research was to identify possible derivatives that may be suited for further development based on a comparison of activities with the current clinical artemisinin derivatives DHA, artemether and artesunate. Most clinically used antimalarial drugs target the asexual life cycle of the malaria parasite which is responsible for the clinical symptoms of the disease. The gametocytes, which are responsible for transmission from host to vector, are overlooked. Therefore, in this work, the gametocytocidal activities of the new compounds would also be assessed against the *Pf* NF54 blood-stage gametocytes using the luciferase and pLDH assays. This activity, namely assessment of transmission blocking potential, is now recommended by the WHO as an essential part of development of new antimalarial drugs.

Synthesis of the target compounds firstly involved ammonolysis of artemisinin to provide the parent compound 11-azaartemisinin which was further derivatized to yield the *N*-11-sulfonyl-azaartemisinin derivatives. Structures were confirmed through use of nuclear magnetic resonance (NMR) and infrared (IR) spectroscopic techniques, and high resolution mass spectrometry (HRMS) techniques. Biological screening of the compounds using as comparator drugs DHA, artemether and artesunate revealed that the *N*-*p*-trifluoromethyl-benzenesulfonyl compound **11** had the best antiplasmodial activity (IC_{50} 2 – 3 nM) and activity equipotent with artemether against the *Pf* NF54 strain. The 3'-thienylsulfonyl derivative **17** also displayed activity equipotent with that of artesunate against this strain. Both compounds **11** and **17** had enhanced activity as well as lower resistance index (RI) values against the W2 cell line when compared to artemether and artesunate. Overall the compounds displayed good selectivity values towards the malaria parasites with respect to mammalian cells and are thus considered to be relatively non-cytotoxic.

Gametocytocidal activity of the compounds alongside artemether and artesunate were also determined. The *N*-*p*-trifluoromethylbenzenesulfonyl compound **11** was active against both early- and late-stage gametocytes and was more stage specific towards later stage gametocytes when compared with artemether and artesunate. However, the 2'-thienylsulfonyl derivative **16** presented the better late-stage gametocytocidal activity and stage-specific value when compared to **11**. Although compound **16** (IC_{50} 4 – 5 nM) is not as potent as **11** towards

asexual blood-stage parasites, it has better activity than artemether against *Pf* W2 strain, and an improved RI value. Compound **16** also demonstrated to be non-cytotoxic.

Therefore, compounds **11** and **16** are identified as potential drug candidates for further evaluation as potential drug development candidates. These compounds will therefore be carried forward for pharmacokinetic studies and *in vivo* evaluation of efficacies.

Chapter 5: "Activities of *N*-sulfonyl-11-azaartemisinin derivatives against the apicomplexan parasite *Neospora caninum* and comparative cytotoxicities" is the third and final article in the thesis. It involved evaluation of the activities of nine of the *N*-sulfonyl-azaartemisinins against the apicomplexan parasite *Neospora caninum in vitro* and comparative antitumor activities. *Neospora caninum* is the causative pathogen of the disease neosporosis that has a major economic effect on the beef- and dairy cattle industries. There is but a handful of treatment options available for neosporosis, each with its own set of challenges. The most common among them are the undesirable residues that are left behind in milk and meat products after treatment. Hence, it is necessary to consider the artemisinin class of compounds; these are notably effective in the treatment of the apicomplexan parasite that causes malaria, as discussed above.

The new 11-azaartemisinin derivatives were screened against human foreskin fibroblasts infected with tachyzoites of *N. caninum*. Artemisone was used as a standard. Evaluation of the cytotoxicities of the compounds against the normal human fetal lung fibroblast WI-38 cell line was also carried out. The most active compounds against neosporosis were the *p*-iodobenzenesulfonyl-11-azaartemisinin derivative **10** and the 2',5'-dichloro-3'-thienylsulfonyl-11-azaartemisinin derivative **13** with IC_{50} values of 45 nM and 40 nM, respectively. Compounds **10** and **13** are 3 – 4 times more active than azaartemisinin and some 2-fold less potent than artemisone. The compounds were screened *in vitro* alongside parthenolide against a panel of the TK-10 (renal), MCF-7 (breast) and UACC-62 (melanoma) cell lines. Overall, the compounds displayed very poor activity against all three cancer cell lines except in the case of the straight chain *N*-alkanesulfonylazaartemisinin **8** against the MCF-7 cell line with an IC_{50} value of 4.21 µM, making it more active than the parthenolide standard. Overall, the compounds proved to be more selective towards the *N. caninum* tachyzoites. It is clear that we have a new set of derivatives that should be carried forward for evaluation of their potential in leading to new treatment options for neosporosis.

To conclude, the compounds synthesized in this study proved to be active against malaria as well as neosporosis. Hit compounds with activities comparable to current artemisinins have

been identified. It is recommended that these compounds should be evaluated further in order to assess their potential for development into new drugs for treatment of malaria or neosporosis.

REFERENCES

Ariey, F., Witkowski, B., Amaratunga, C., Beghain, J., Langlois, A.-C., Khim, N., Kim, S., Duru, V., Bouchier, C. & Ma, L. (2014) A molecular marker of artemisinin-resistant *Plasmodium falciparum* malaria. *Nature,* 505**,** 50-55.

Avery, M. A., Bonk, J. D., Chong, W. K. M., Mehrotra, S., Miller, R., Milhous, W., Goins, D. K., Venkatesan, S. & Wyandt, C. (1995) Structure-activity relationships of the antimalarial agent artemisinin. 2. Effect of heteroatom substitution at O-11: synthesis and bioassay of *N*-Alkyl-11-aza-9-desmethylartemisinins. *Journal of Medicinal Chemistry,* 38**,** 5038-5044.

Avery, M. A., Wesley, J. D. B., Chong, K. M., Mehrotra, S., Miller, R., Milhous, W., Goins, D. K., Venkatesan, S., Wyandt, C., Khan, I. & Avery, B. A. (1996) Structure-activity relationships of the antimalarial agent artemisinin. 2. Effect of heteratom substitution at O-11: Synthesis and bioassay of *N*-alkyl-11-aza-9-desmethylartemisinins. *Journal of Medicinal Chemistry,* 38**,** 5038-5044.

Brewer, T. G., Grate, S. J., Peggins, J. O., Weina, P. J., Petras, J. M., Levine, B. S., Heiffer, M. H. & Schuster, B. G. (1994) Fatal neurotoxicity of arteether and artemether. *The American Journal of Tropical Medicine and Hygiene,* 51**,** 251-259.

Bruce-Chwatt, L. J. (1981) Alphonse Laveran's discovery 100 years ago and today's global fight against malaria. *Journal of the Royal Society of Medicine,* 74**,** 531.

China Cooperative Research Group. (1982) The chemistry and synthesis of qinghaosu derivatives. *Journal of Traditional Chinese Medicine,* 2**,** 1-16.

Cox, F. E. G. (2010) History of the discovery of the malaria parasites and their vectors. *Parasite Vectors,* 3**,** 5.

Genovese, R. F., Newman, D. B. & Brewer, T. G. (2000) Behavioral and neural toxicity of the artemisinin antimalarial, arteether, but not artesunate and artelinate, in rats. *Pharmacology Biochemistry and Behavior,* 67**,** 37-44.

Haynes, R. K., Wong, H.-N., Lee, K.-W., Lung, C.-M., Shek, L. Y., Williams, I. D., Croft, S. L., Vivas, L., Rattray, L. & Stewart, L. (2007) Preparation of *N*-sulfonyl- and *N*-carbonyl-11-azaartemisinins with greatly enhanced thermal stabilities: *in vitro* antimalarial activities. *ChemMedChem,* 2**,** 1464-1479.

Le, T. N., De Borggraeve, W. M., Grellier, P., Pham, V. C. & Dehaen, W. (2014) Synthesis of 11-aza-artemisinin derivatives using the Ugi reaction and an evaluation of their antimalarial activity. *Tetrahedron Letters,* 55**,** 4892-4894.

Li, Y. (1979) Synthesis of some derivatives of artemisinin. *Reprinted from Kexue Tongbao,* 24, 667-669.

Li, Y., Yu, P.-L., Chen, Y.-X., Li, L.-Q., Gai, Y.-Z., Wang, D.-S. & Zheng, Y.-P. (1981) Studies on analogs of artemisinin. I. The synthesis of ethers, carboxylic esters and carbonates of dihydroartemisinin. *Acta Pharmaceutica Sinica,* 16**,** 429.

Lubell, Y., Dondorp, A., Guérin, P. J., Drake, T., Meek, S., Ashley, E., Day, N. P. J., White, N. J. & White, L. J. (2014) Artemisinin resistance - modelling the potential human and economic costs. *Malaria Journal,* 13**,** 1.

Nontprasert, A., Nosten-Bertrand, M., Pukrittayakamee, S., Vanijanonta, S., Angus, B. J. & White, N. J. (1998) Assessment of the neurotoxicity of parenteral artemisinin derivatives in mice. *The American Journal of Tropical Medicine and Hygiene,* 59**,** 519-522.

Phyo, A. P., Nkhoma, S., Stepniewska, K., Ashley, E. A., Nair, S., McGready, R., Ler Moo, C., Al-Saai, S., Dondorp, A. M. & Lwin, K. M. (2012) Emergence of artemisinin-resistant malaria on the western border of Thailand: a longitudinal study. *The Lancet,* 379**,** 1960-1966.

Schmuck, G. & Haynes, R. K. (2000) Establishment of an *in vitro* screening model for neurodegeneration induced by antimalarial drugs of the artemisinin-type. *Neurotoxicity Research,* 2**,** 37-49.

Schmuck, G., Roehrdanz, E., Haynes, R. K. & Kahl, R. (2002) Neurotoxic mode of action of artemisinin. *Antimicrobial Agents and Chemotherapy,* 46**,** 821-827.

Wellems, T. E. & Plowe, C. V. (2001) Chloroquine-resistant malaria. *Journal of Infectious Diseases,* 184**,** 770-776.

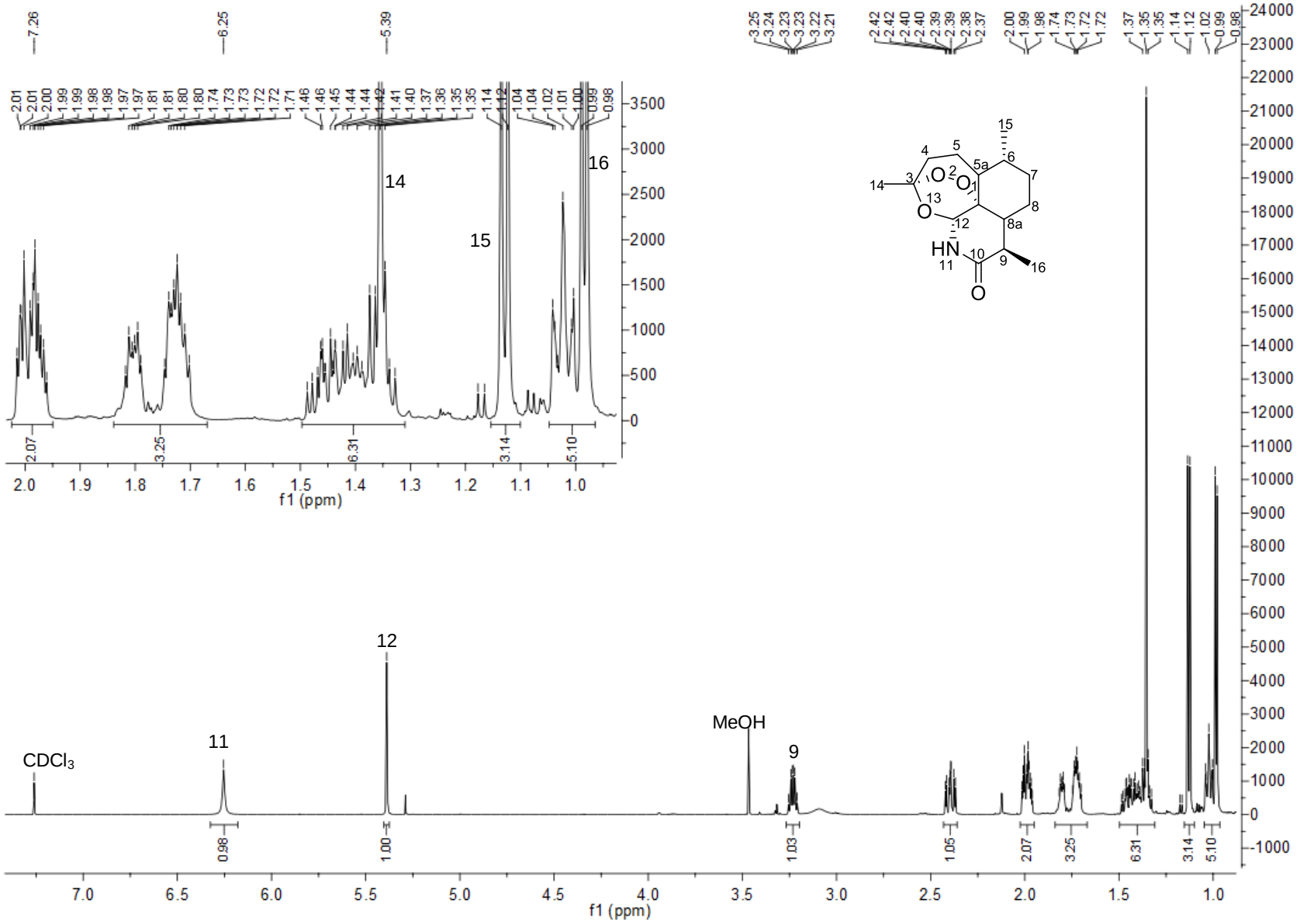
Who (2015) Global Malaria Programme Fact Sheet: World Malaria Report 2015 Geneva, World Health Organization, http://www.who.int/malaria/media/world-malaria-report-2015/en/.

ADDENDUM A
Analytical data for Chapter 4

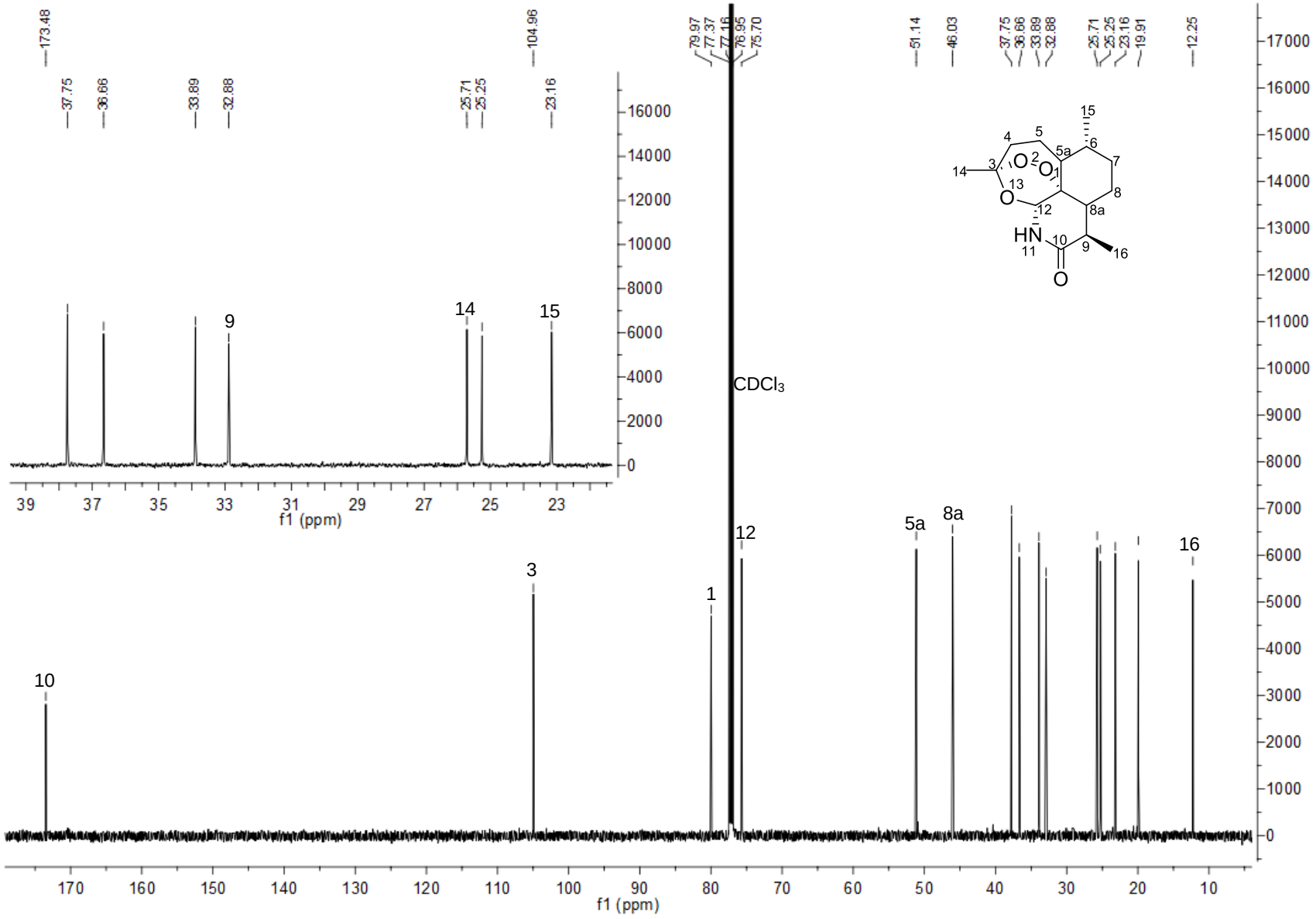
This addendum contains the following analytical data for compounds **5** – **18** in Chapter 4:

^{1}H NMR, ^{13}C NMR, DEPT 90, DEPT 135, COSY, HSQC, IR, HRMS

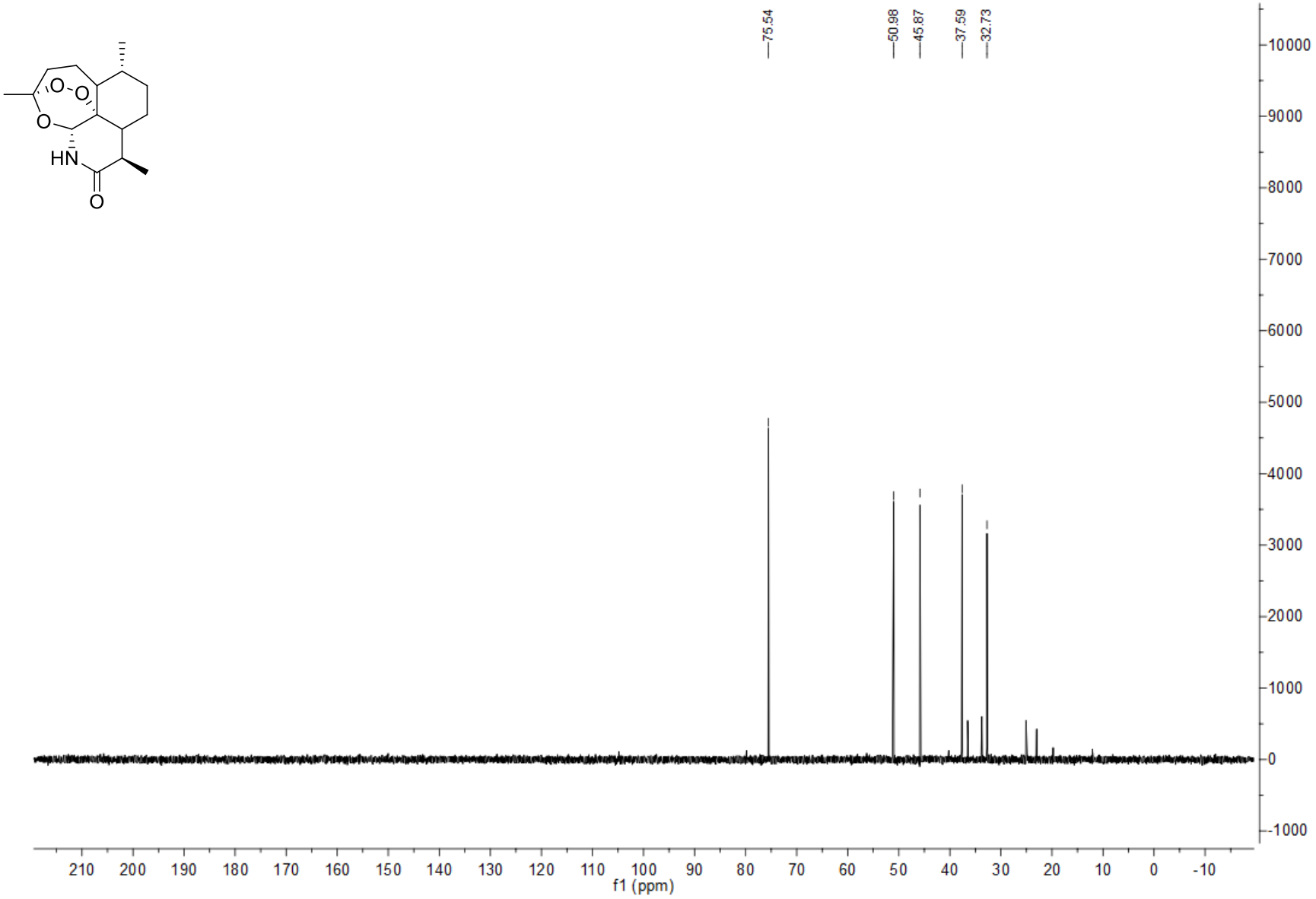
^{1}H NMR $CDCl_3$ 11-azaartemisinin (**5**)



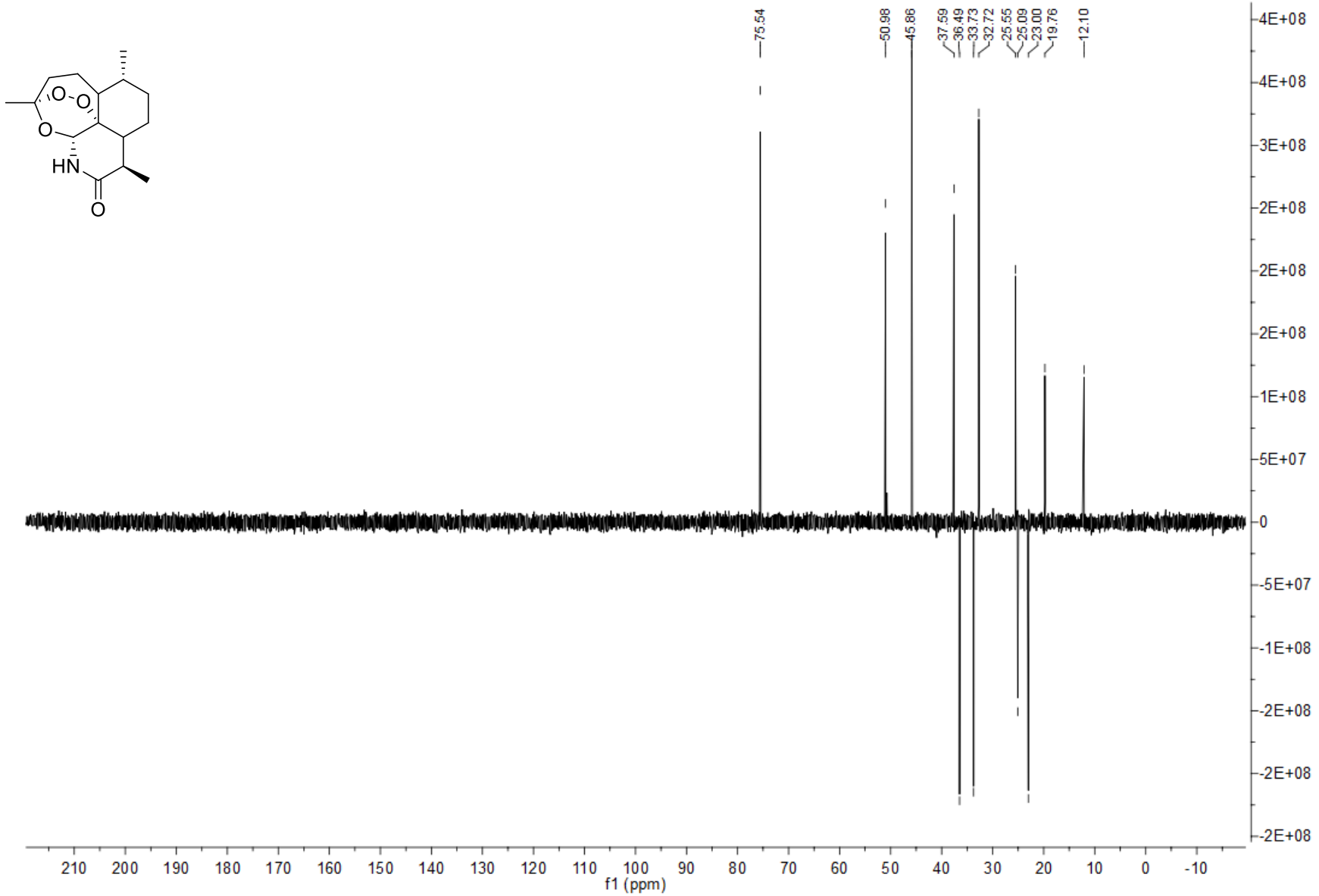
^{13}C NMR $CDCl_3$ 11-azaartemisinin (**5**)



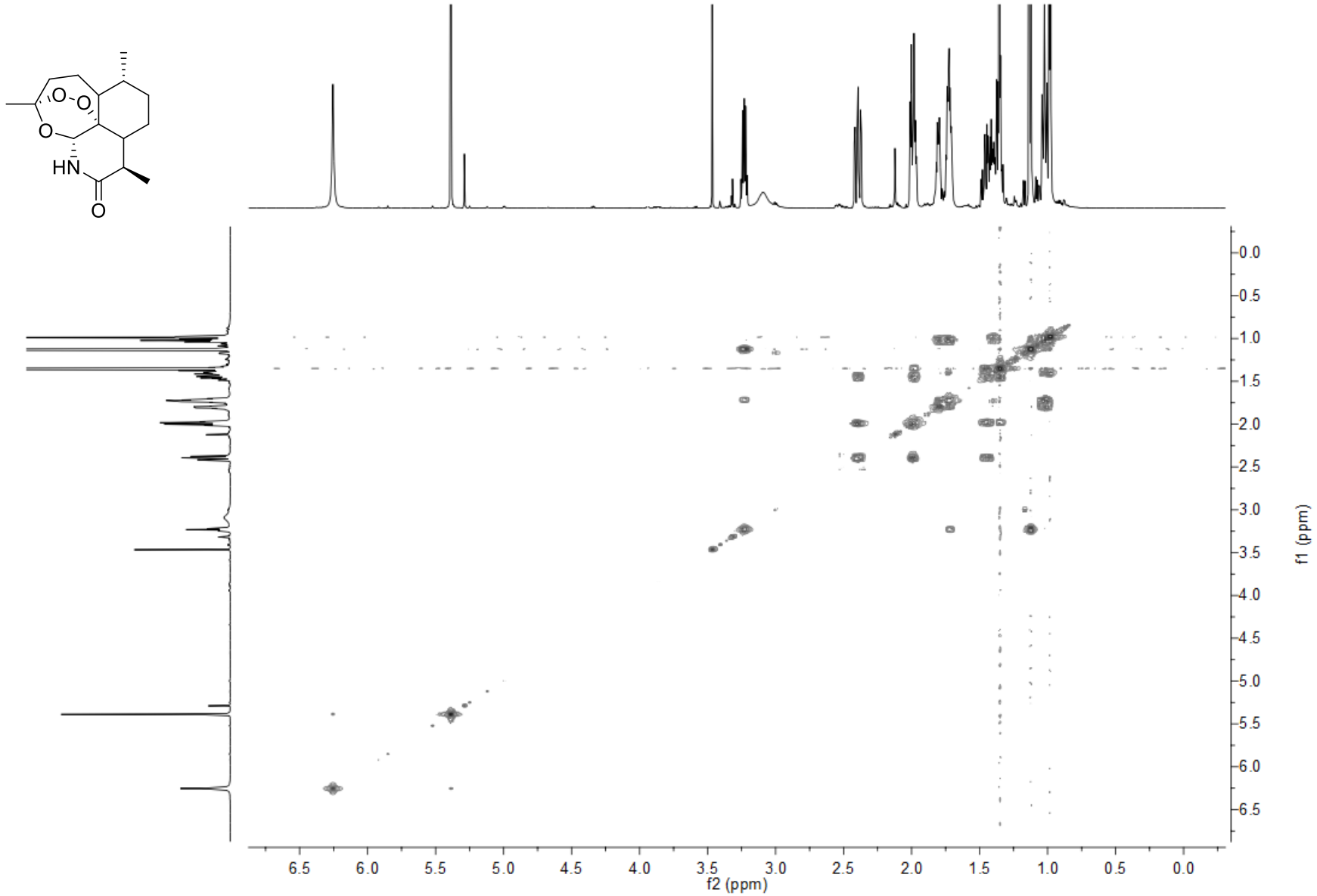
DEPT90 NMR $CDCl_3$ 11-azaartemisinin (**5**)



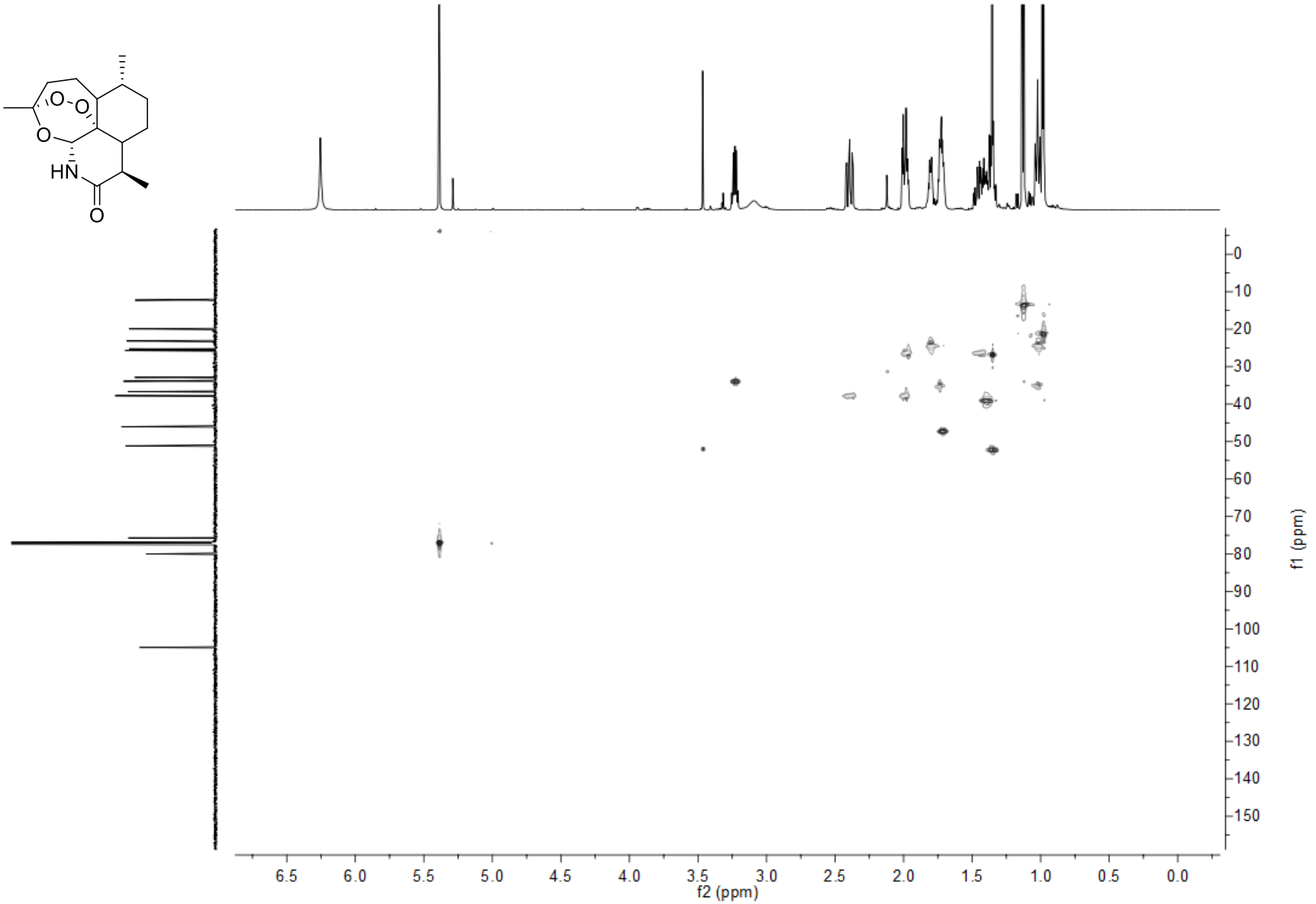
DEPT135 NMR $CDCl_3$ 11-azaartemisinin (**5**)



COSY NMR $CDCl_3$ 11-azaartemisinin (**5**)



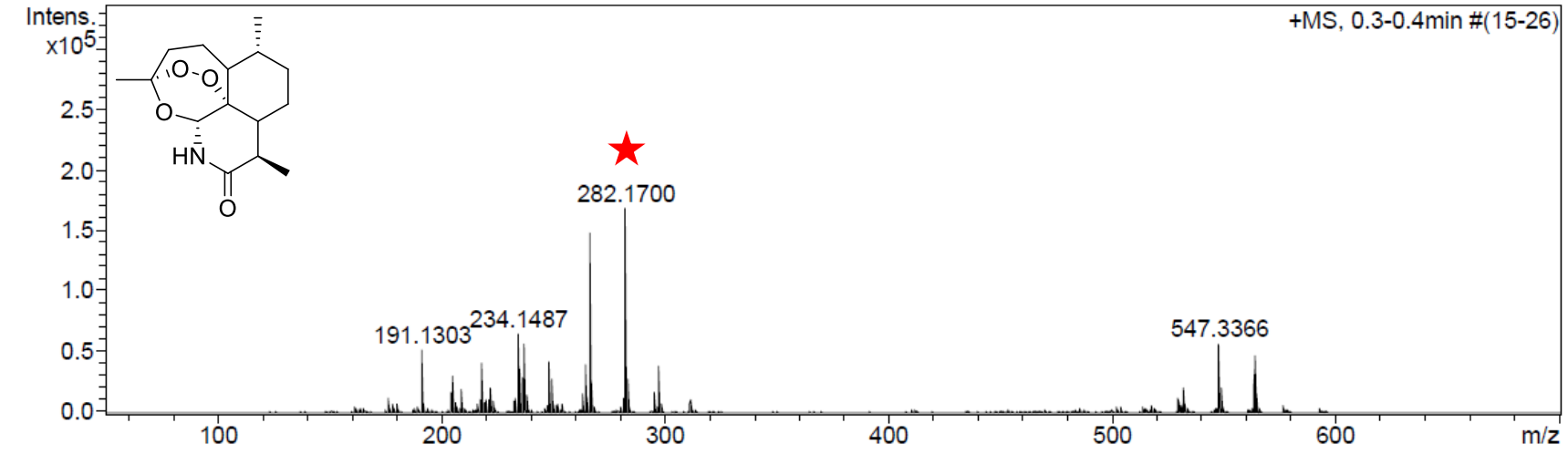
HSQC NMR $CDCl_3$ 11-azaartemisinin (**5**)



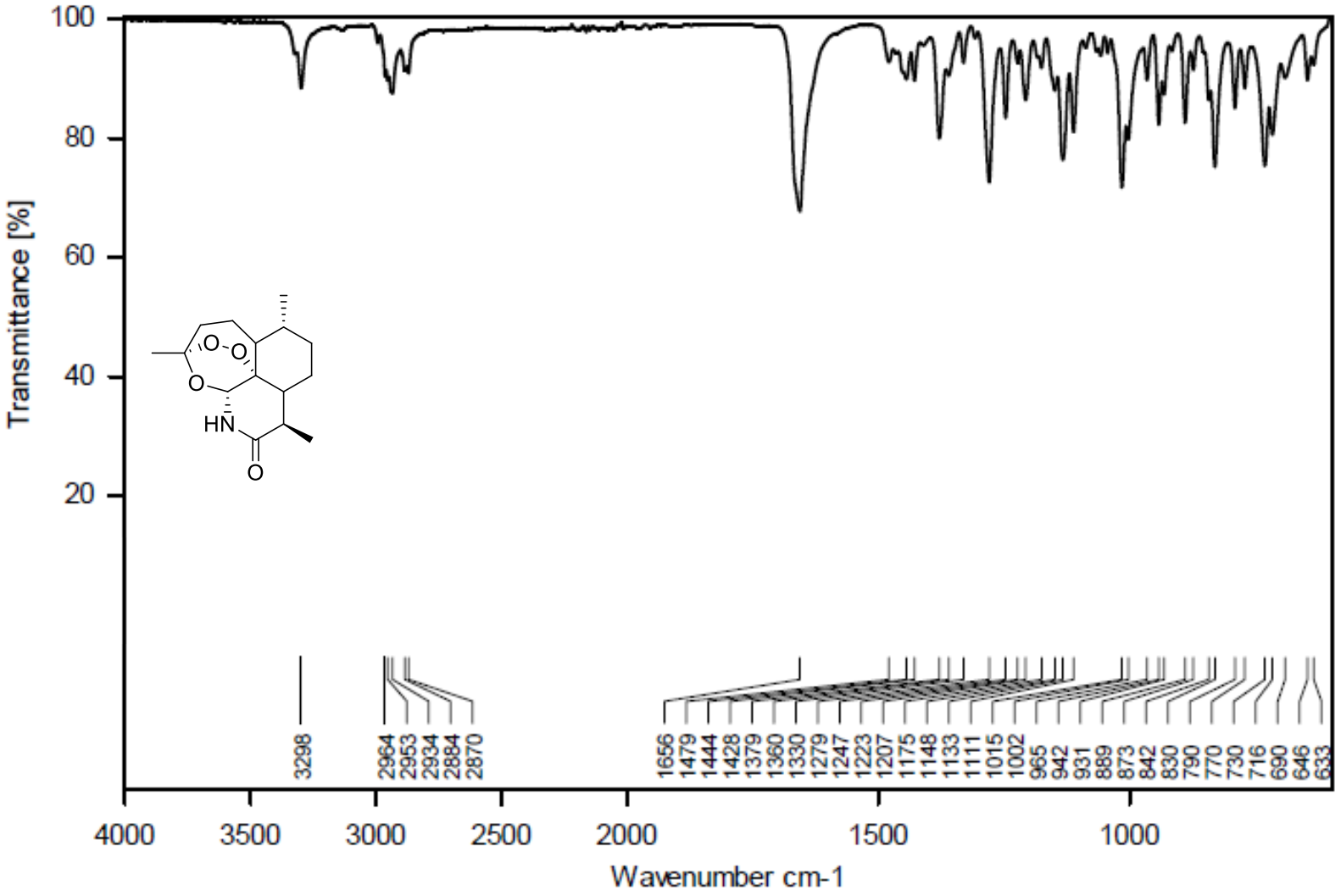
HRMS 11-azaartemisinin (**5**)

Acquisition Parameter

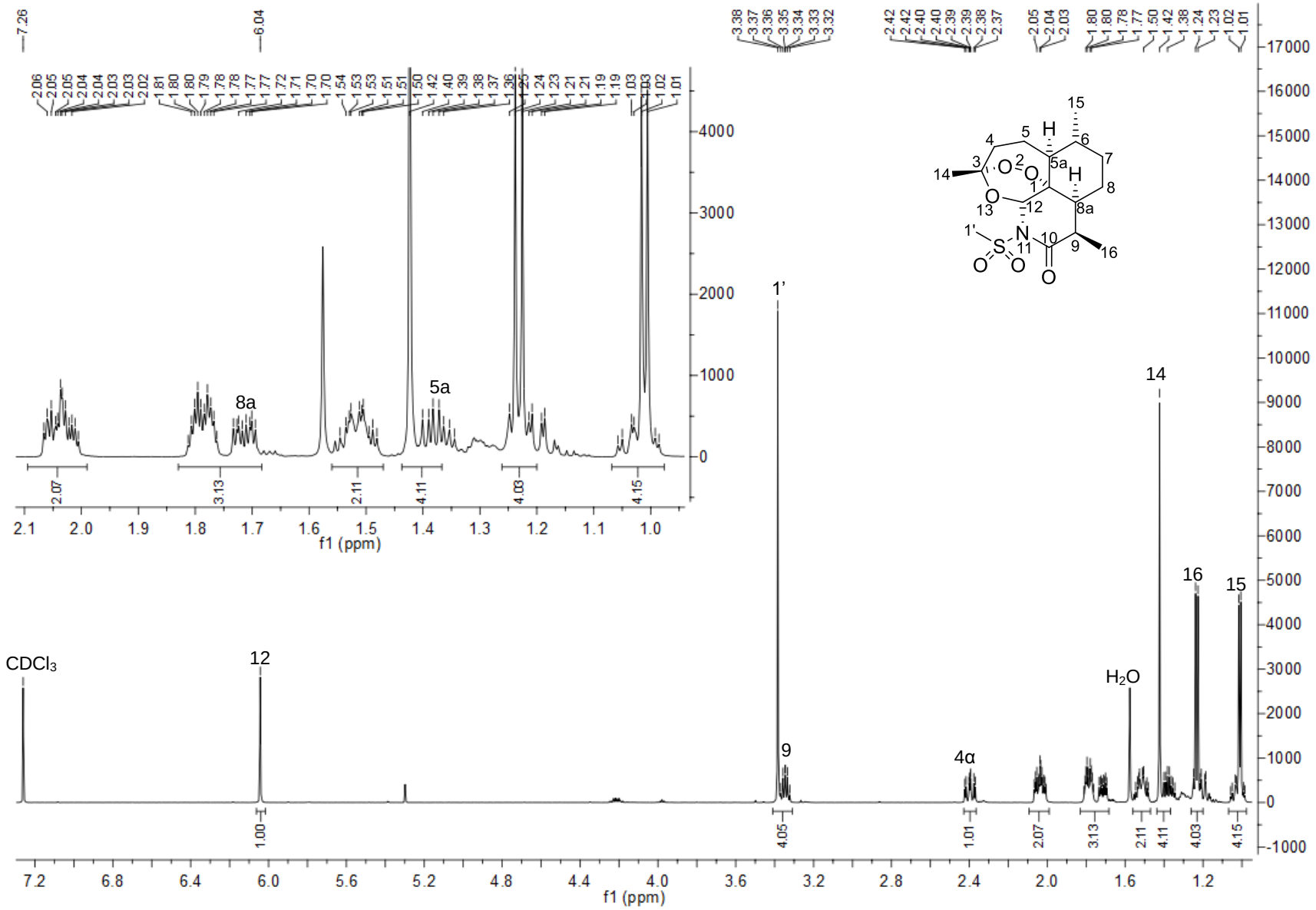
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



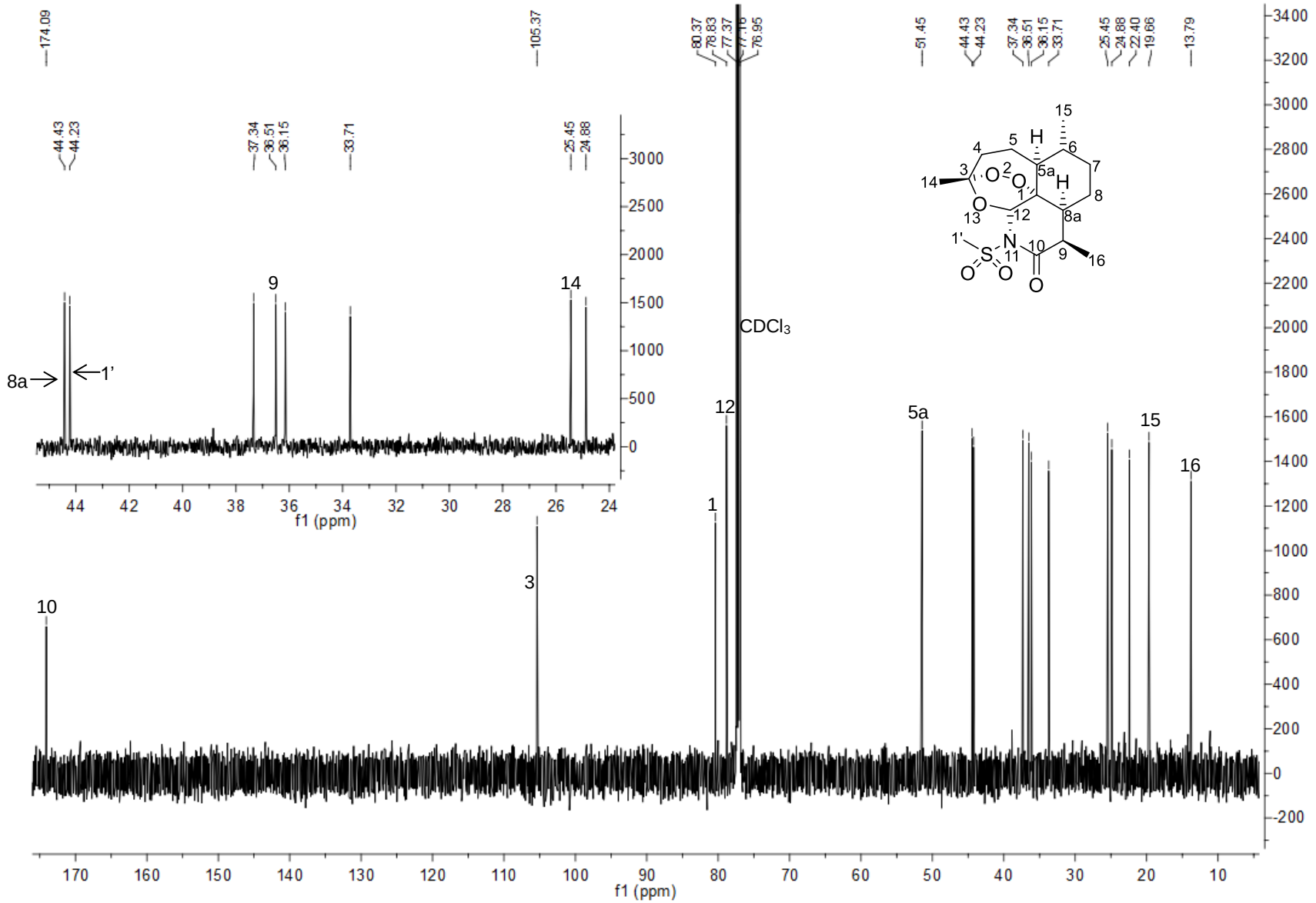
IR 11-azaartemisinin (**5**)



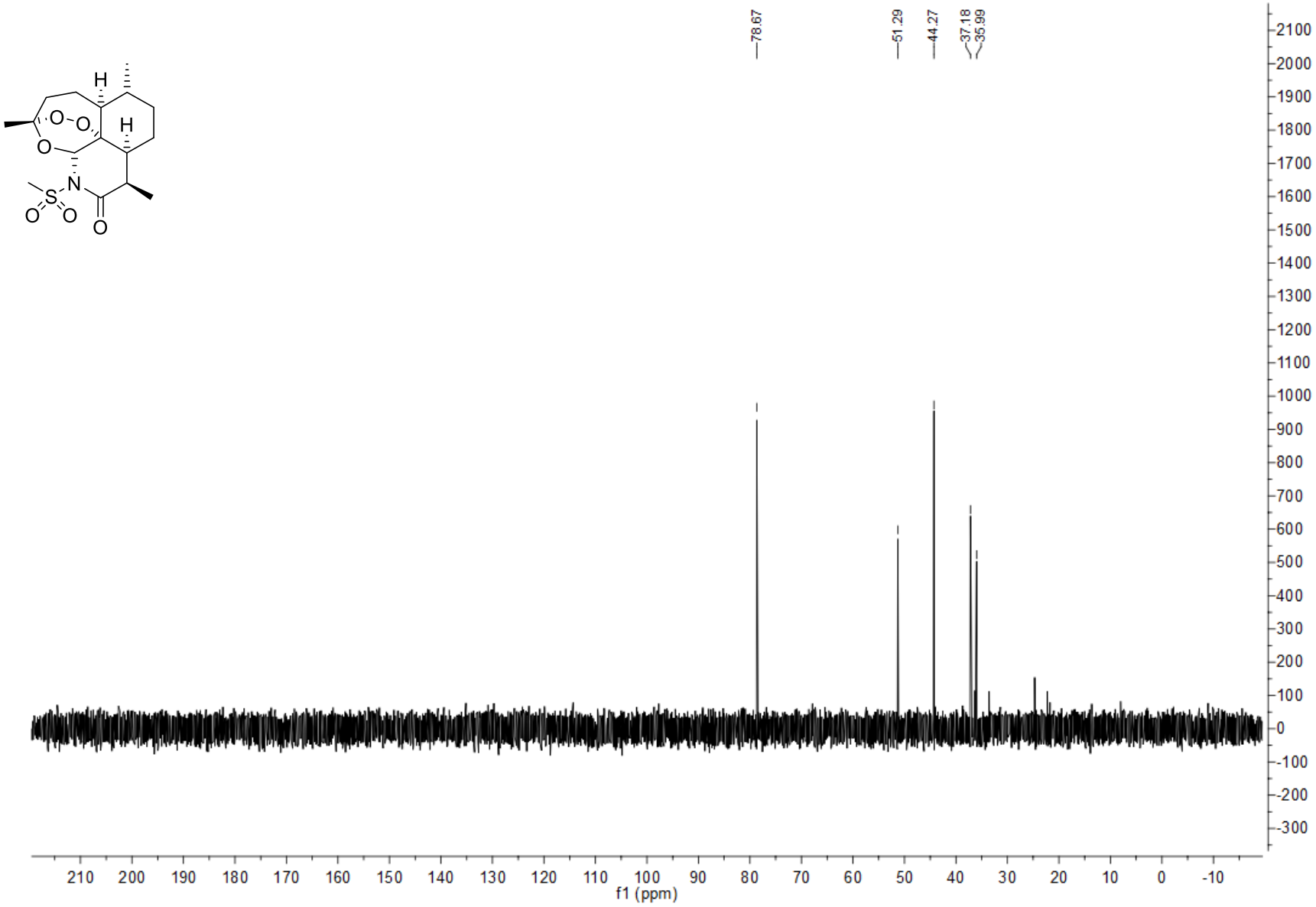
^{1}H NMR $CDCl_3$ *N*-Methanesulfonylazaartemisinin (**6**)



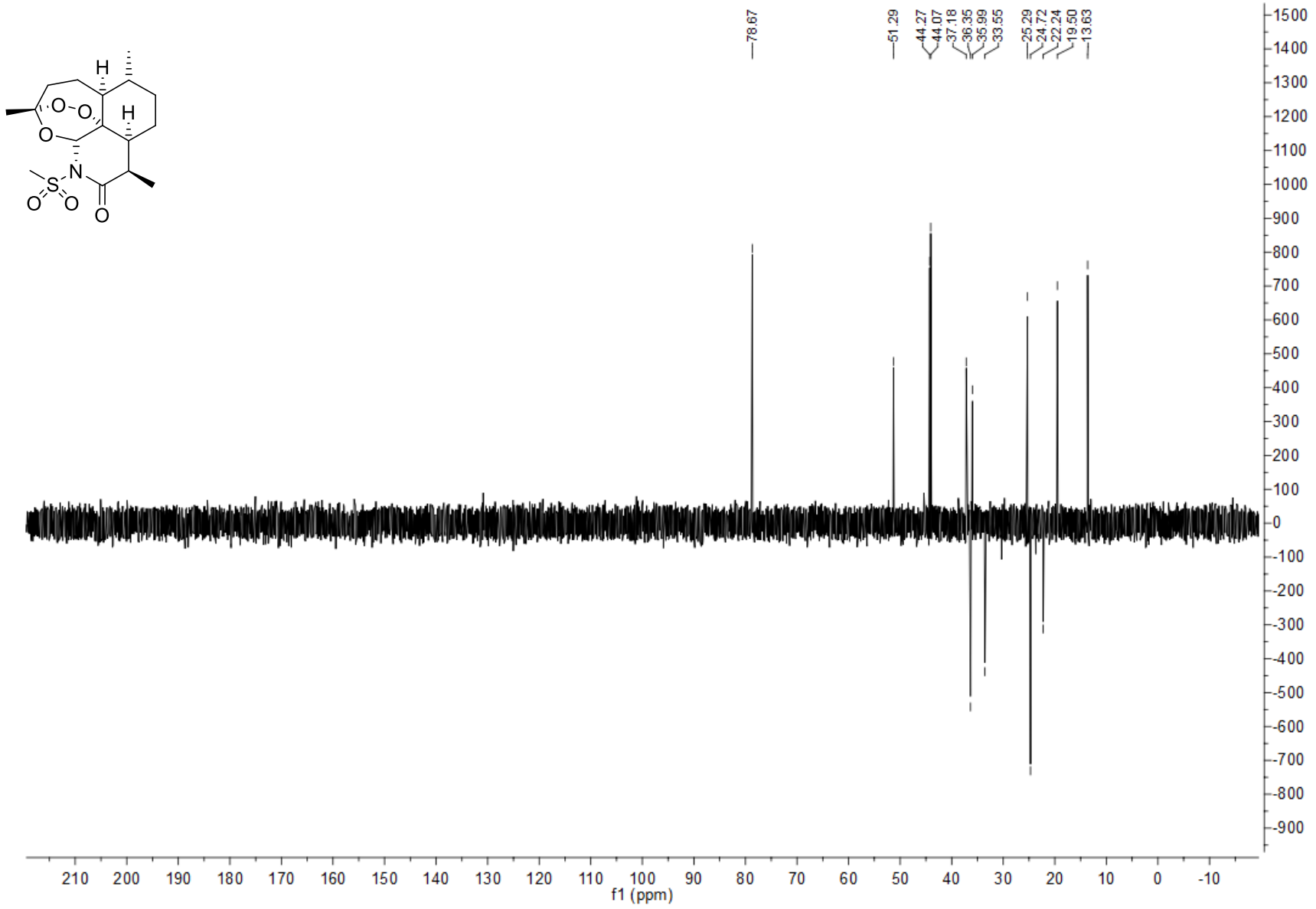
^{13}C NMR $CDCl_3$ *N*-Methanesulfonylazaartemisinin (**6**)



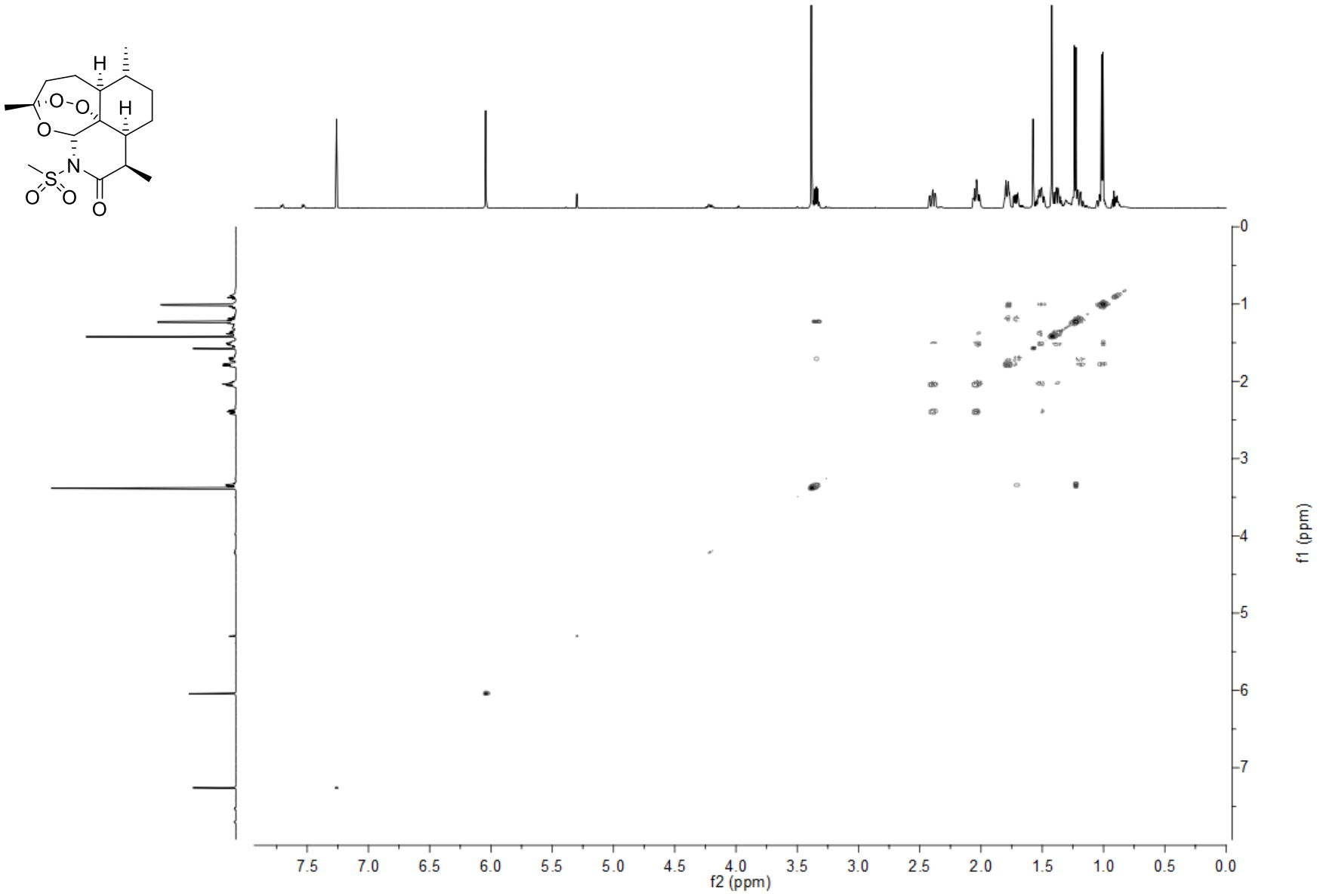
DEPT90 $CDCl_3$ *N*-Methanesulfonylazaartemisinin (**6**)



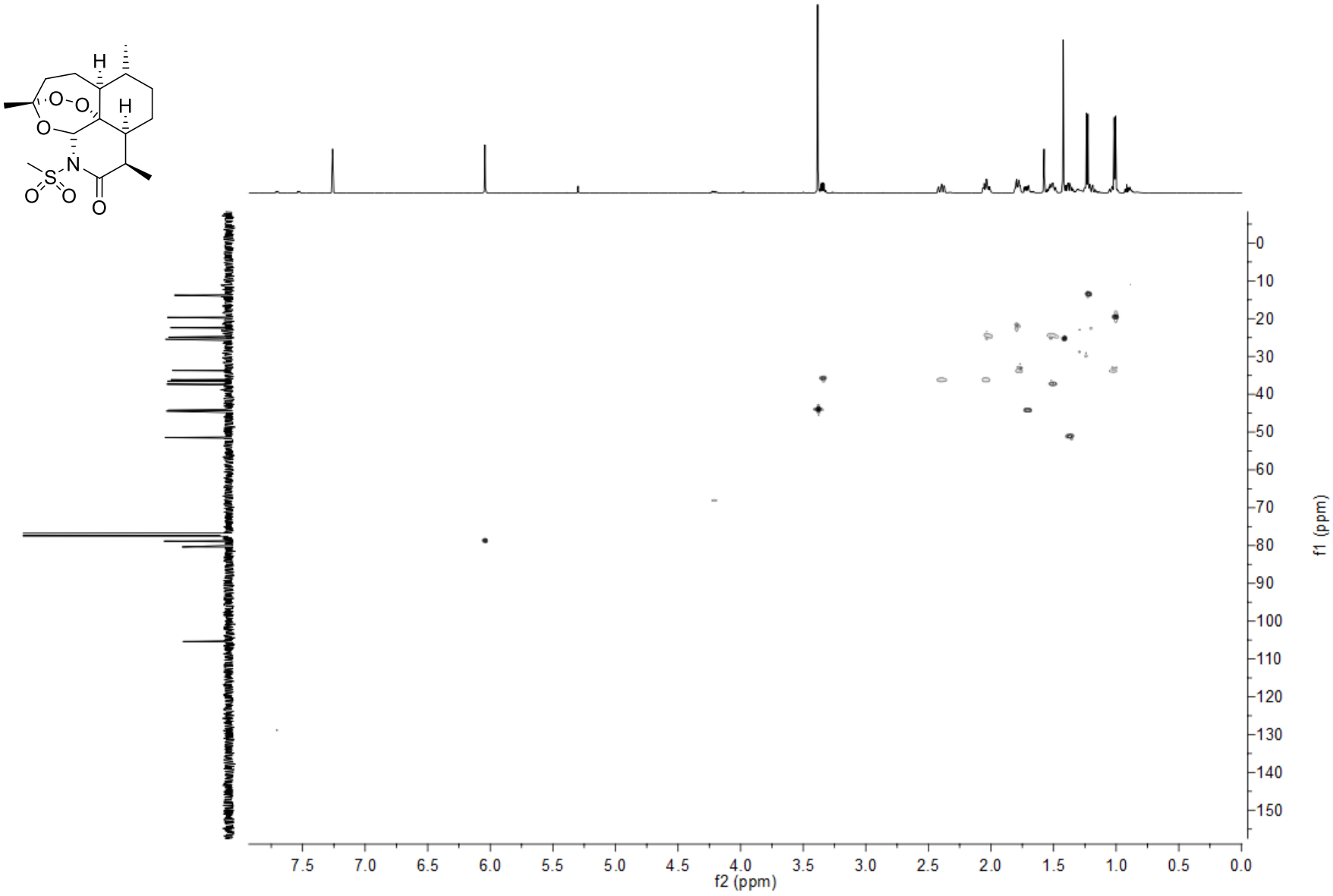
DEPT135 $CDCl_3$ *N*-Methanesulfonylazaartemisinin (**6**)



COSY $CDCl_3$ *N*-Methanesulfonylazaartemisinin (**6**)



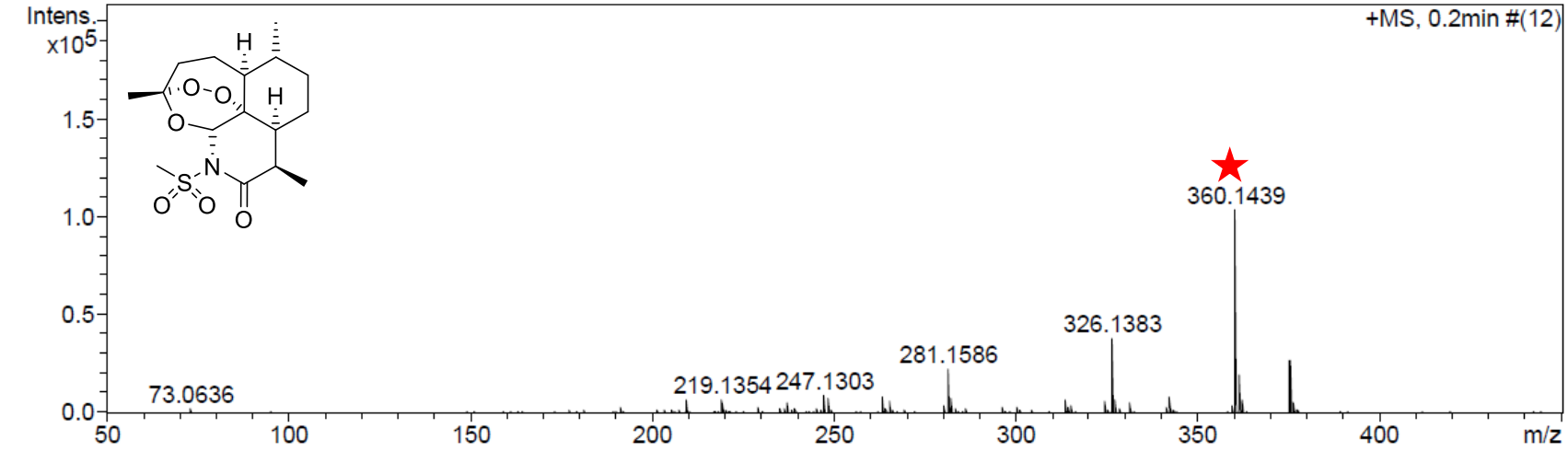
HSQC $CDCl_3$ *N*-Methanesulfonylazaartemisinin (**6**)



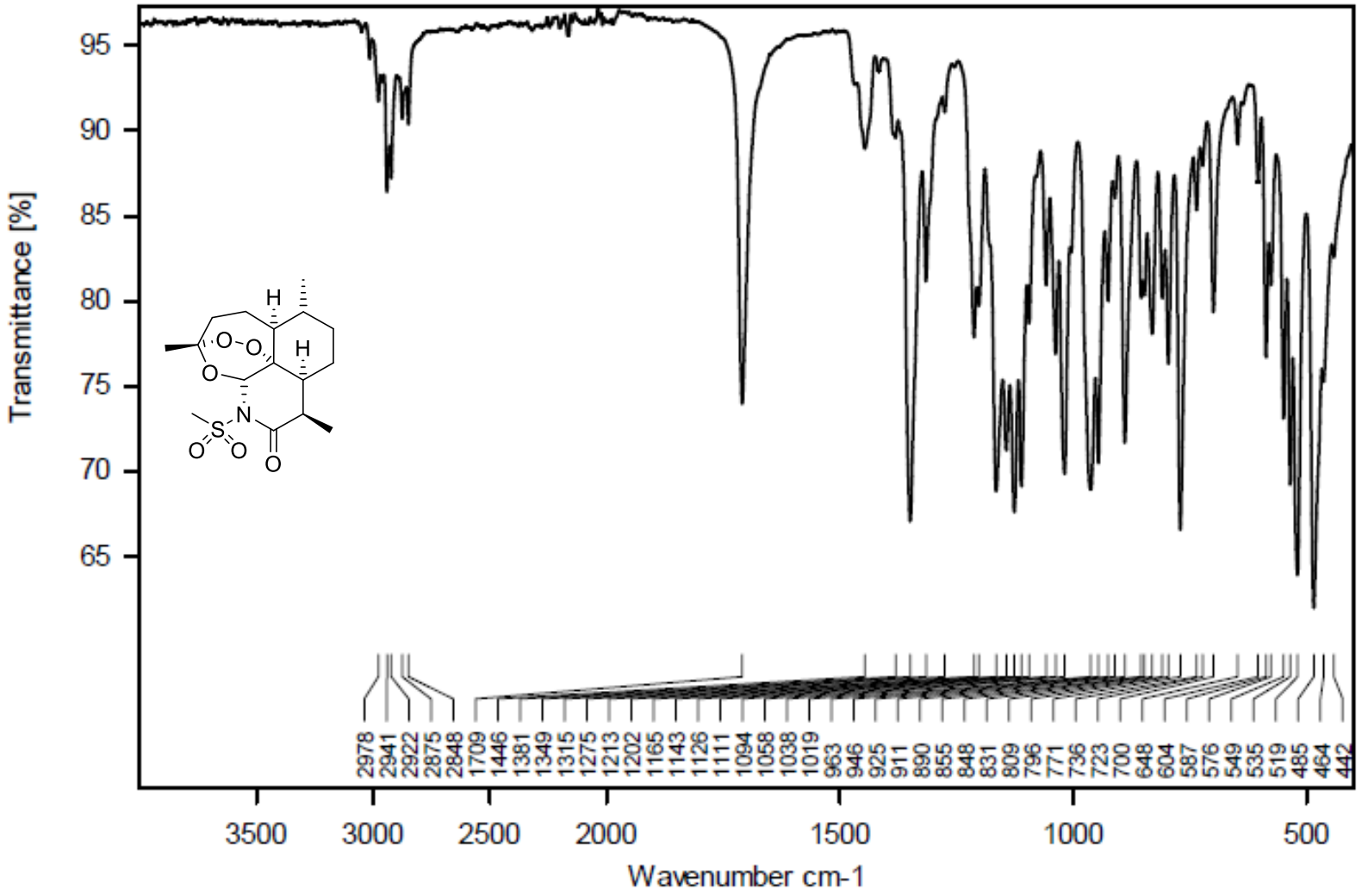
HRMS *N*-Methanesulfonylazaartemisinin (**6**)

Acquisition Parameter

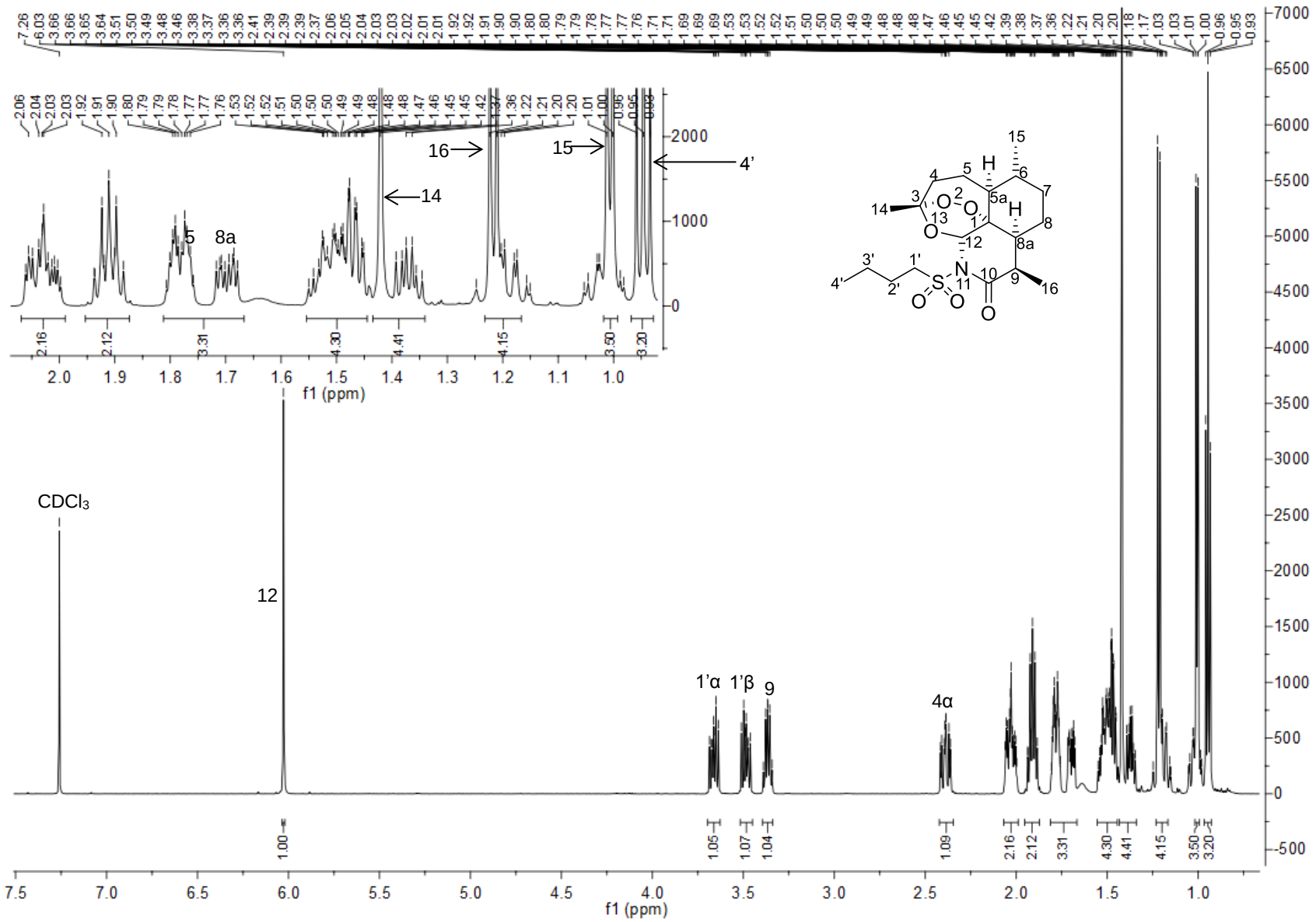
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



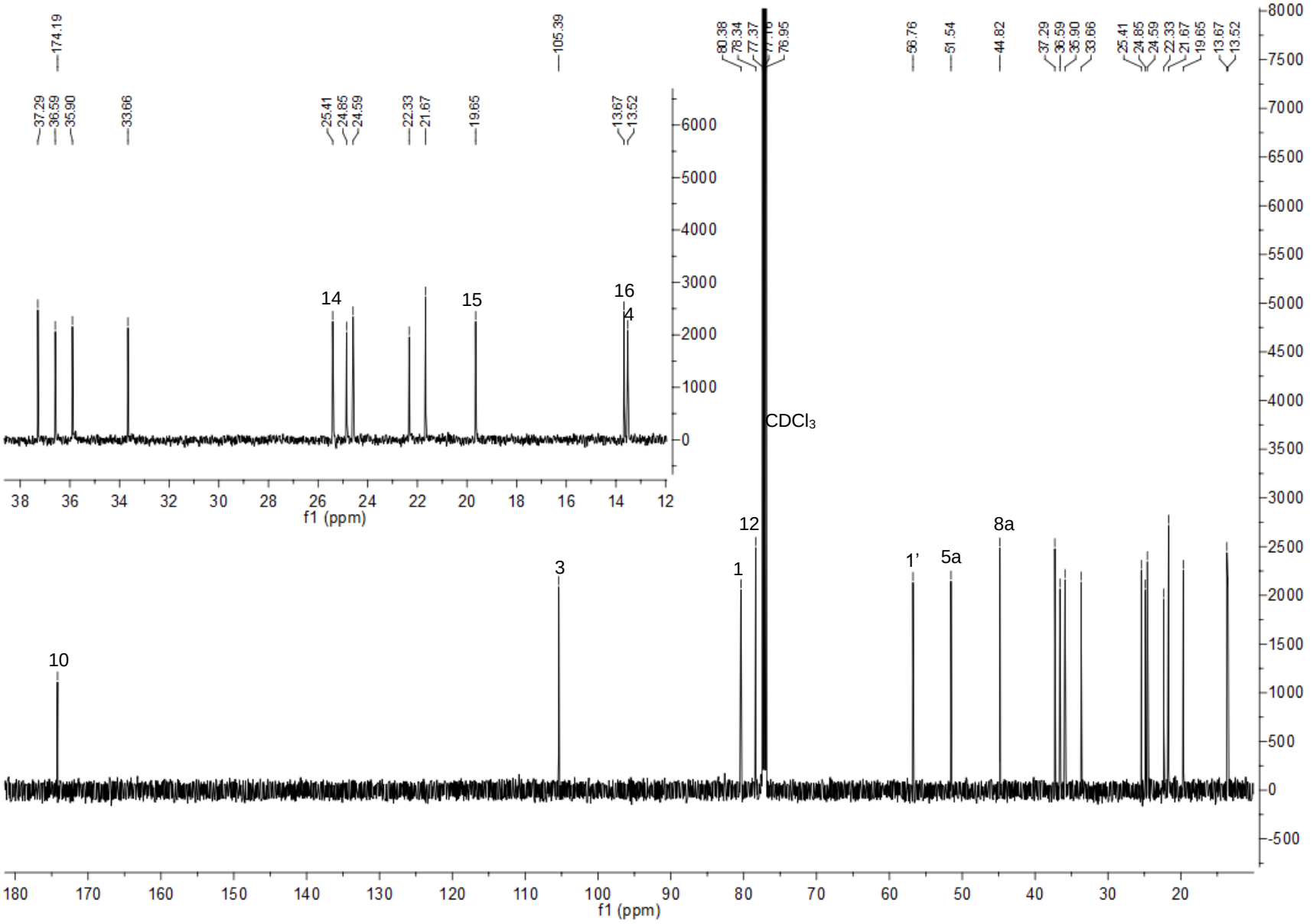
IR *N*-Methanesulfonylazaartemisinin (**6**)



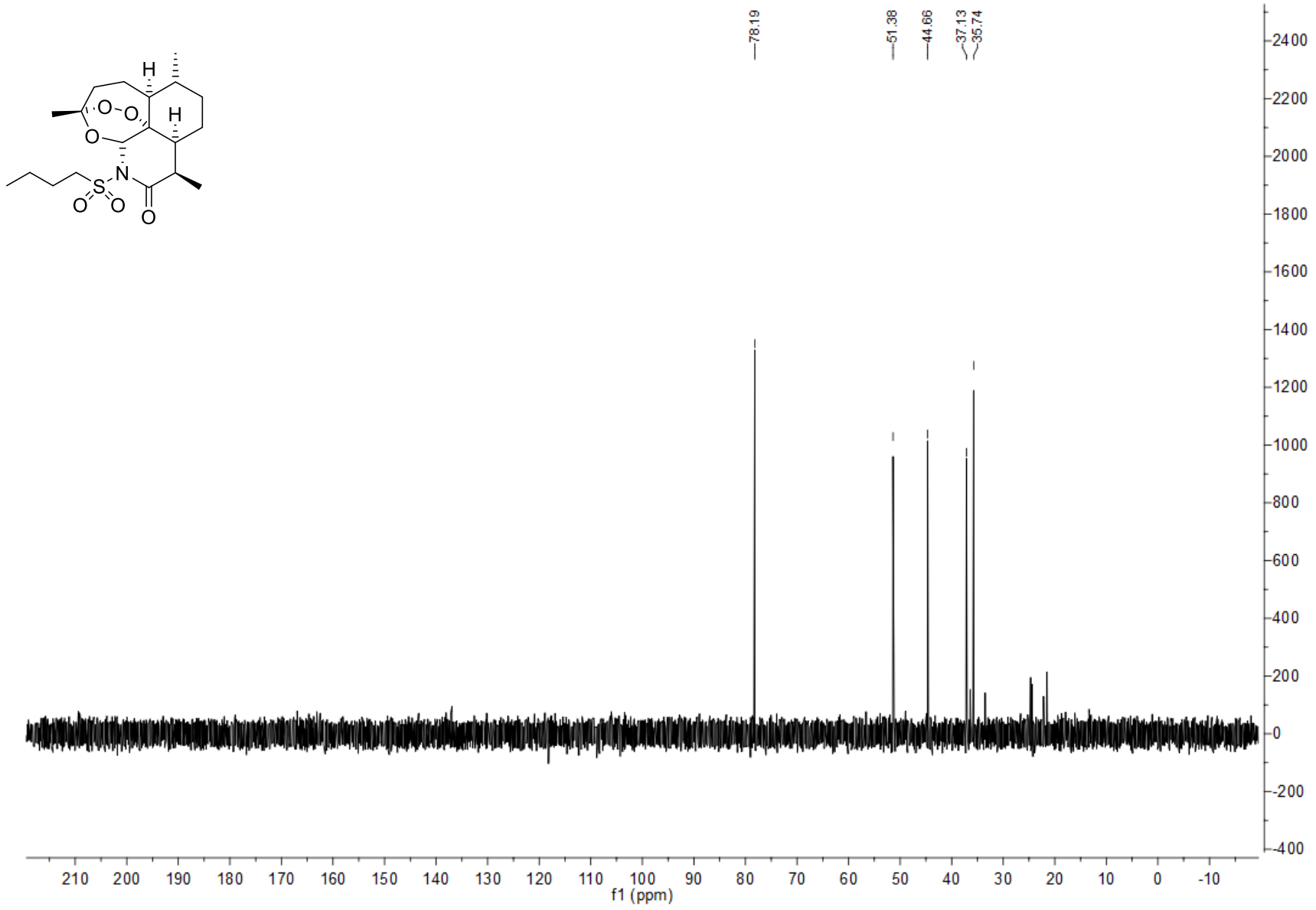
^{1}H NMR $CDCl_3$ Butanesulfonyl-azaartemisinin (**7**)



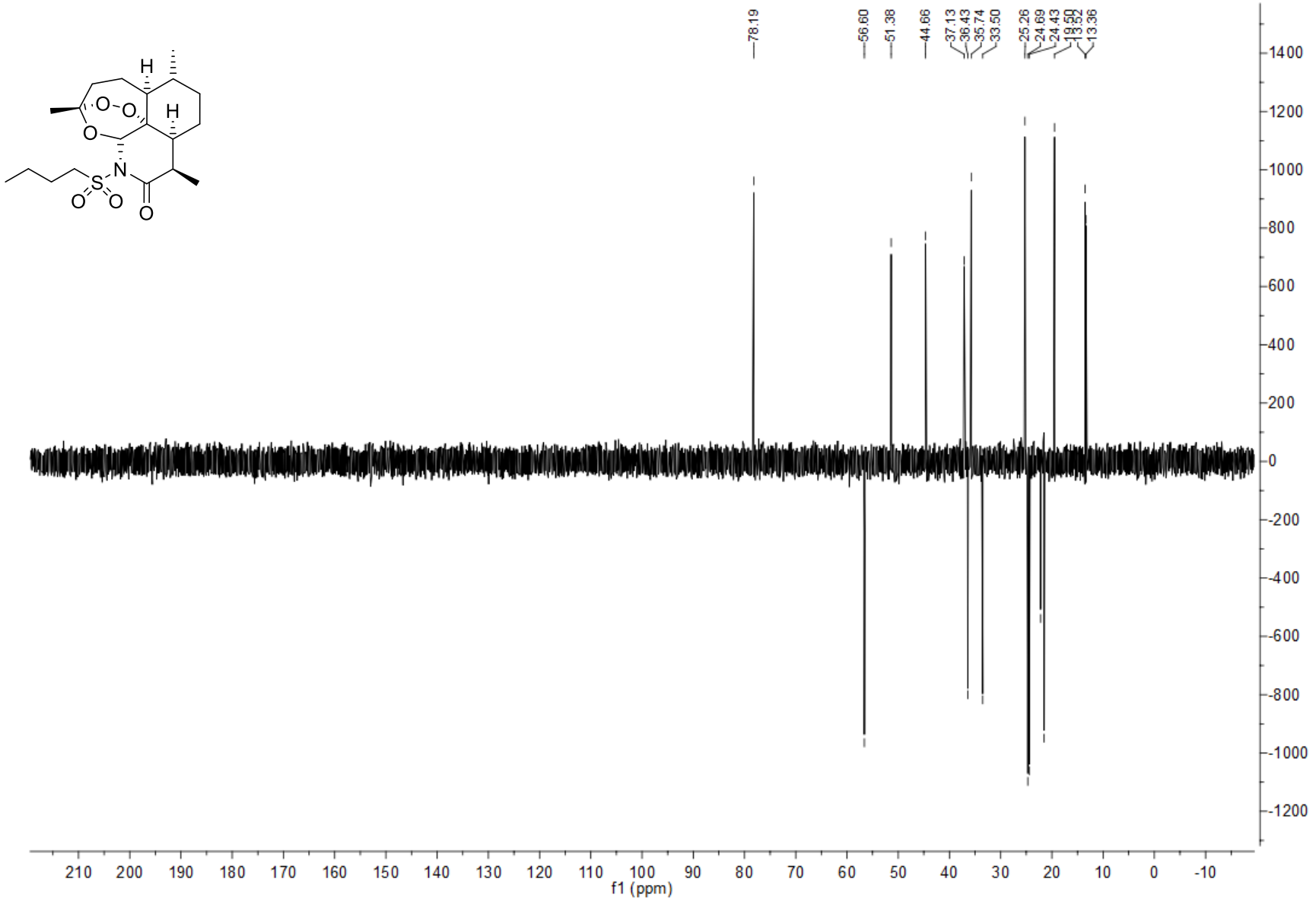
^{13}C NMR $CDCl_3$ Butanesulfonyl-azaartemisinin (**7**)



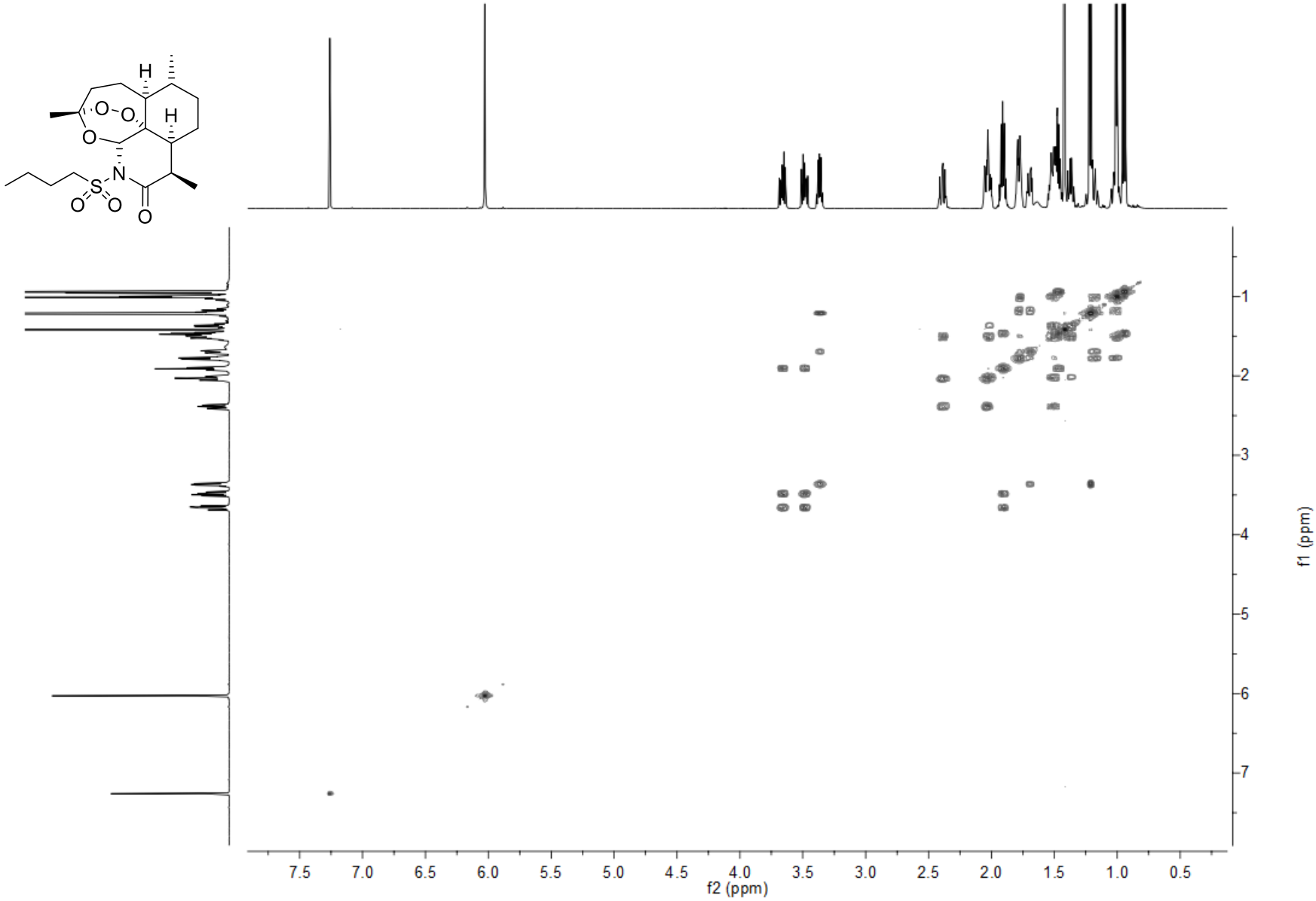
DEPT90 $CDCl_3$ Butanesulfonyl-azaartemisinin (**7**)



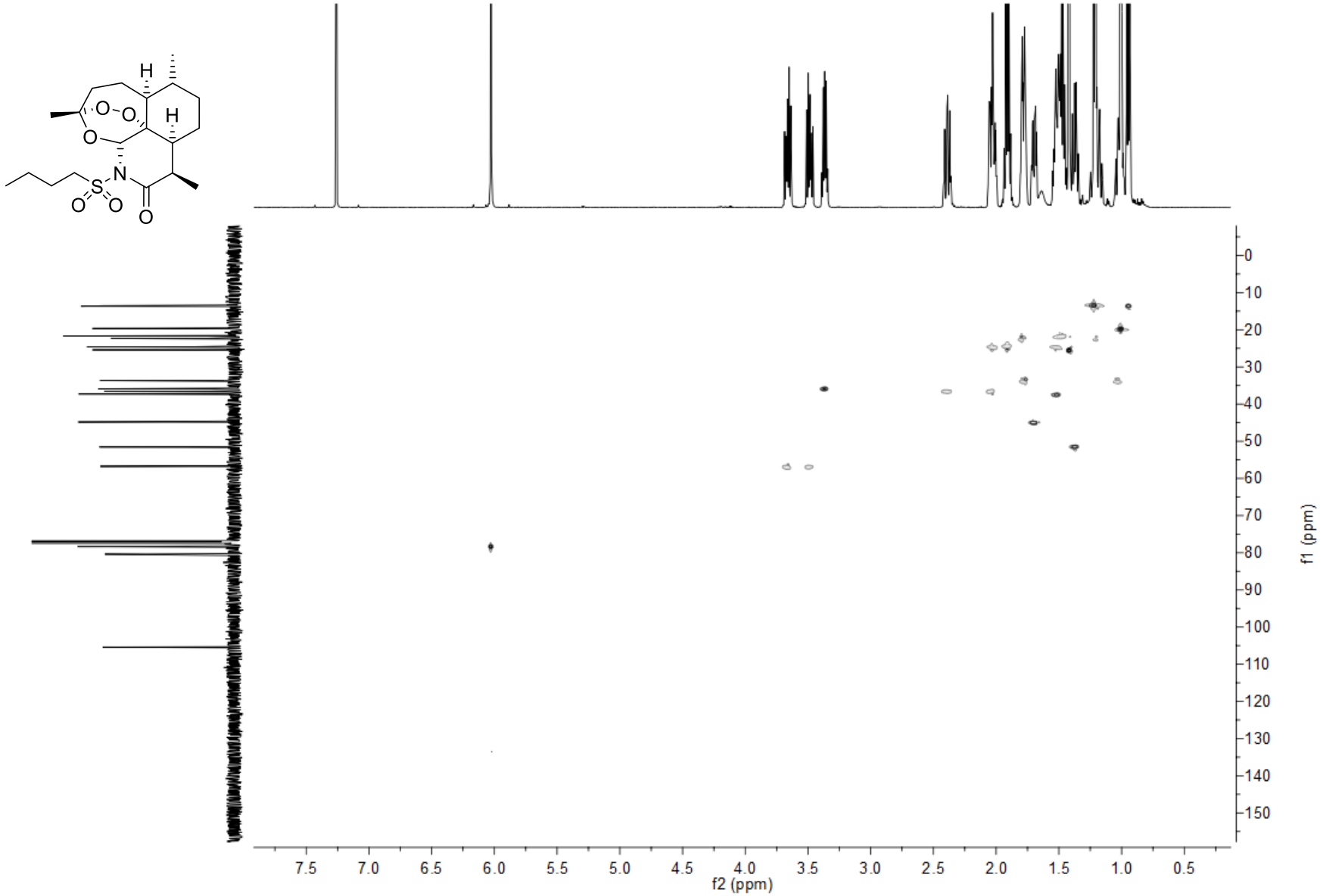
DEPT135 $CDCl_3$ Butanesulfonyl-azaartemisinin (**7**)



COSY $CDCl_3$ Butanesulfonyl-azaartemisinin (**7**)



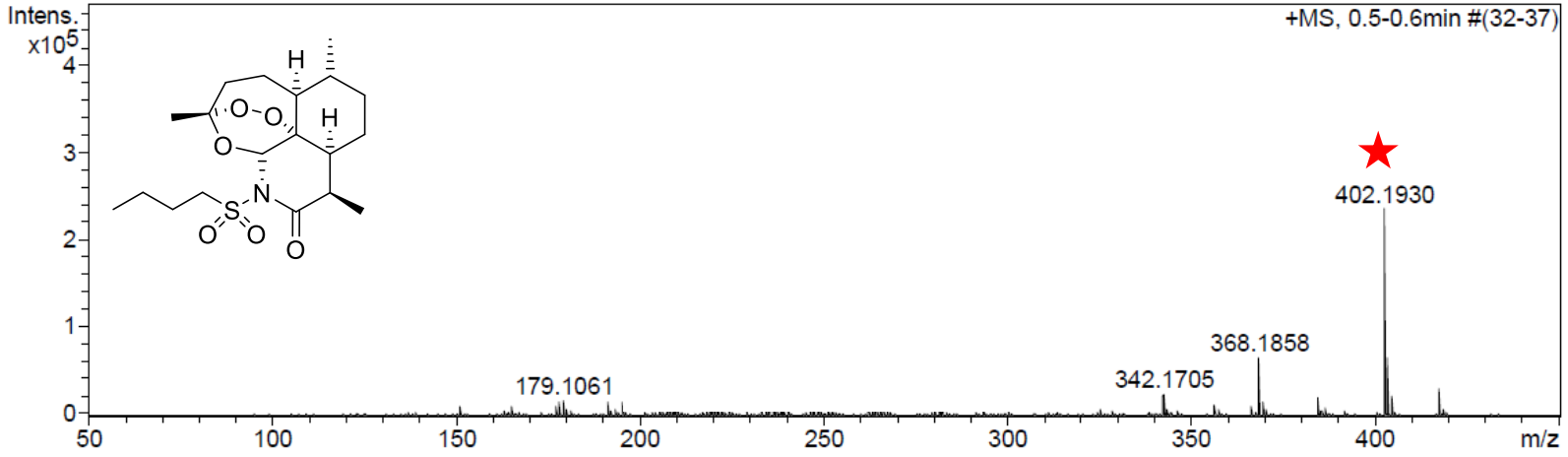
HSQC $CDCl_3$ Butanesulfonyl-azaartemisinin (**7**)



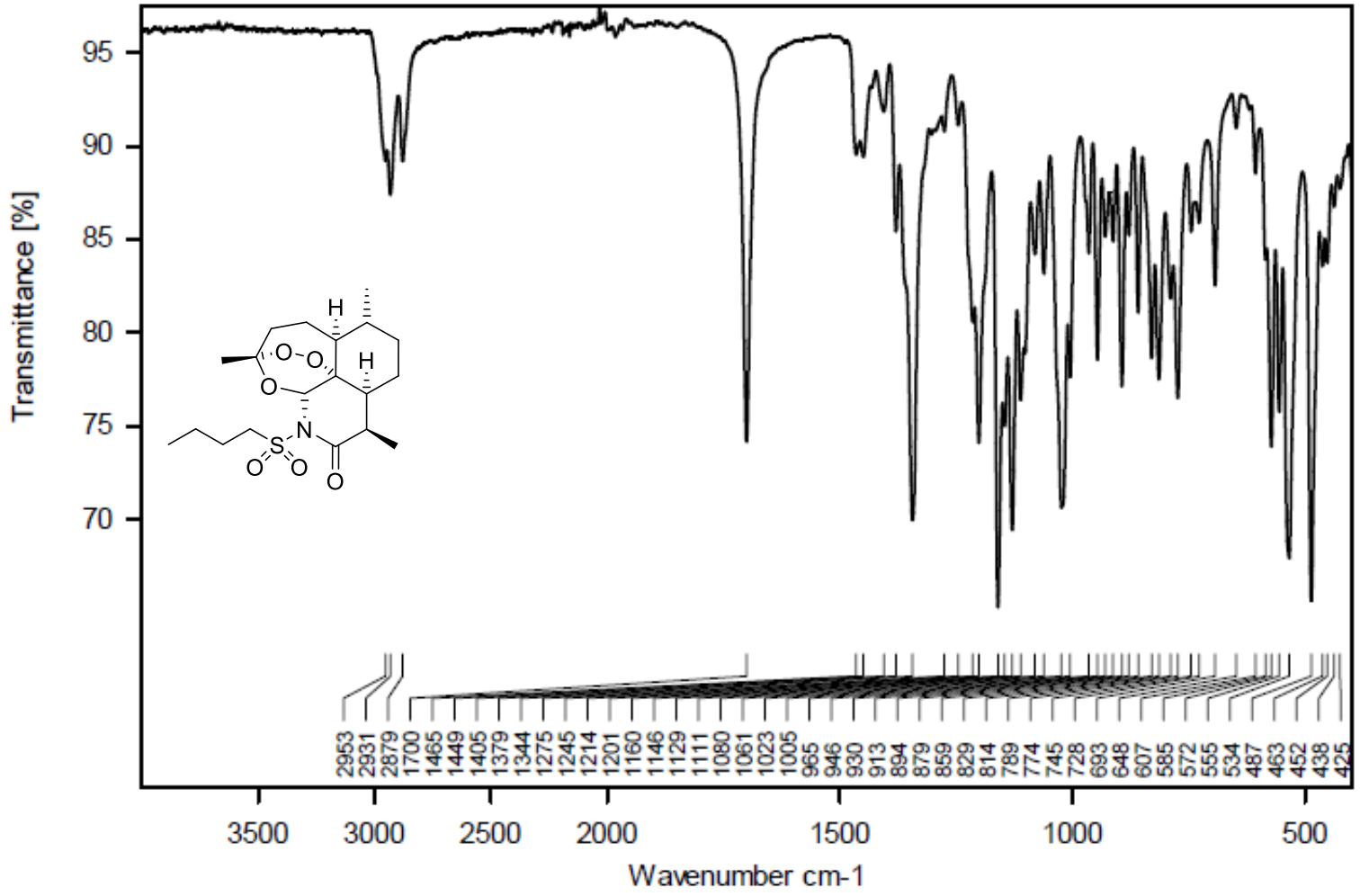
HRMS Butanesulfonyl-azaartemisinin (**7**)

Acquisition Parameter

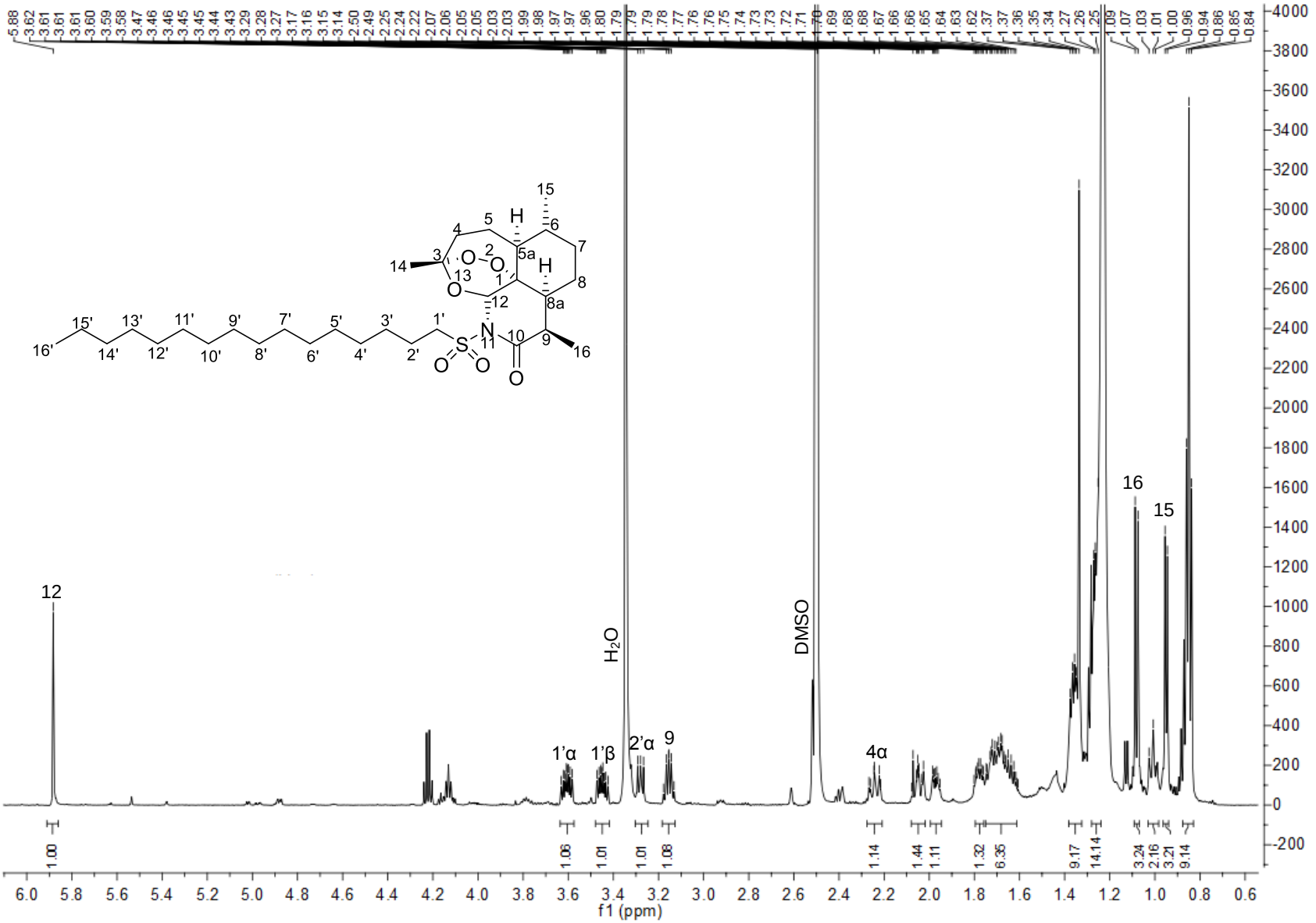
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	3000 m/z	Set Collision Cell RF	150.0 Vpp	Set Divert Valve	Waste



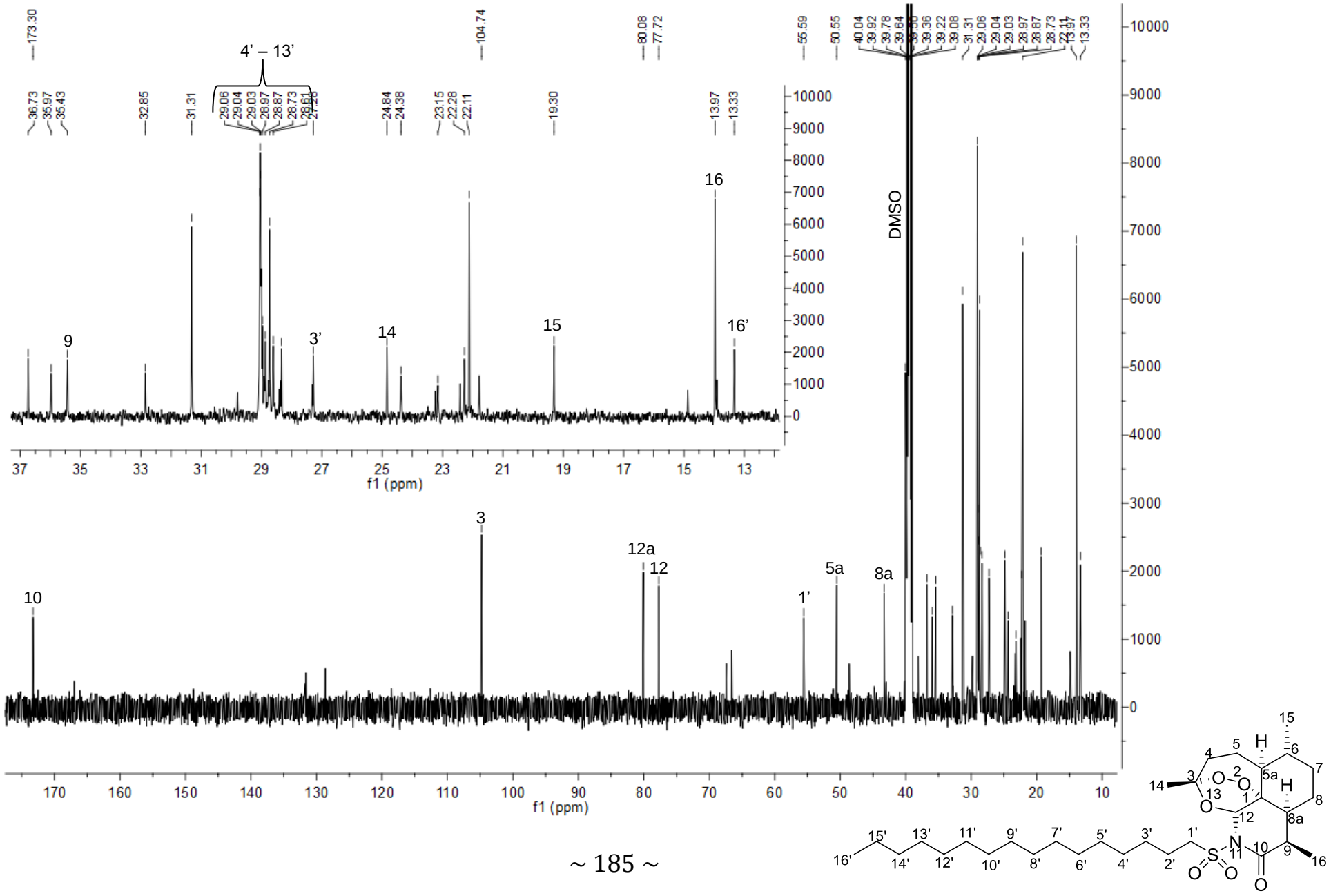
IR Butanesulfonyl-azaartemisinin (**7**)



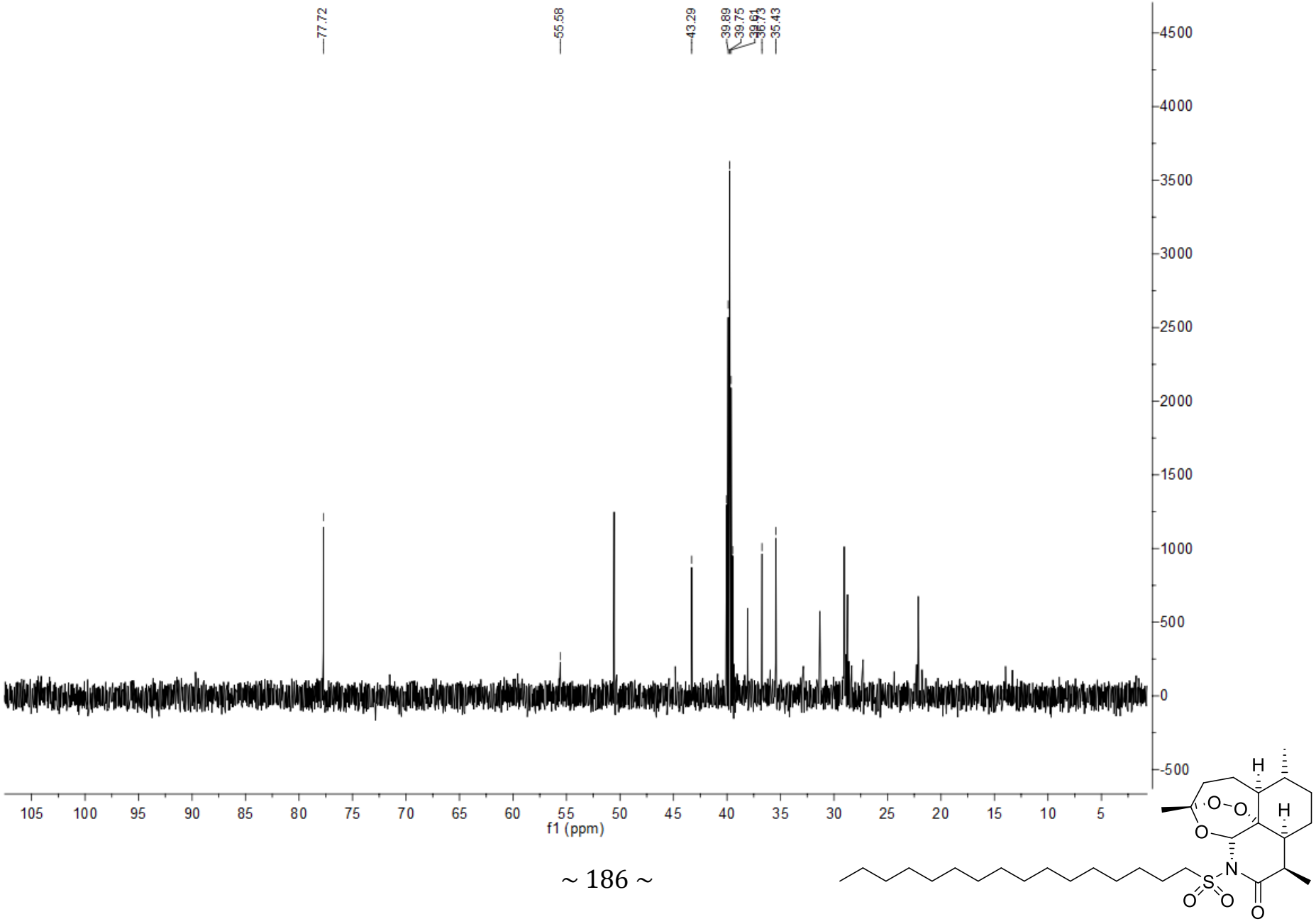
[1]H NMR DMSO 1-Hexadecanesulfonyl-azaartemisinin (**8**)



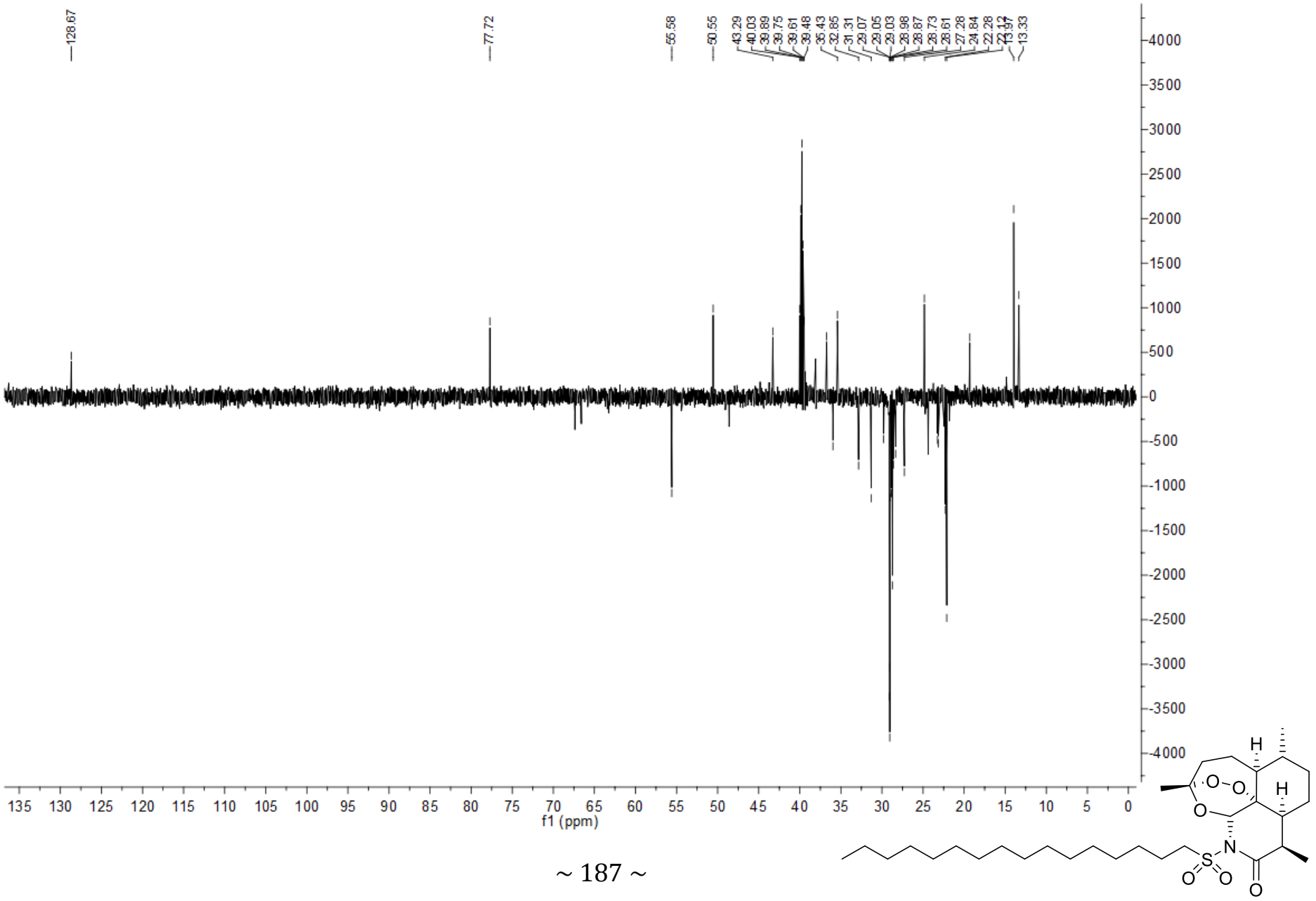
^{13}C NMR DMSO 1-Hexadecanesulfonyl-azaartemisinin (**8**)



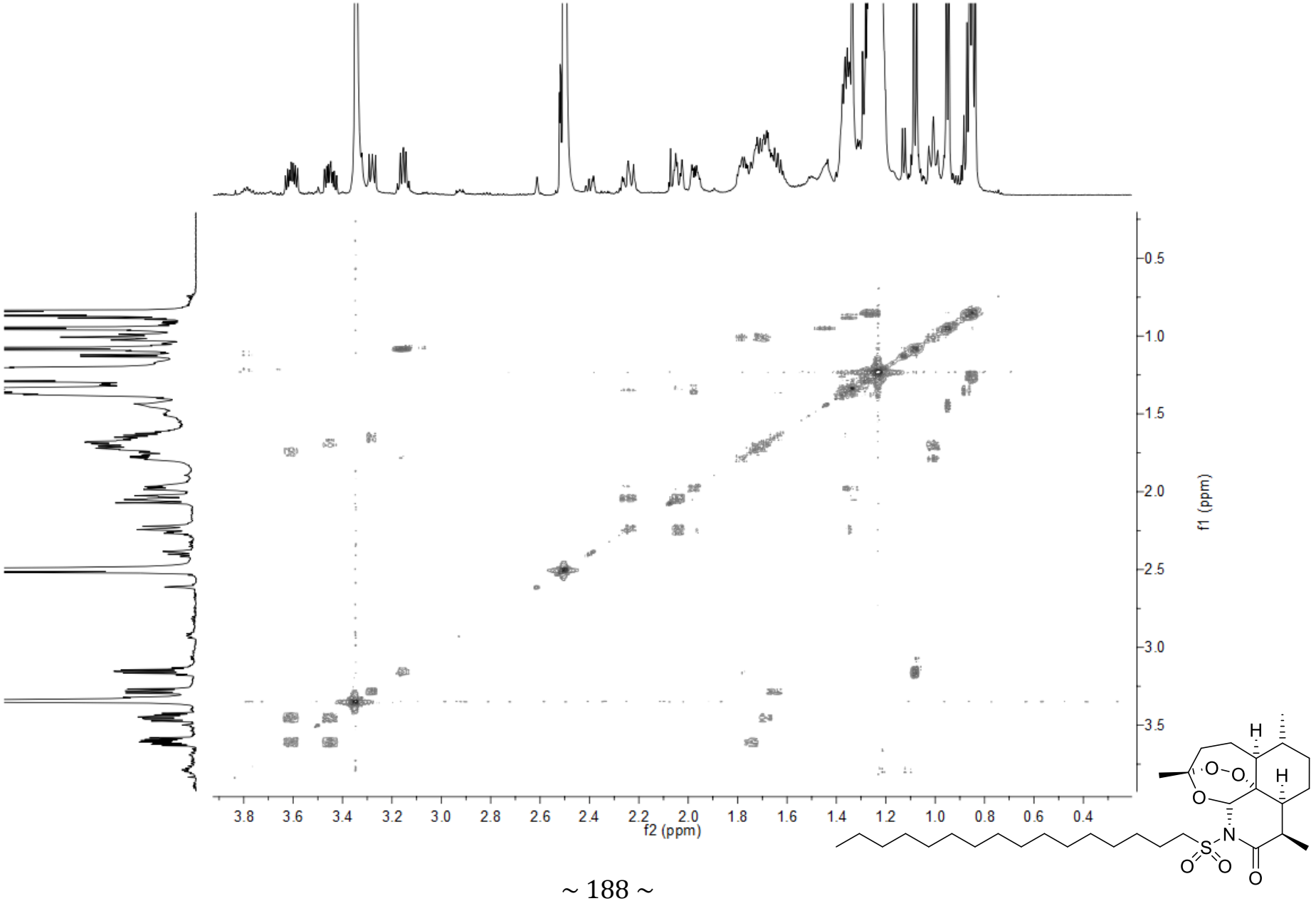
DEPT90 DMSO 1-Hexadecanesulfonyl-azaartemisinin (**8**)



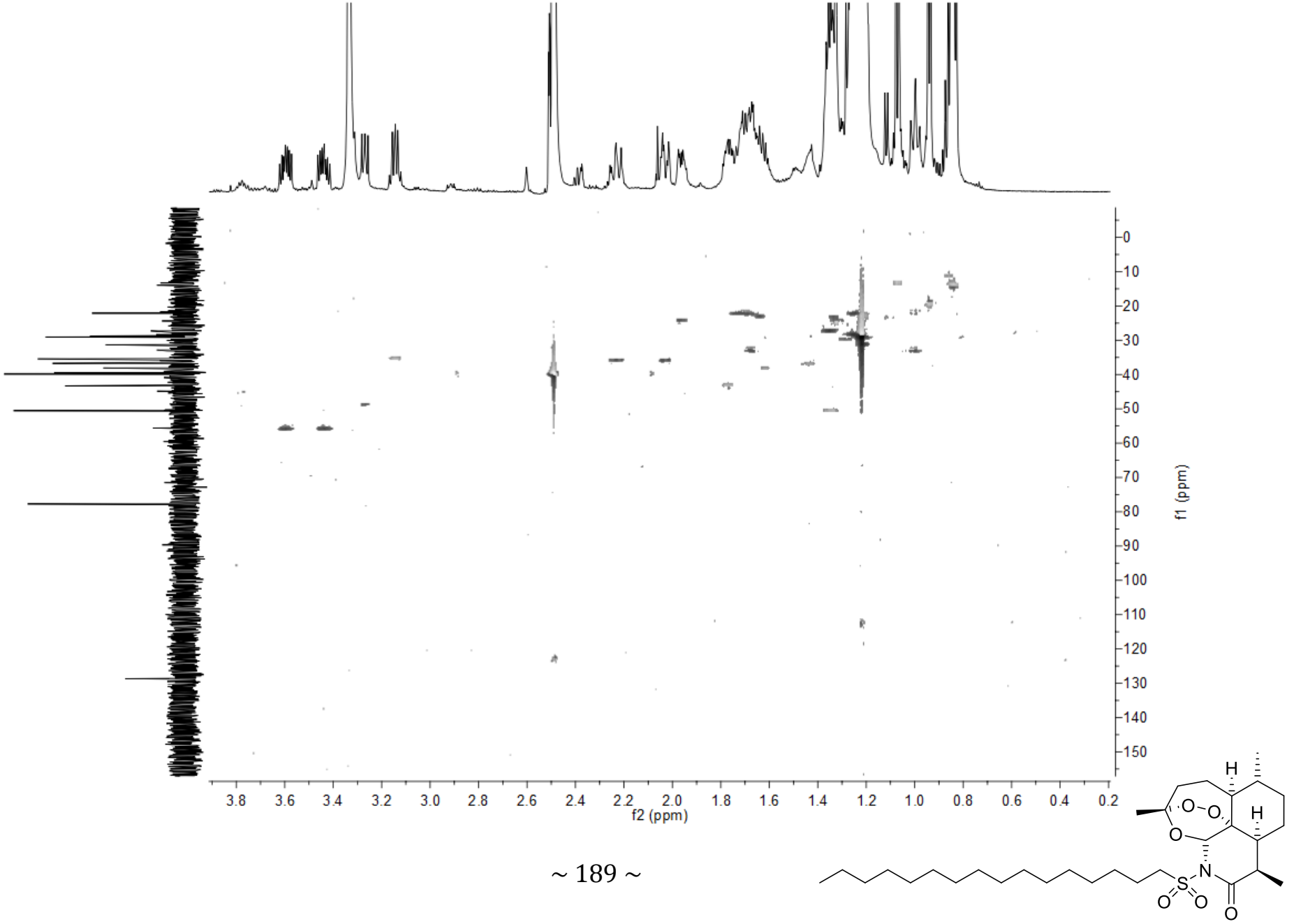
DEPT135 DMSO 1-Hexadecanesulfonyl-azaartemisinin (**8**)



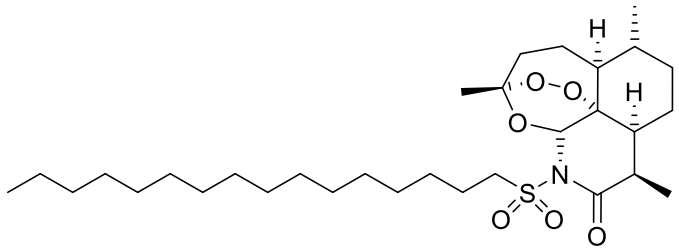
COSY DMSO 1-Hexadecanesulfonyl-azaartemisinin (**8**)



HSQC DMSO 1-Hexadecanesulfonyl-azaartemisinin (**8**)

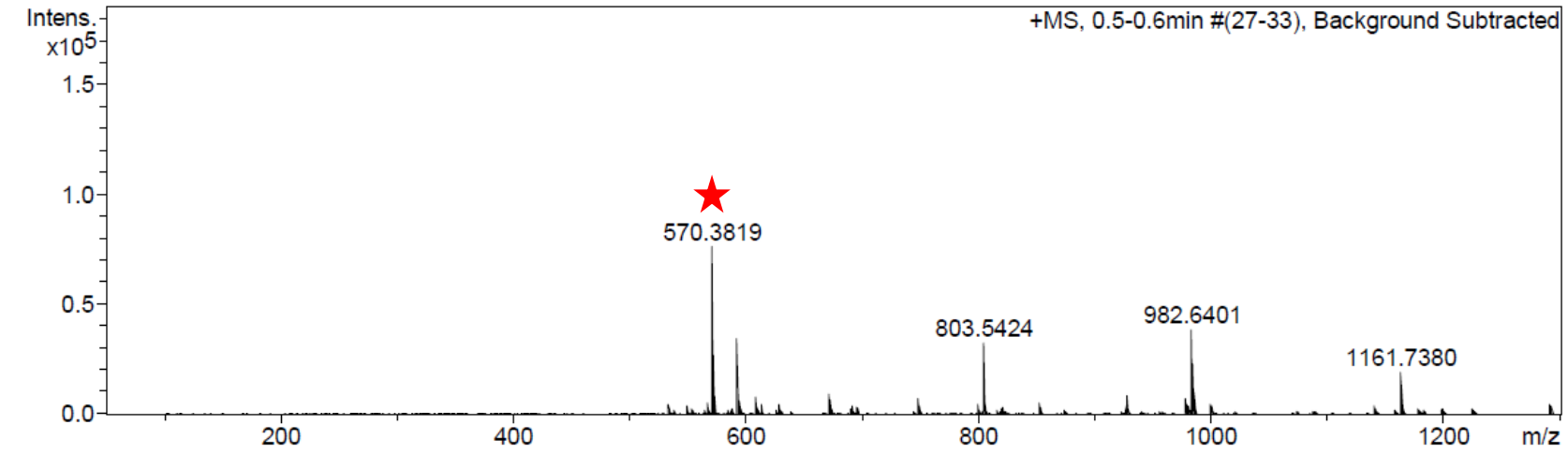


HRMS 1-Hexadecanesulfonyl-azaartemisinin (**8**)

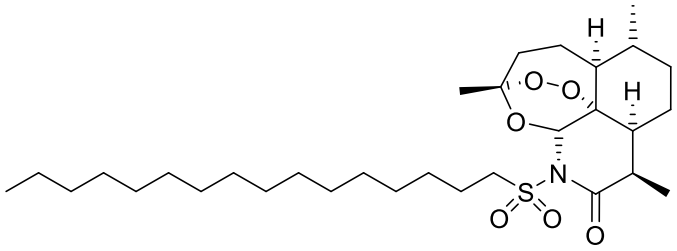


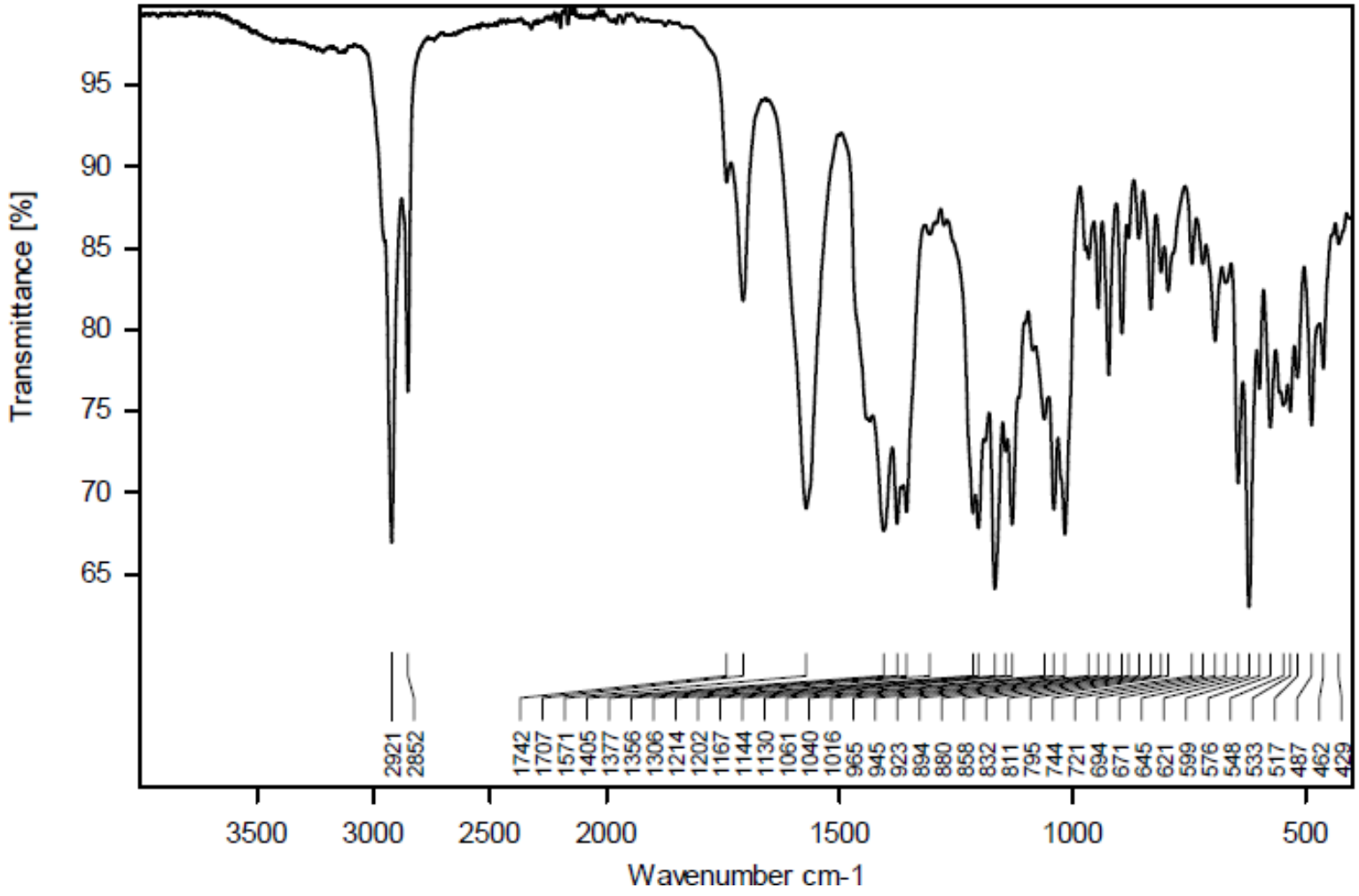
Acquisition Parameter

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	3000 m/z	Set Collision Cell RF	150.0 Vpp	Set Divert Valve	Waste

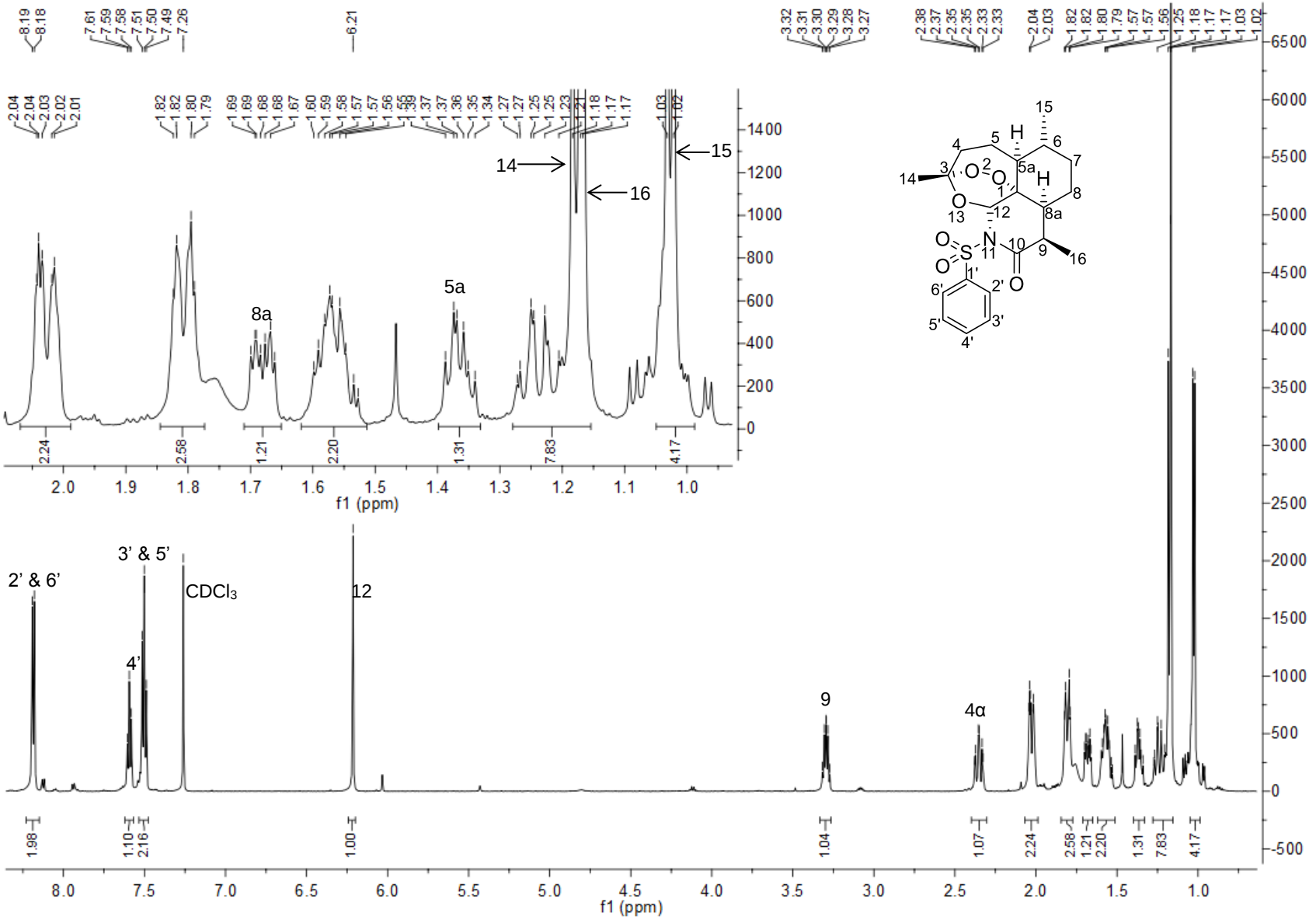


IR 1-Hexadecanesulfonyl-azaartemisinin (**8**)

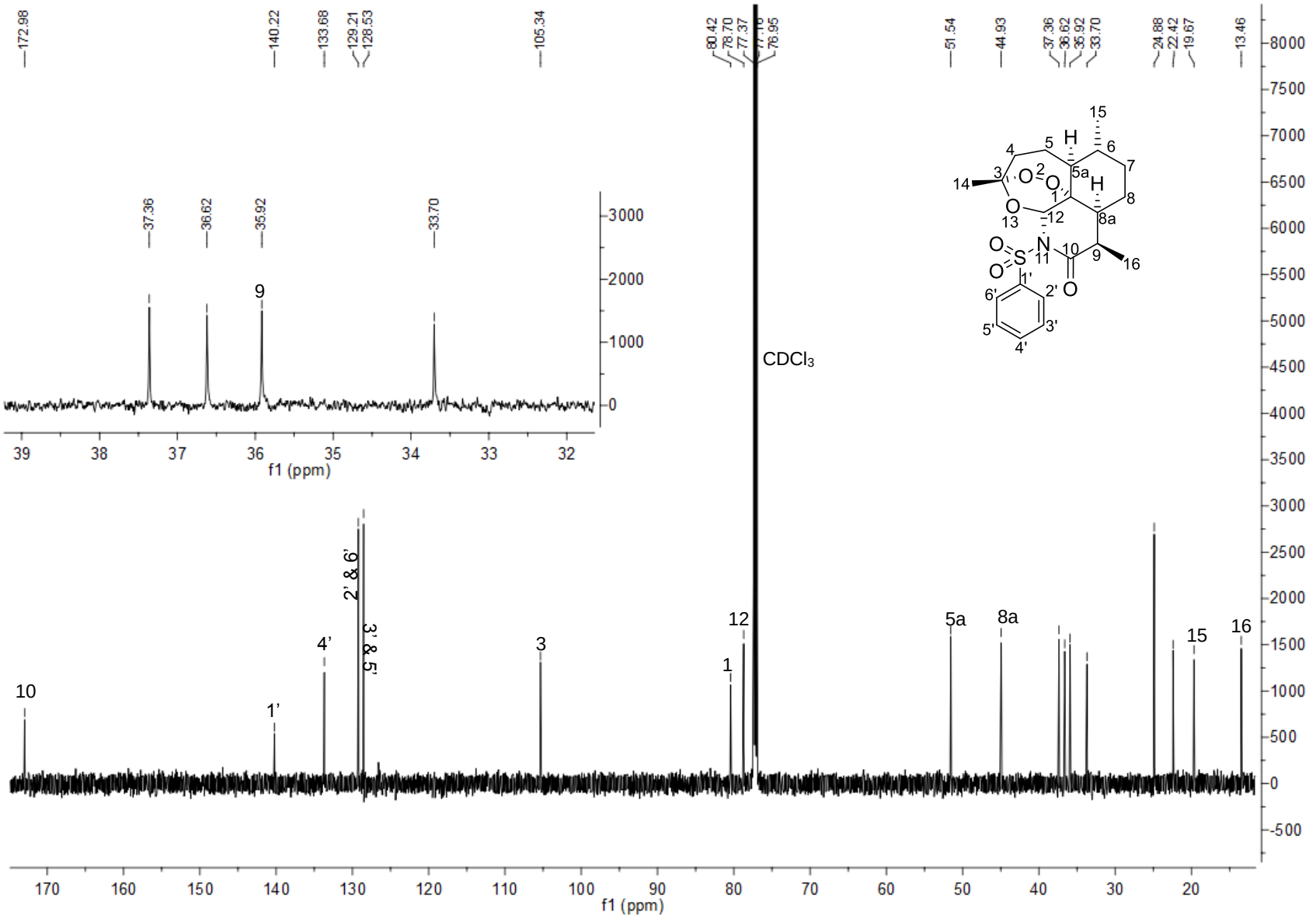




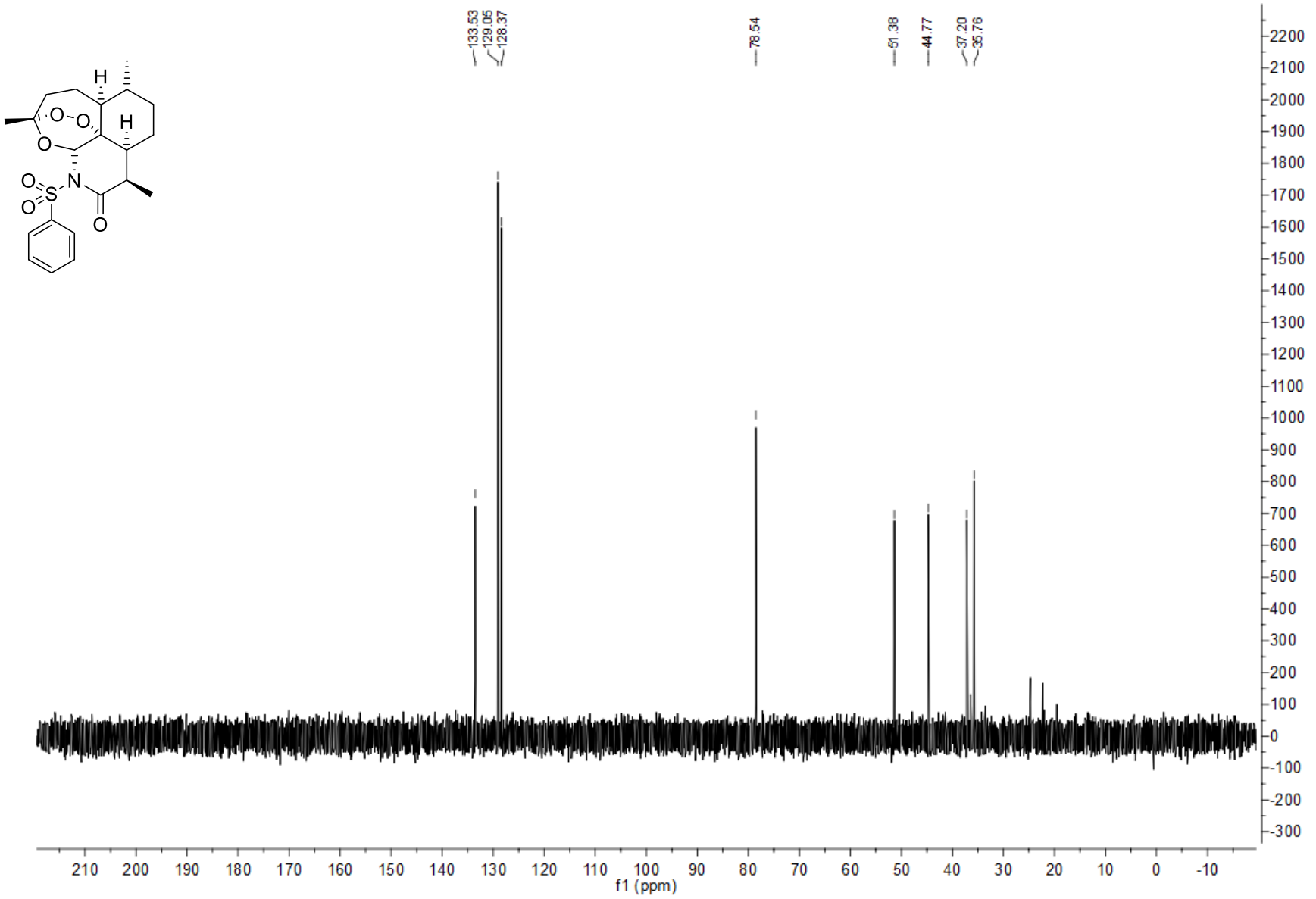
^{1}H NMR $CDCl_3$ Benzenesulfonyl-azaartemisinin (**9**)



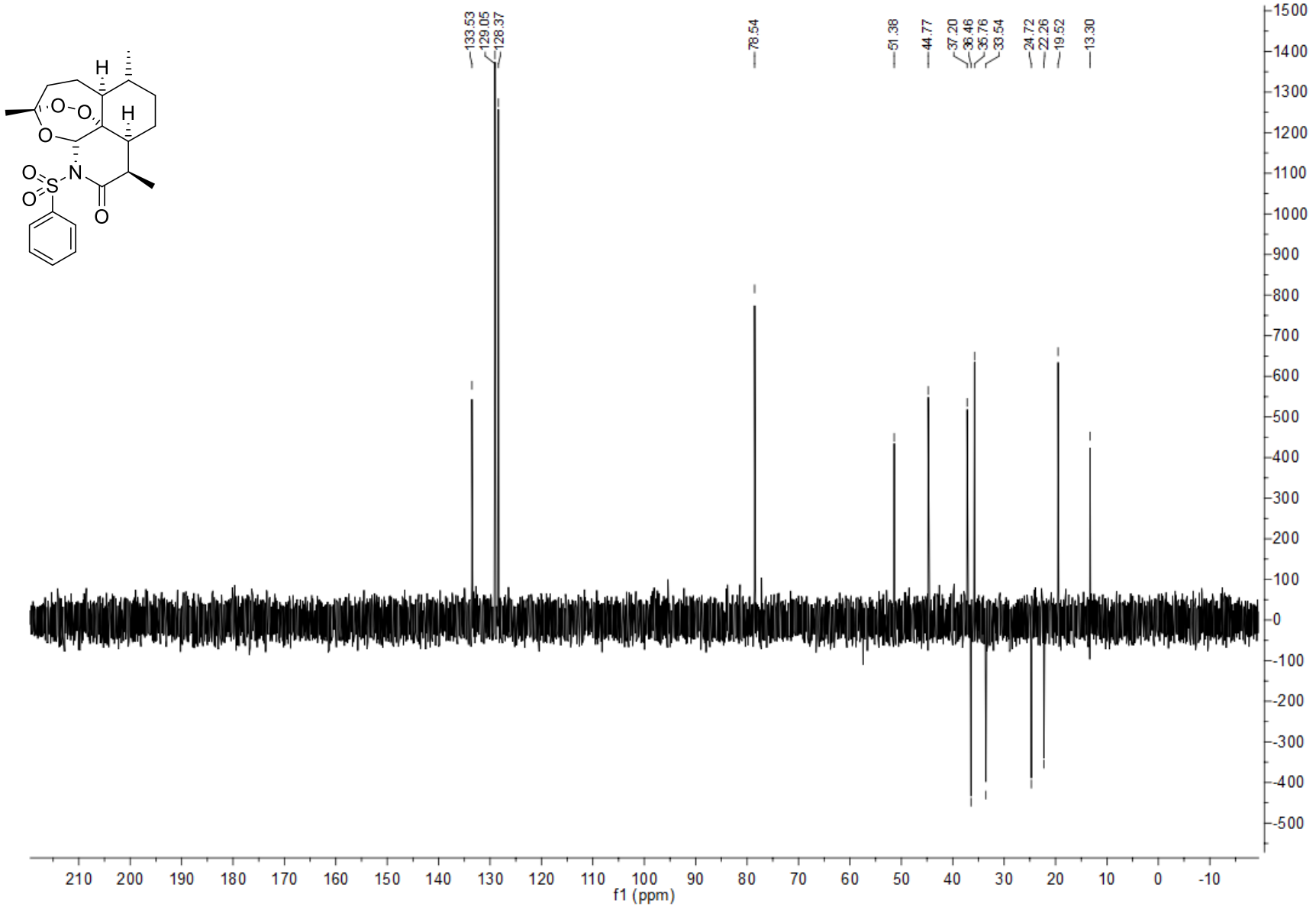
^{13}C NMR $CDCl_3$ Benzenesulfonyl-azaartemisinin (**9**)



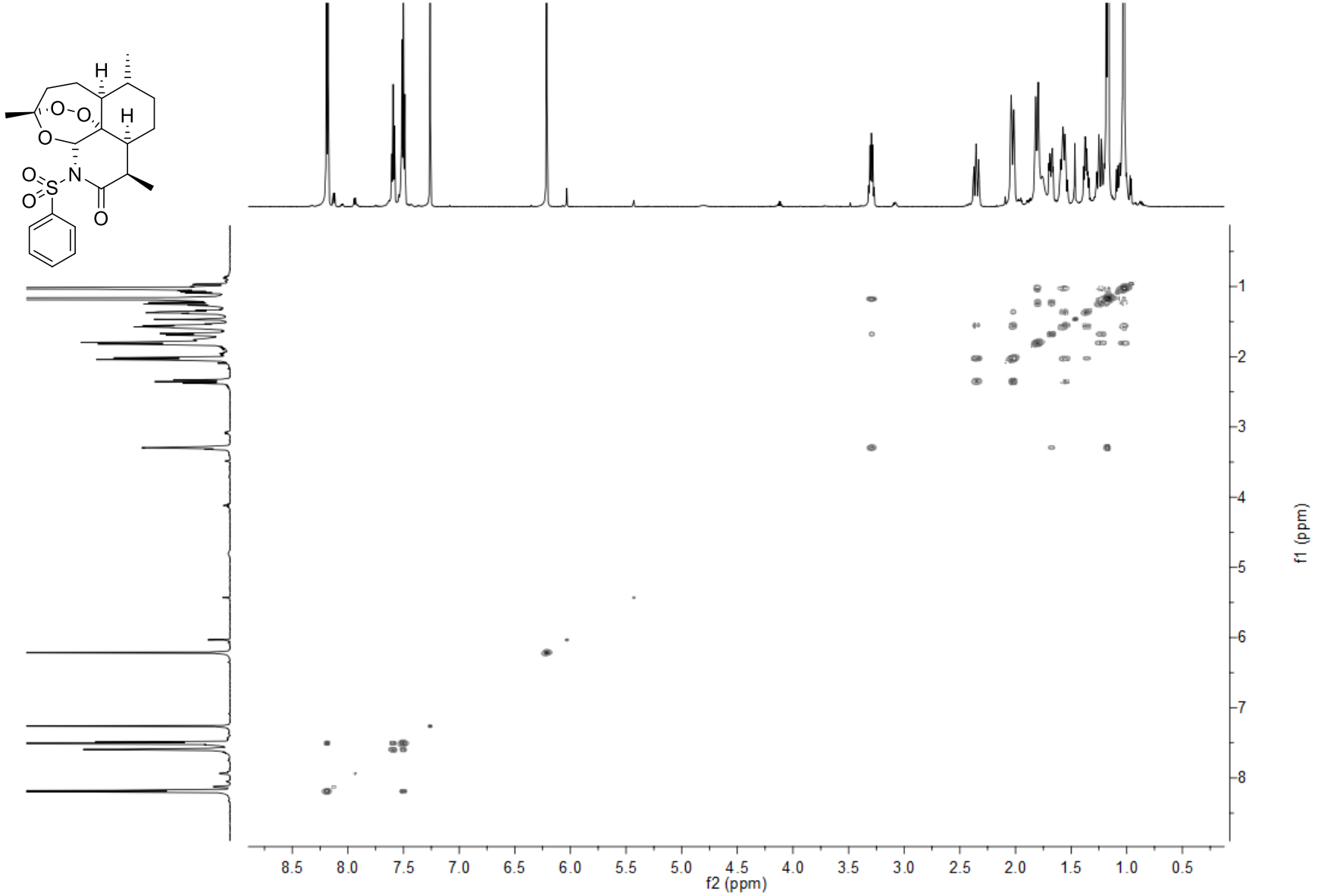
DEPT90 $CDCl_3$ Benzenesulfonyl-azaartemisinin (**9**)



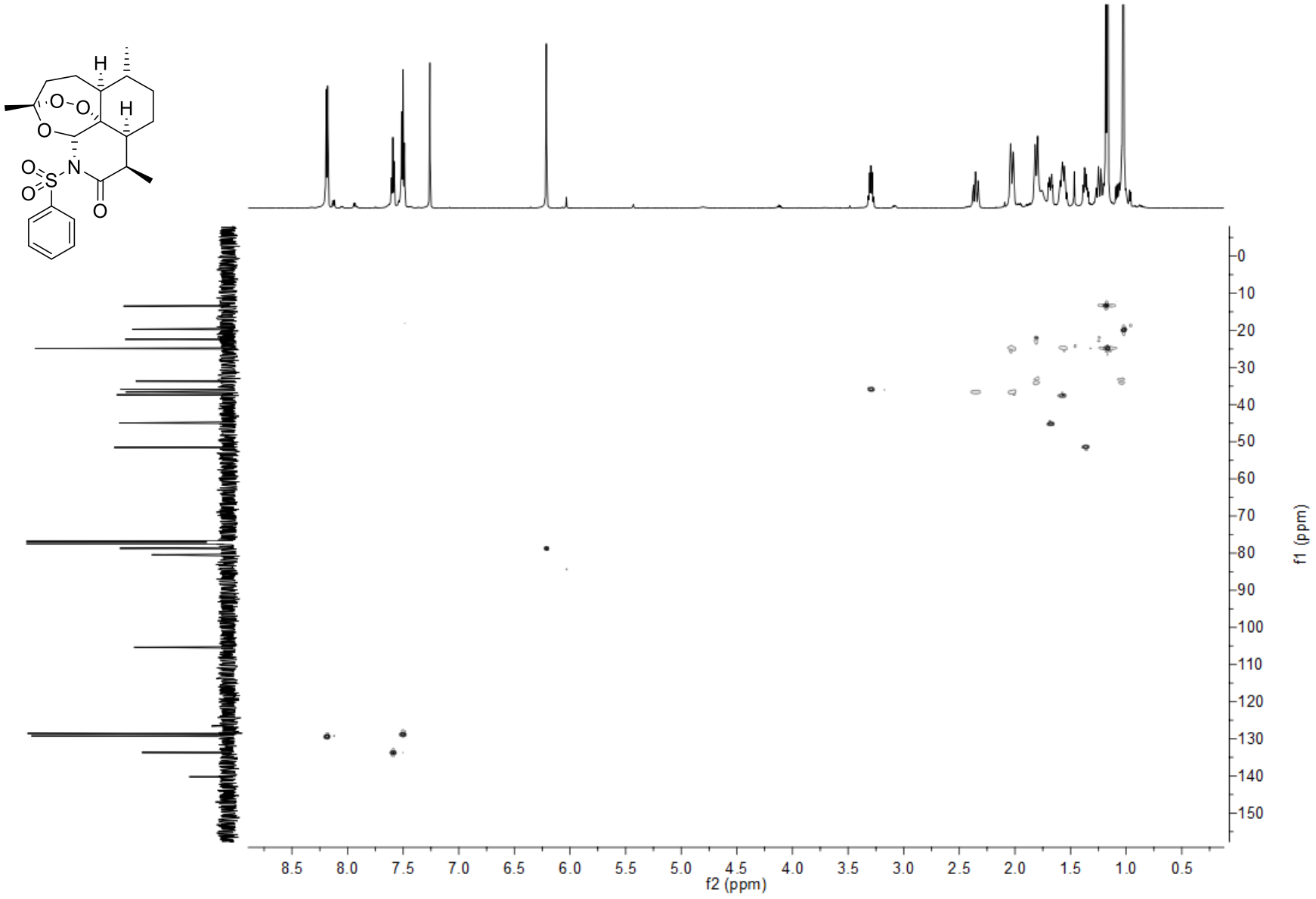
DEPT135 $CDCl_3$ Benzenesulfonyl-azaartemisinin (**9**)



COSY $CDCl_3$ Benzenesulfonyl-azaartemisinin (**9**)



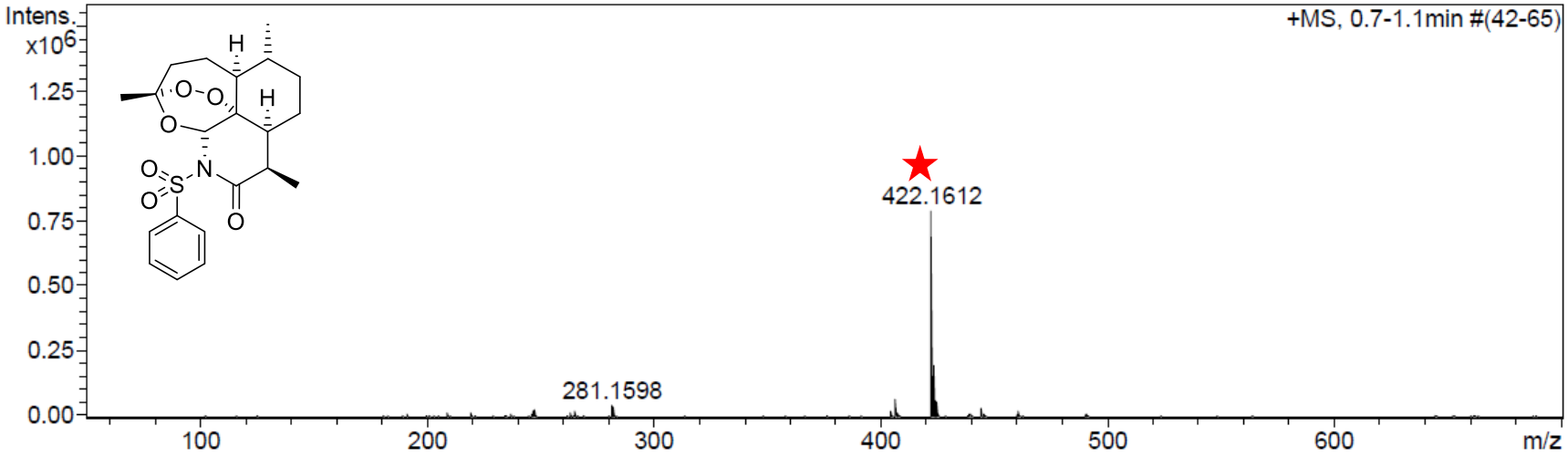
HSQC $CDCl_3$ Benzenesulfonyl-azaartemisinin (**9**)



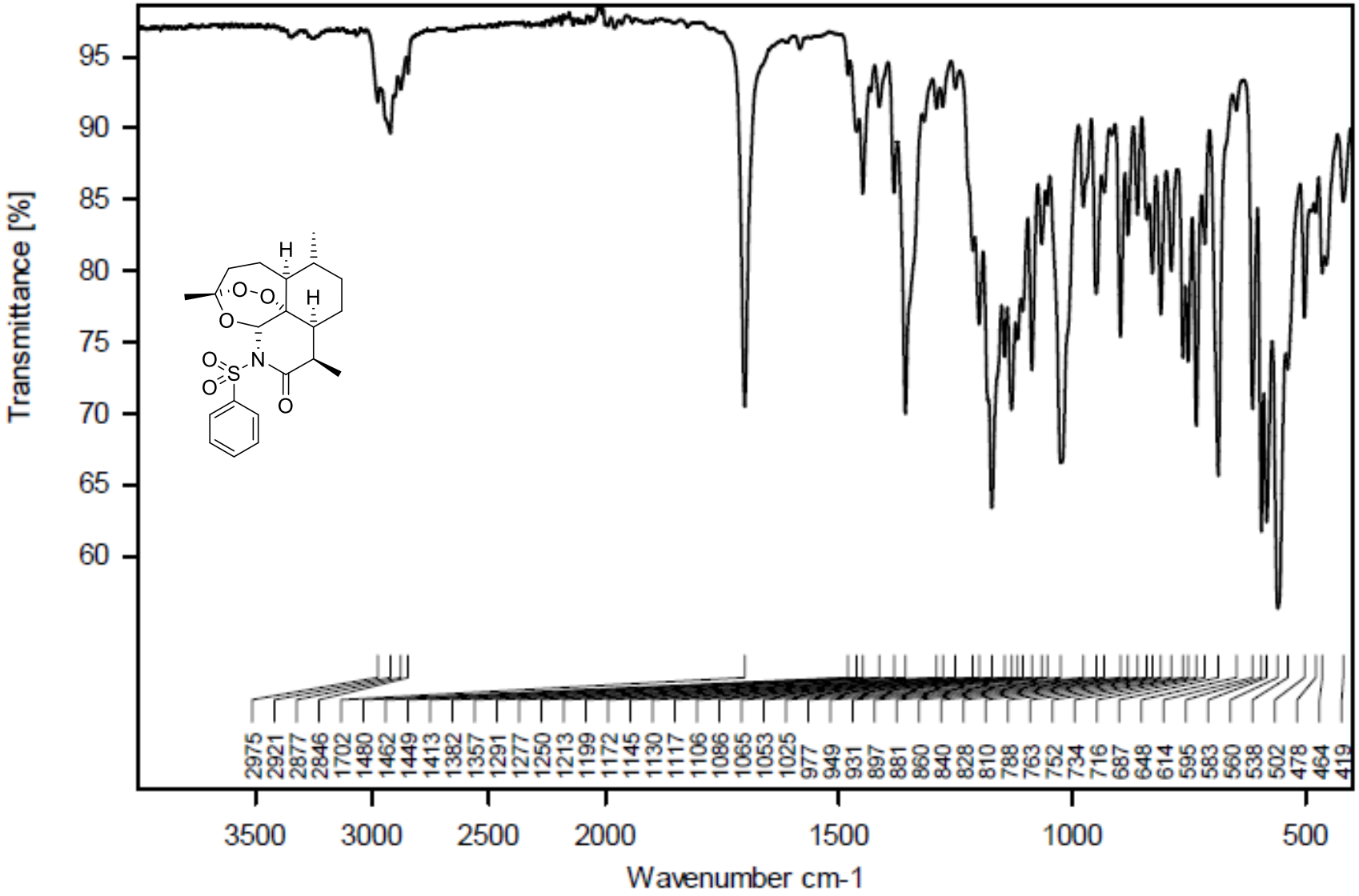
HRMS Benzenesulfonyl-azaartemisinin (**9**)

Acquisition Parameter

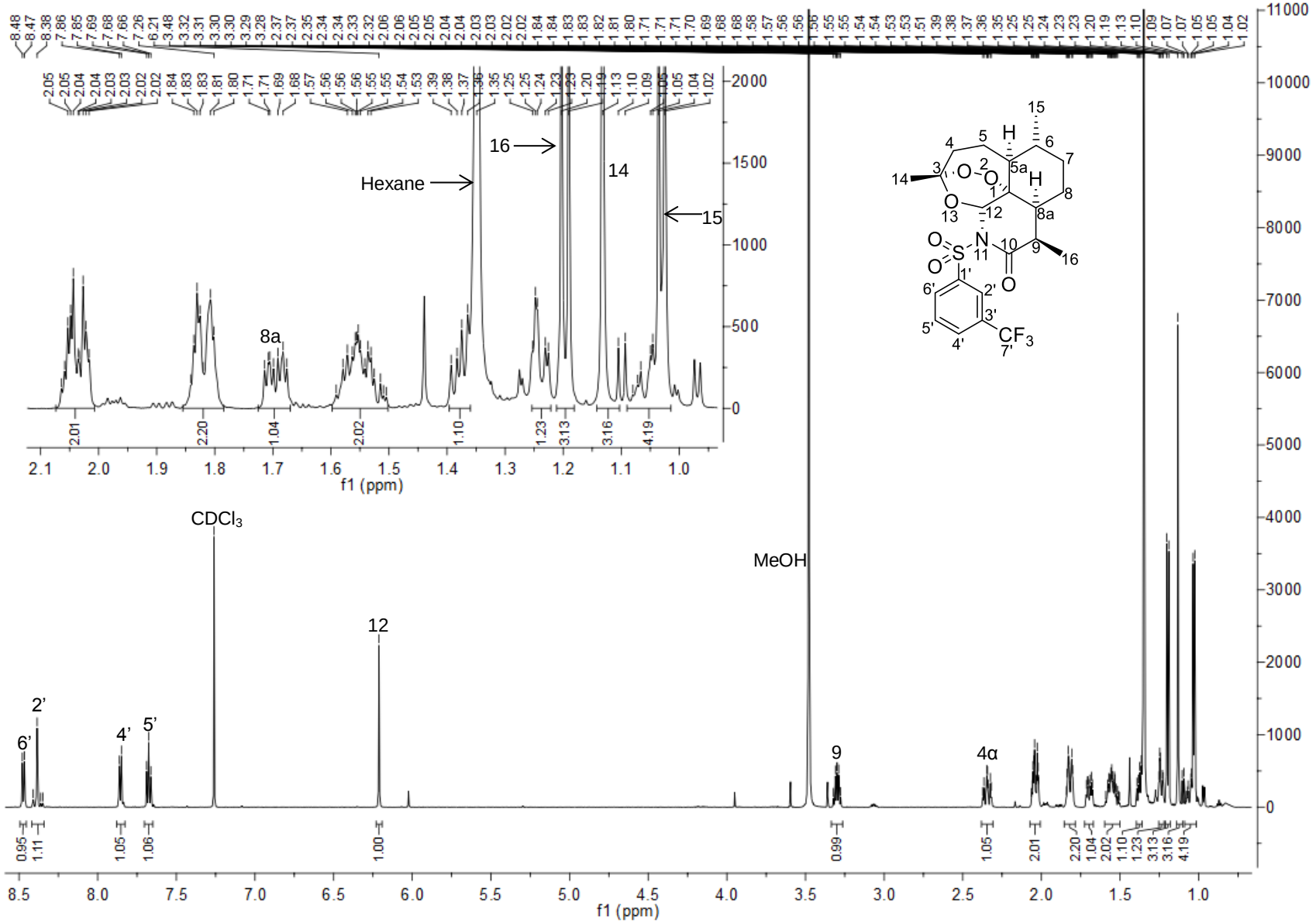
Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	0.4 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	180 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	4.0 l/min
Scan End	3000 m/z	Set Collision Cell RF	150.0 Vpp	Set Divert Valve	Waste



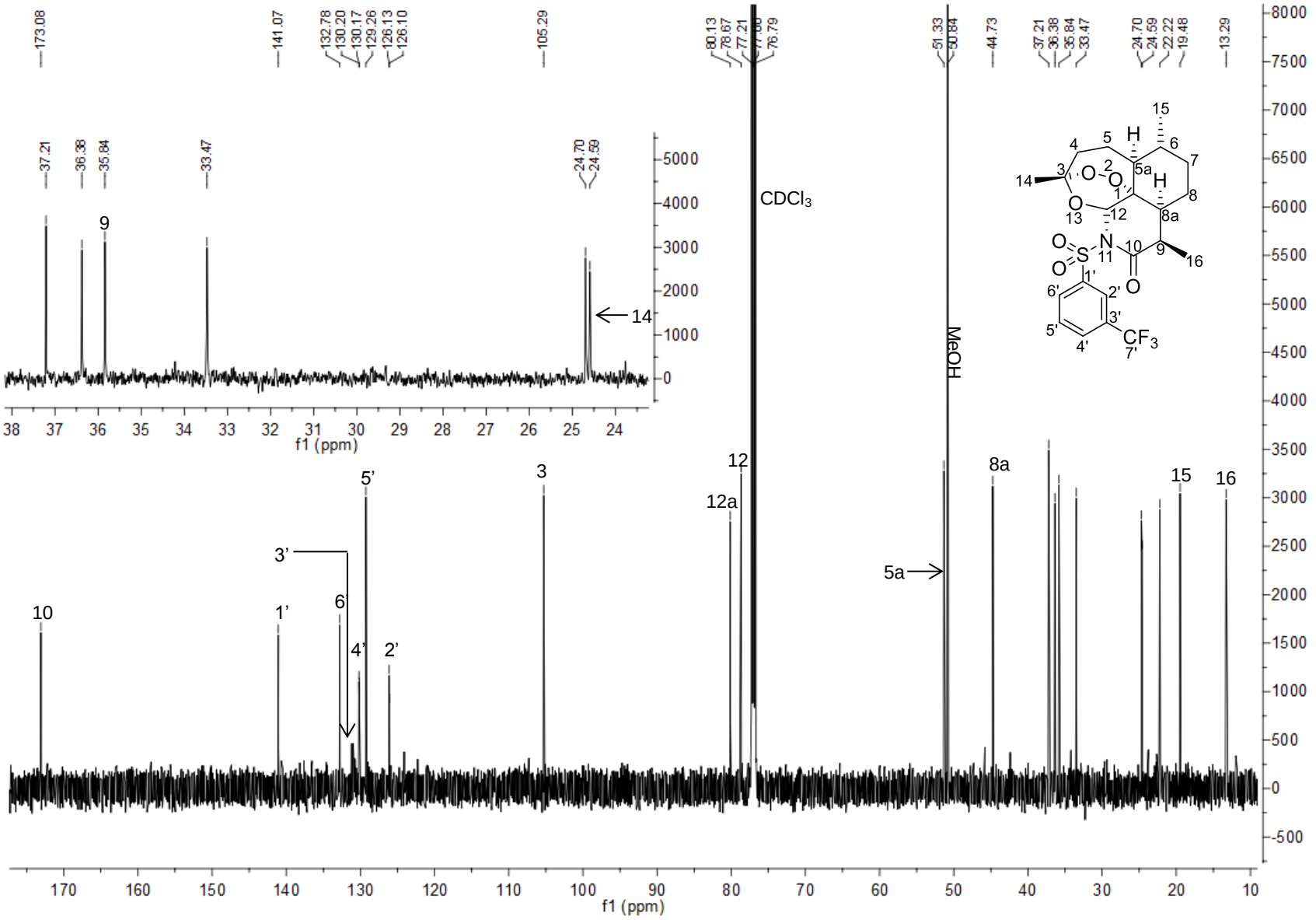
IR Benzenesulfonyl-azaartemisinin (**9**)



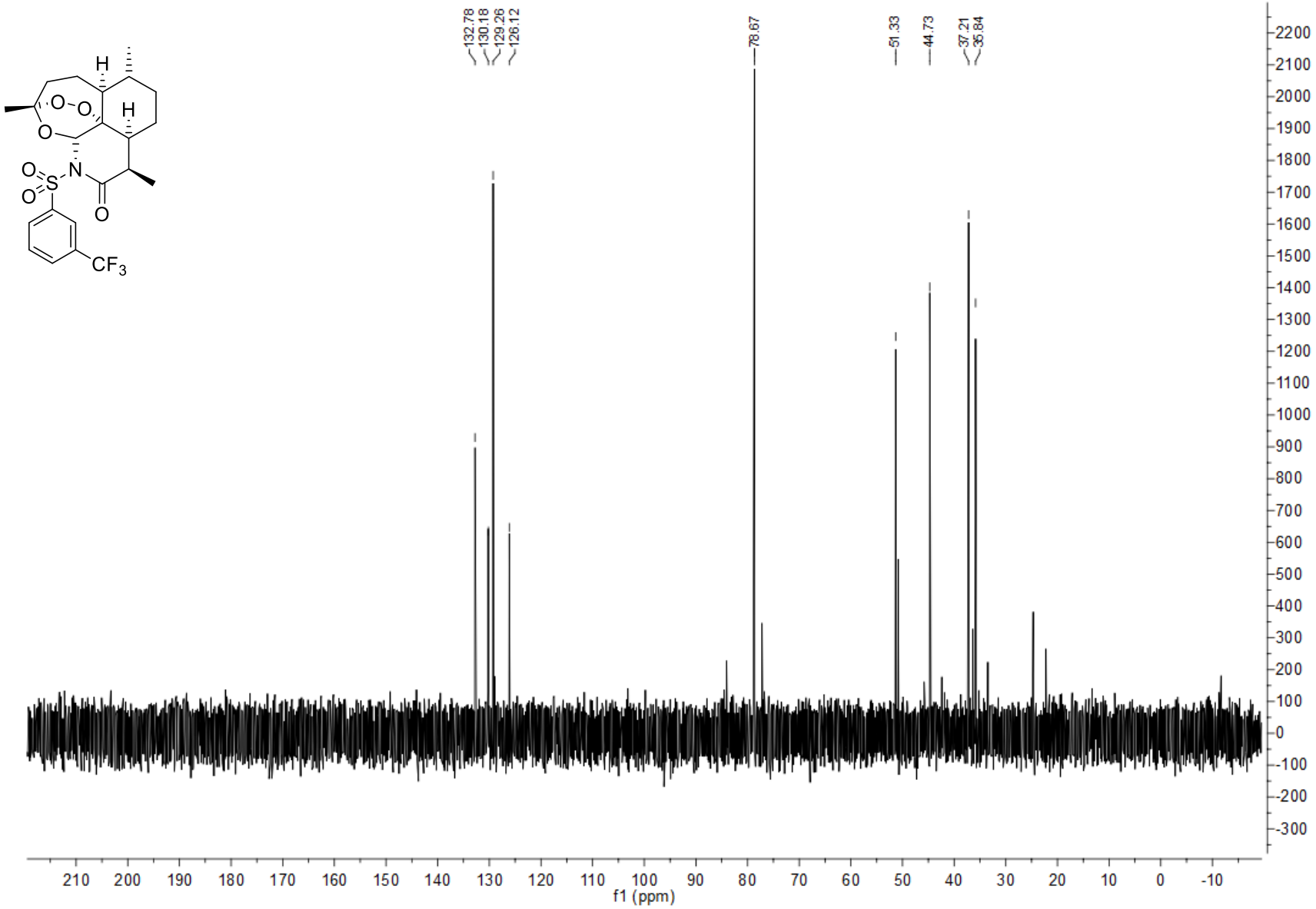
^{1}H NMR $CDCl_3$ 3-(Trifluoromethyl)benzenesulfonyl-azaartemisinin (**10**)



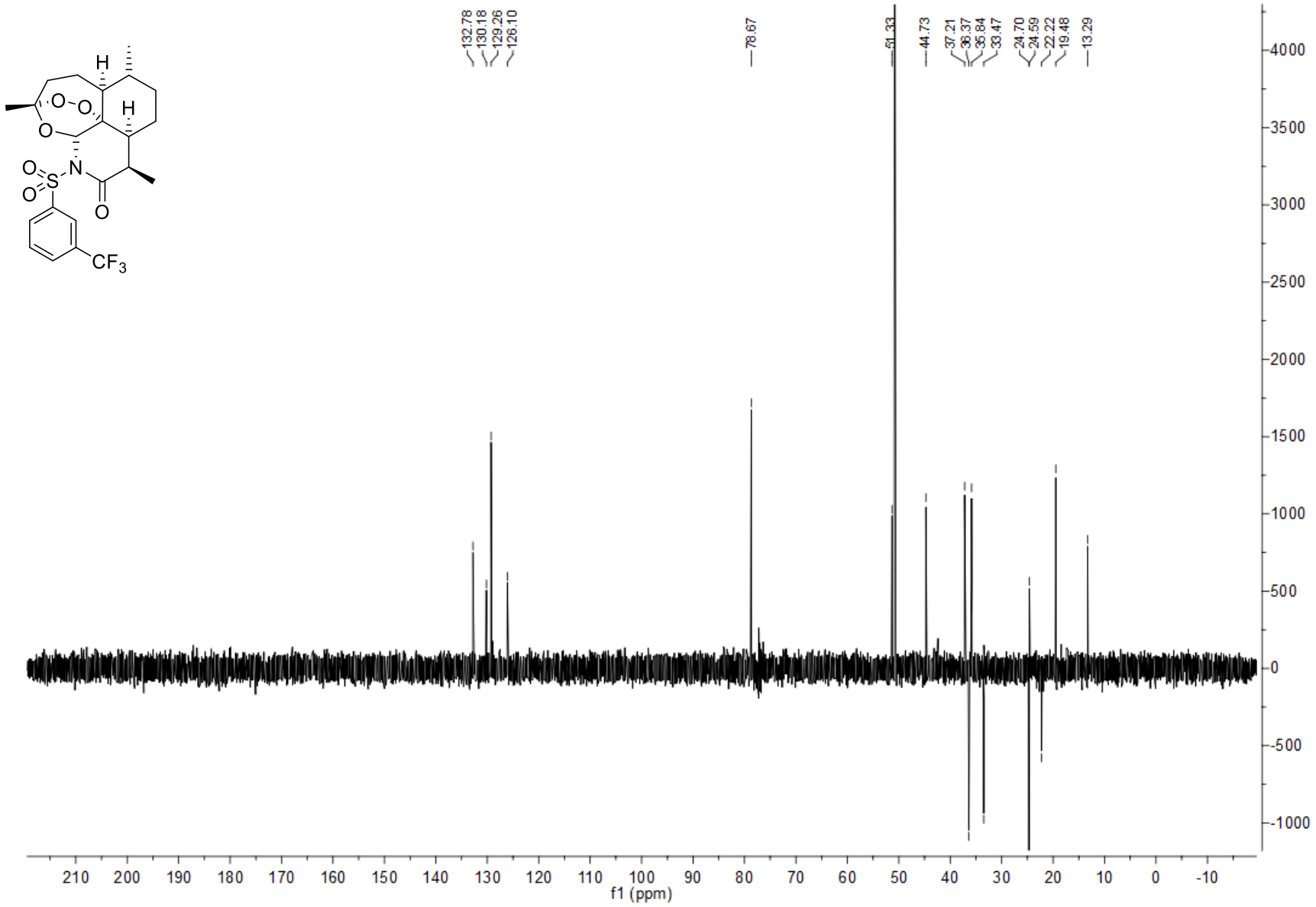
^{13}C NMR $CDCl_3$ 3-(Trifluoromethyl)benzenesulfonyl-azaartemisinin (**10**)



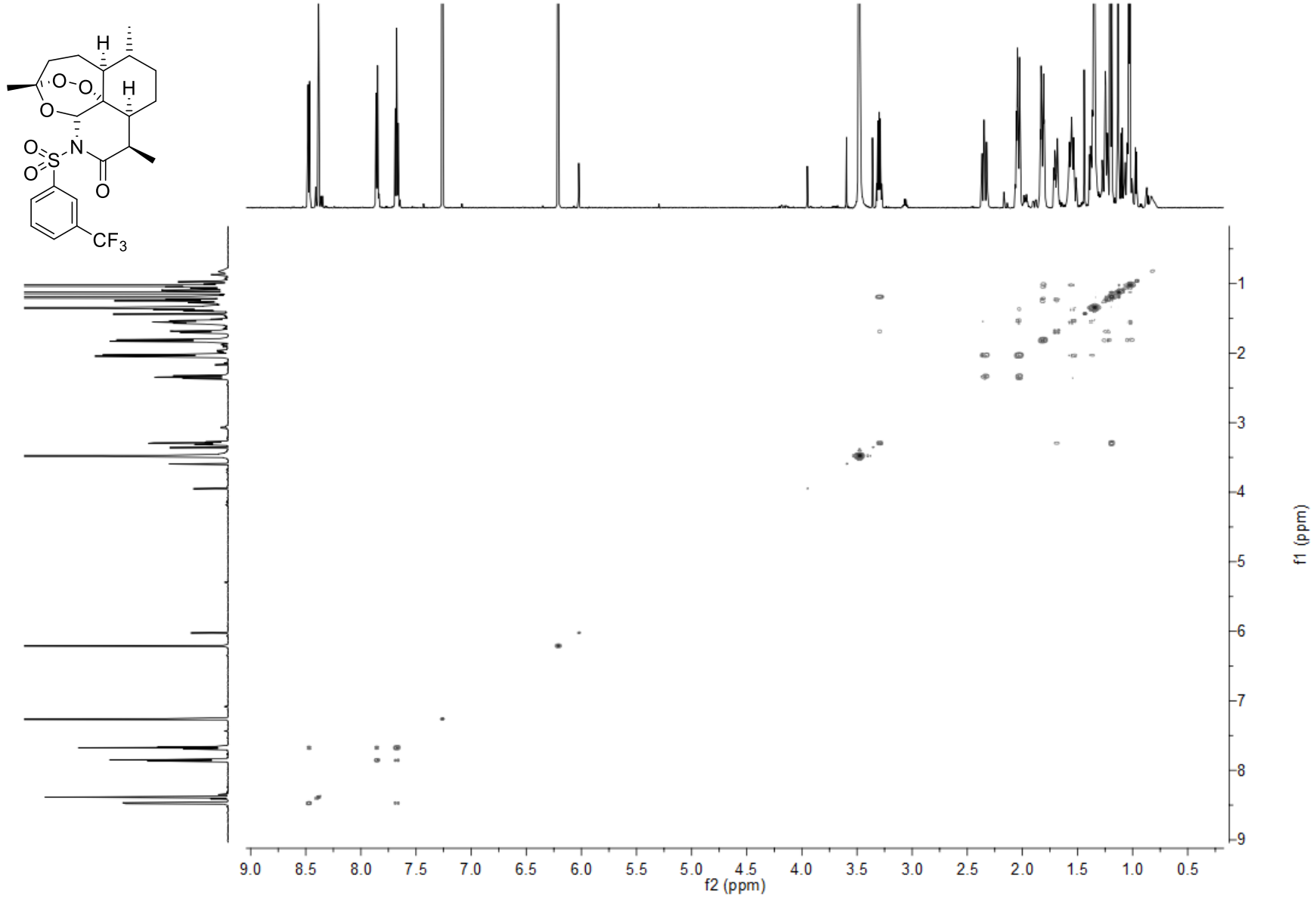
DEPT90 $CDCl_3$ 3-(Trifluoromethyl)benzenesulfonyl-azaartemisinin (**10**)



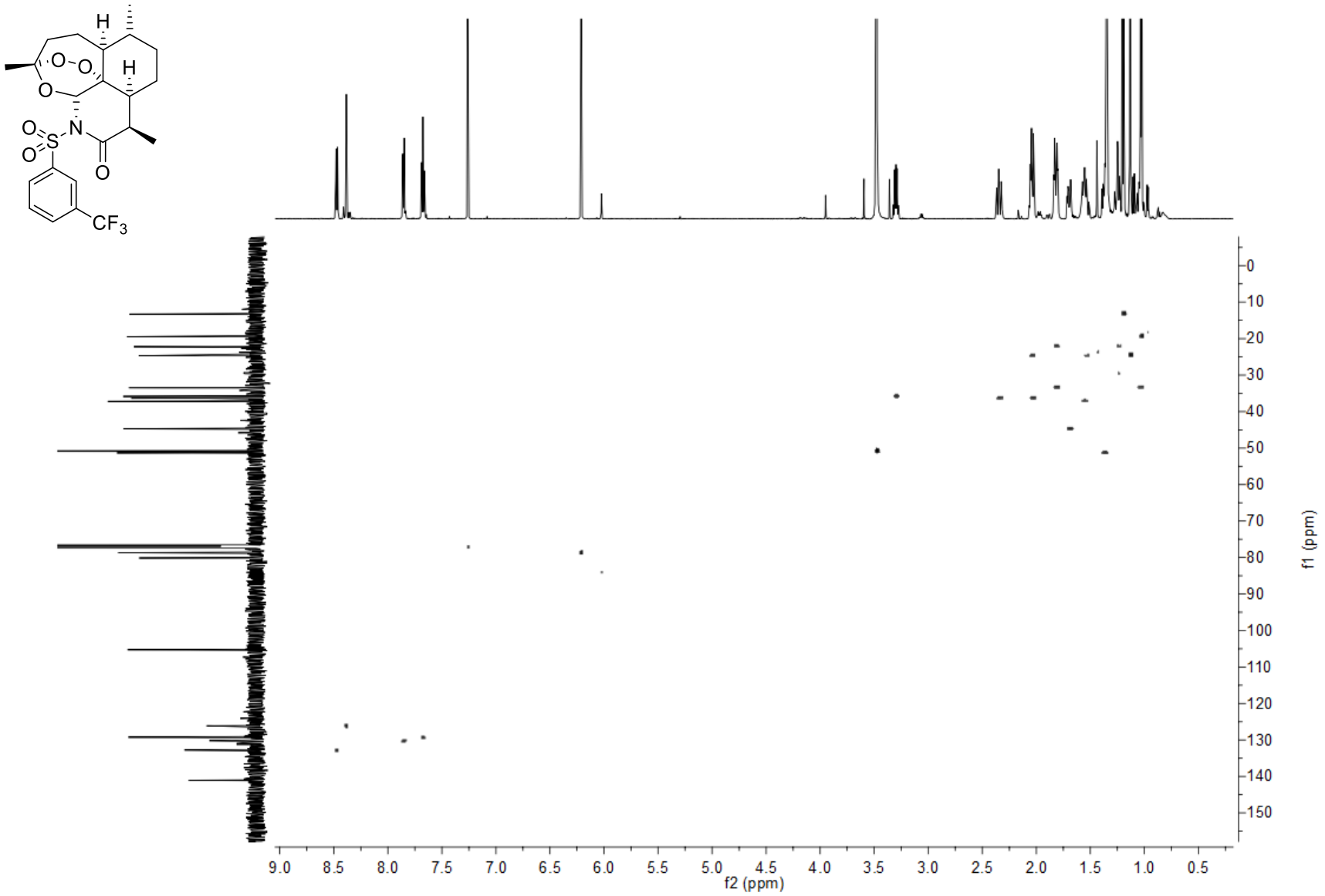
DEPT135 $CDCl_3$ 3-(Trifluoromethyl)benzenesulfonyl-azaartemisinin (**10**)



COSY $CDCl_3$ 3-(Trifluoromethyl)benzenesulfonyl-azaartemisinin (**10**)



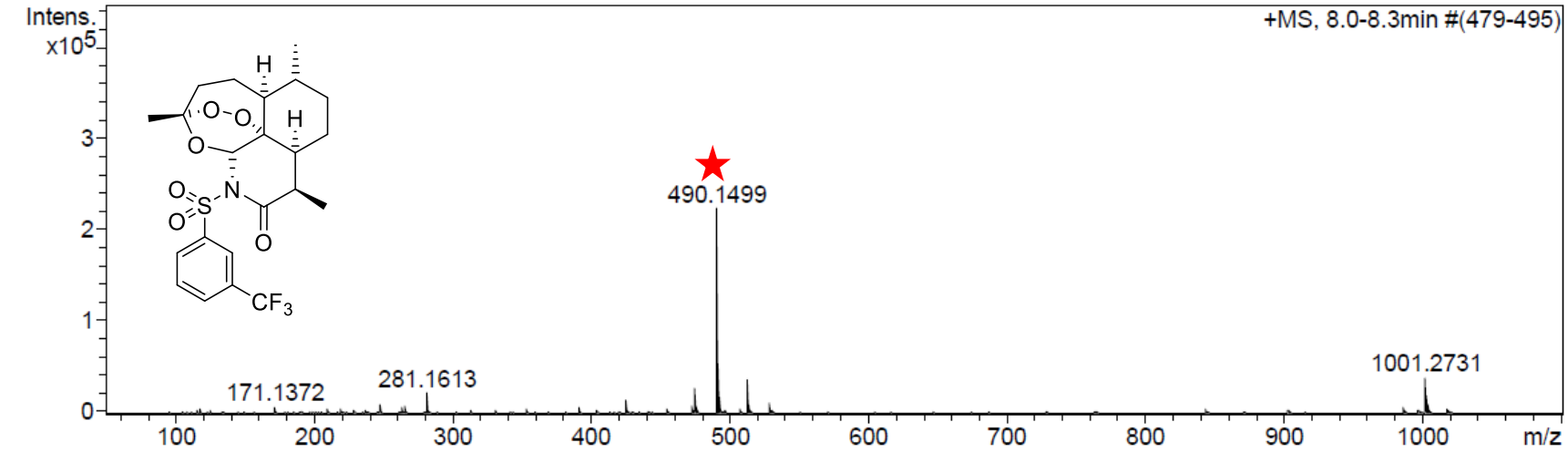
HSQC $CDCl_3$ 3-(Trifluoromethyl)benzenesulfonyl-azaartemisinin (**10**)



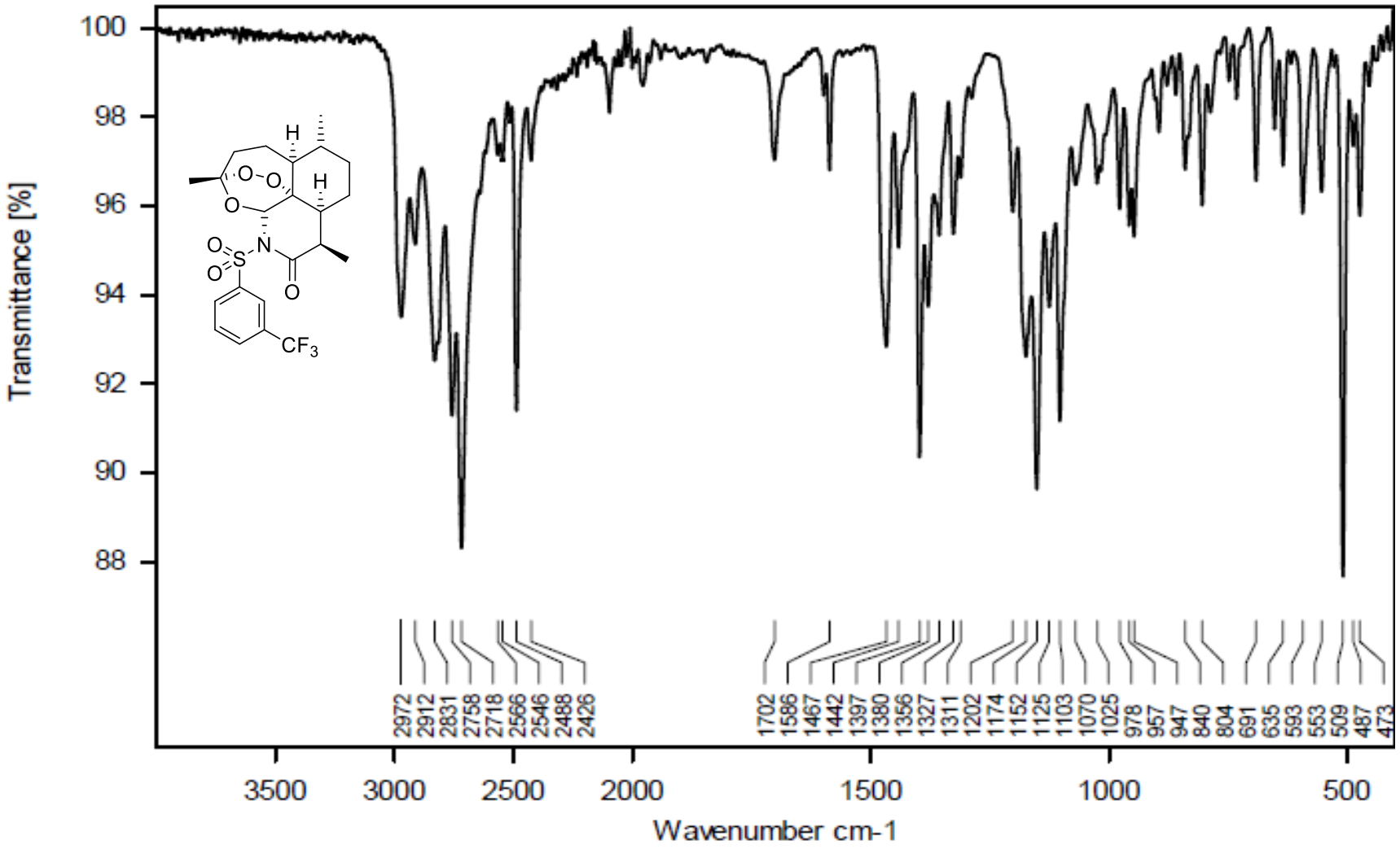
HRMS 3-(Trifluoromethyl)benzenesulfonyl-azaartemisinin (**10**)

Acquisition Parameter

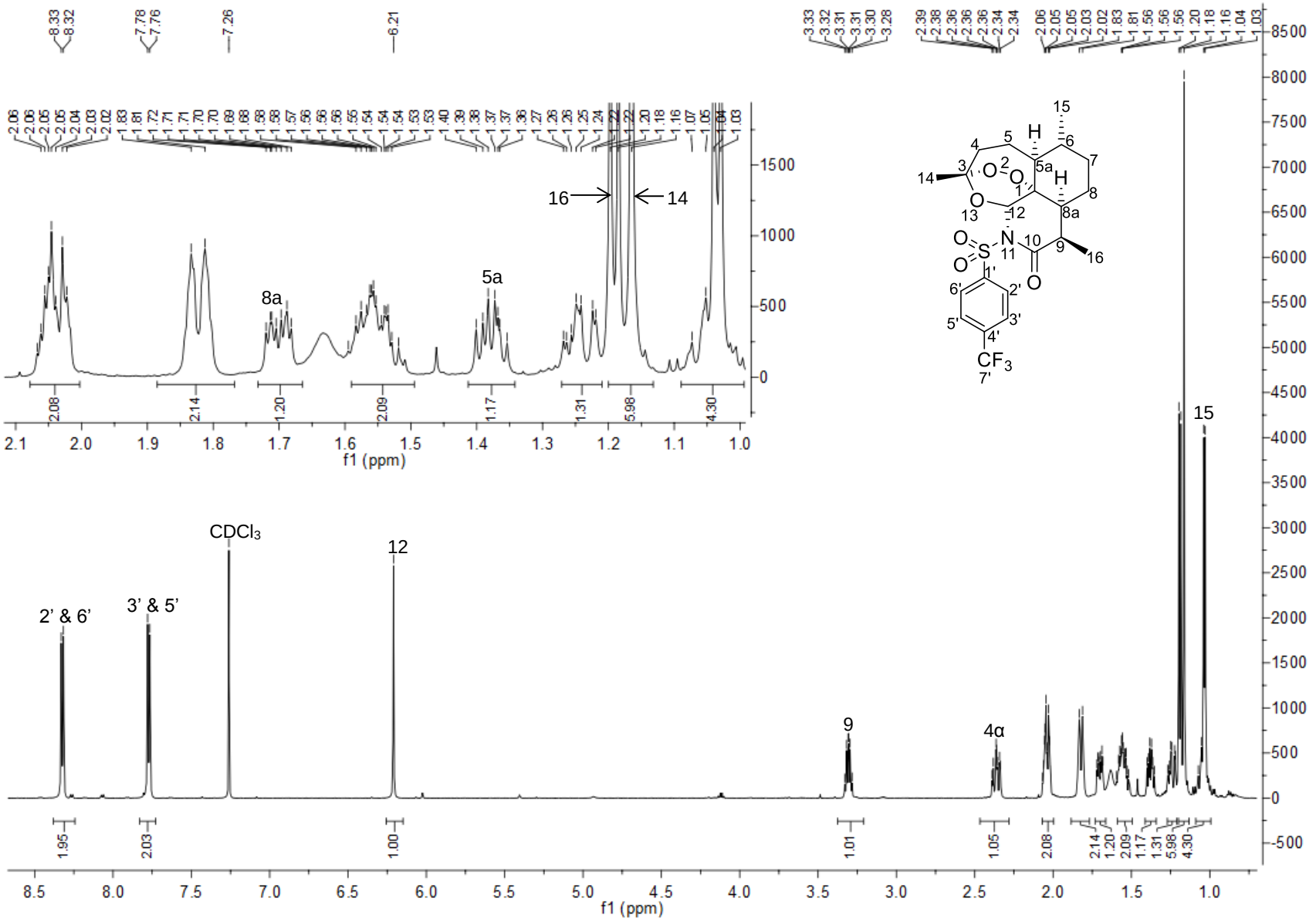
Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	0.4 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	180 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	4.0 l/min
Scan End	3000 m/z	Set Collision Cell RF	150.0 Vpp	Set Divert Valve	Waste



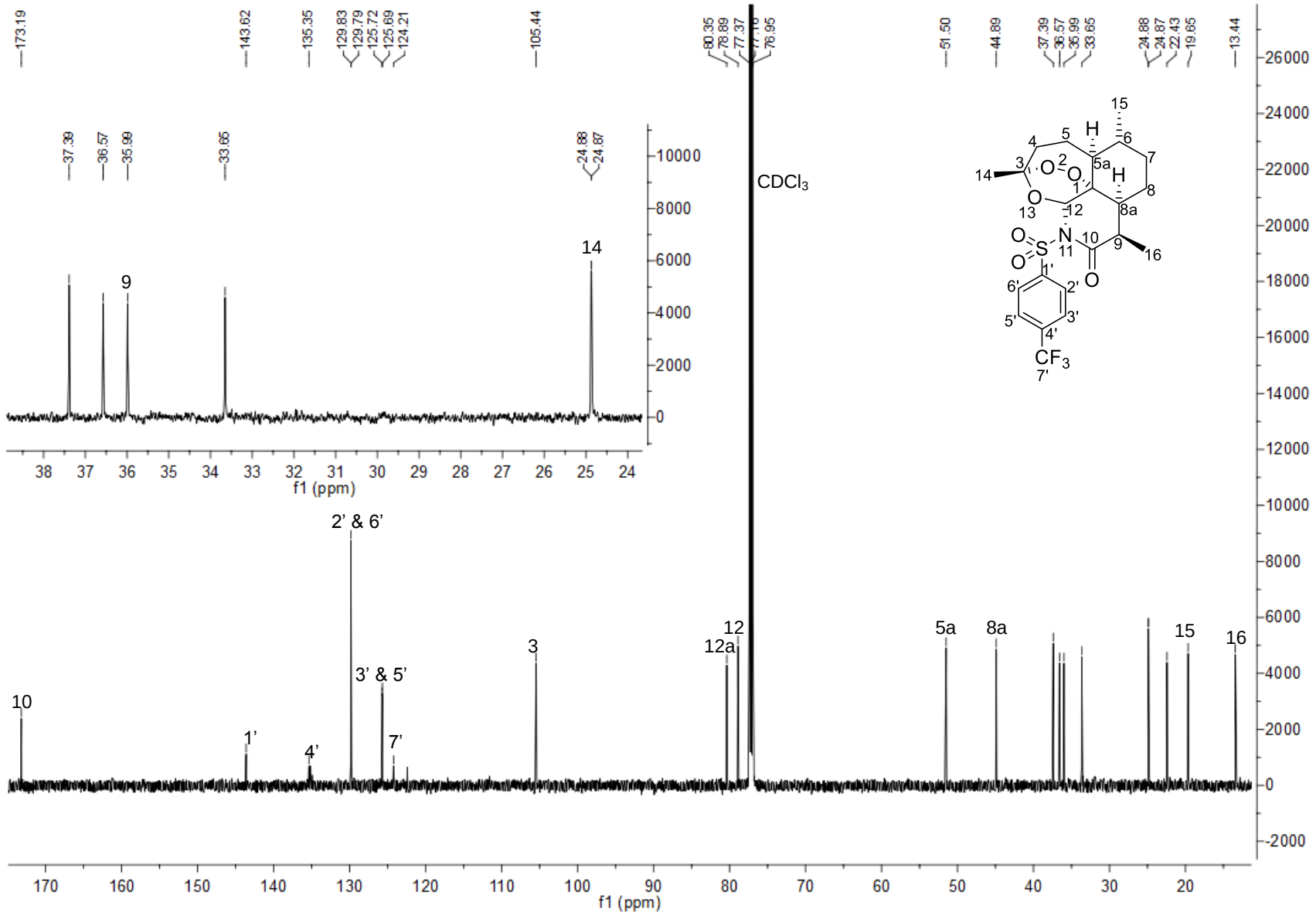
IR 3-(Trifluoromethyl)benzenesulfonyl-azaartemisinin (**10**)



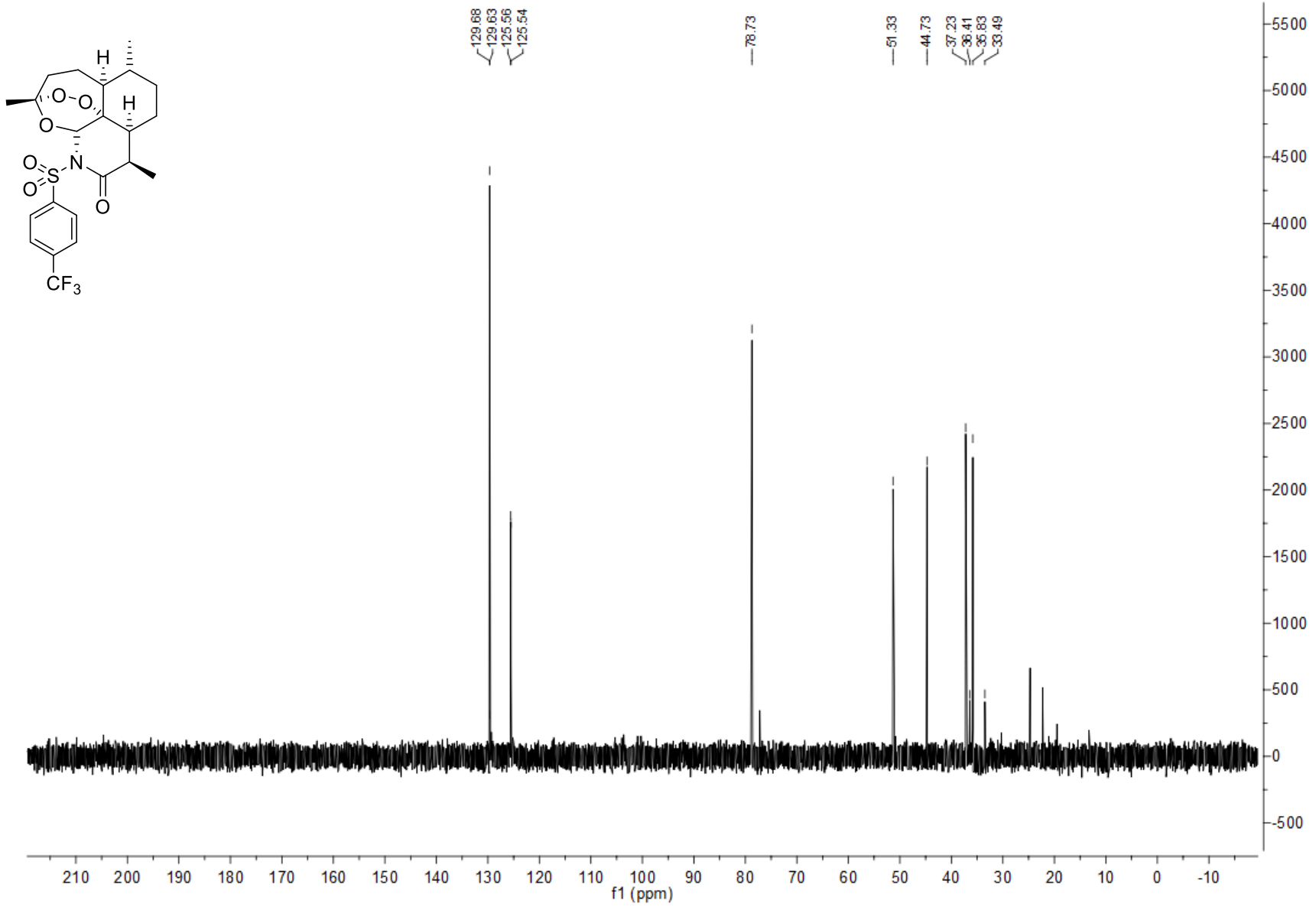
^{1}H NMR $CDCl_3$ 4-(Trifluoromethyl)benzenesulfonyl-azaartemisinin (**11**)



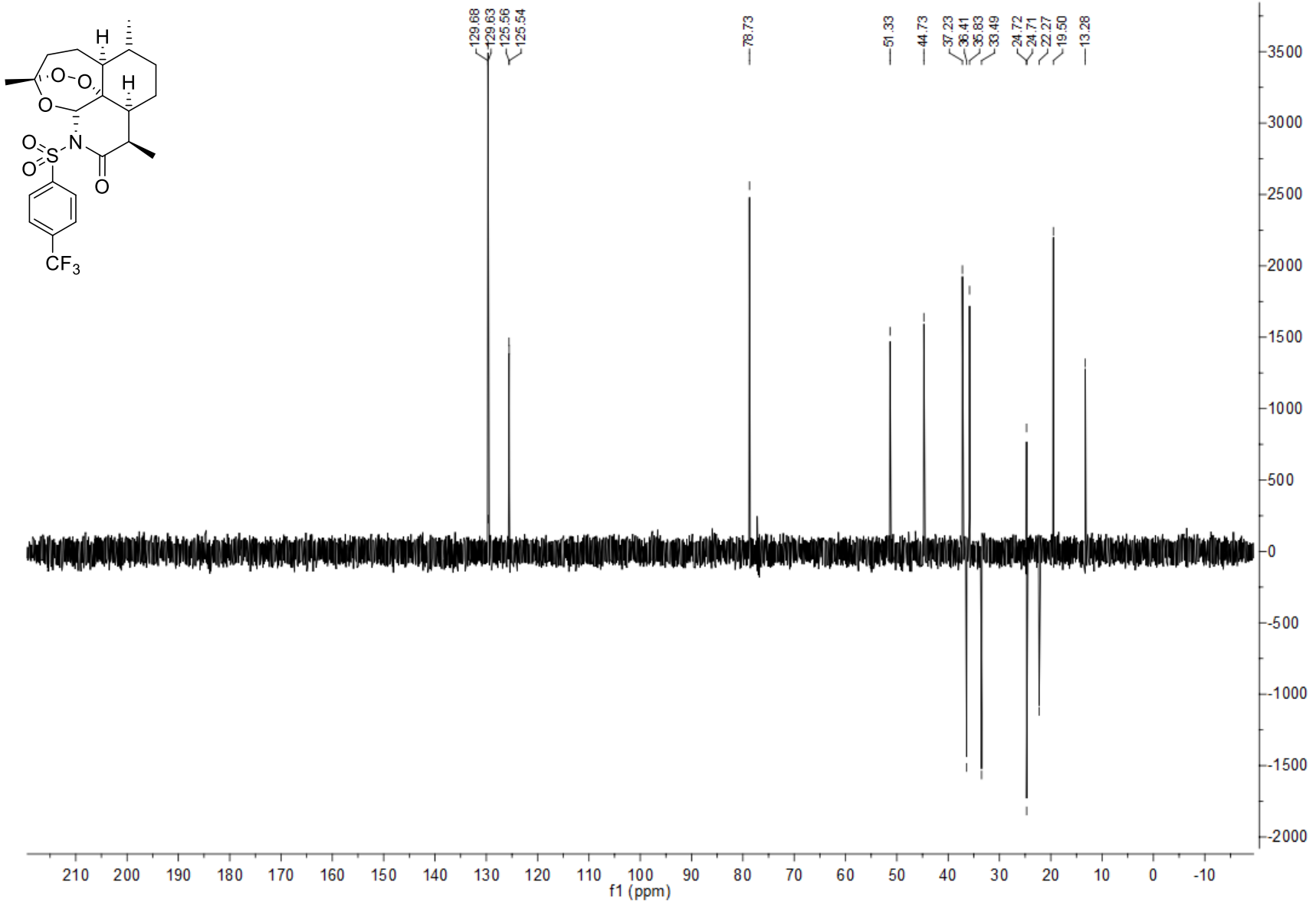
^{13}C NMR $CDCl_3$ 4-(Trifluoromethyl)benzenesulfonyl-azaartemisinin (**11**)



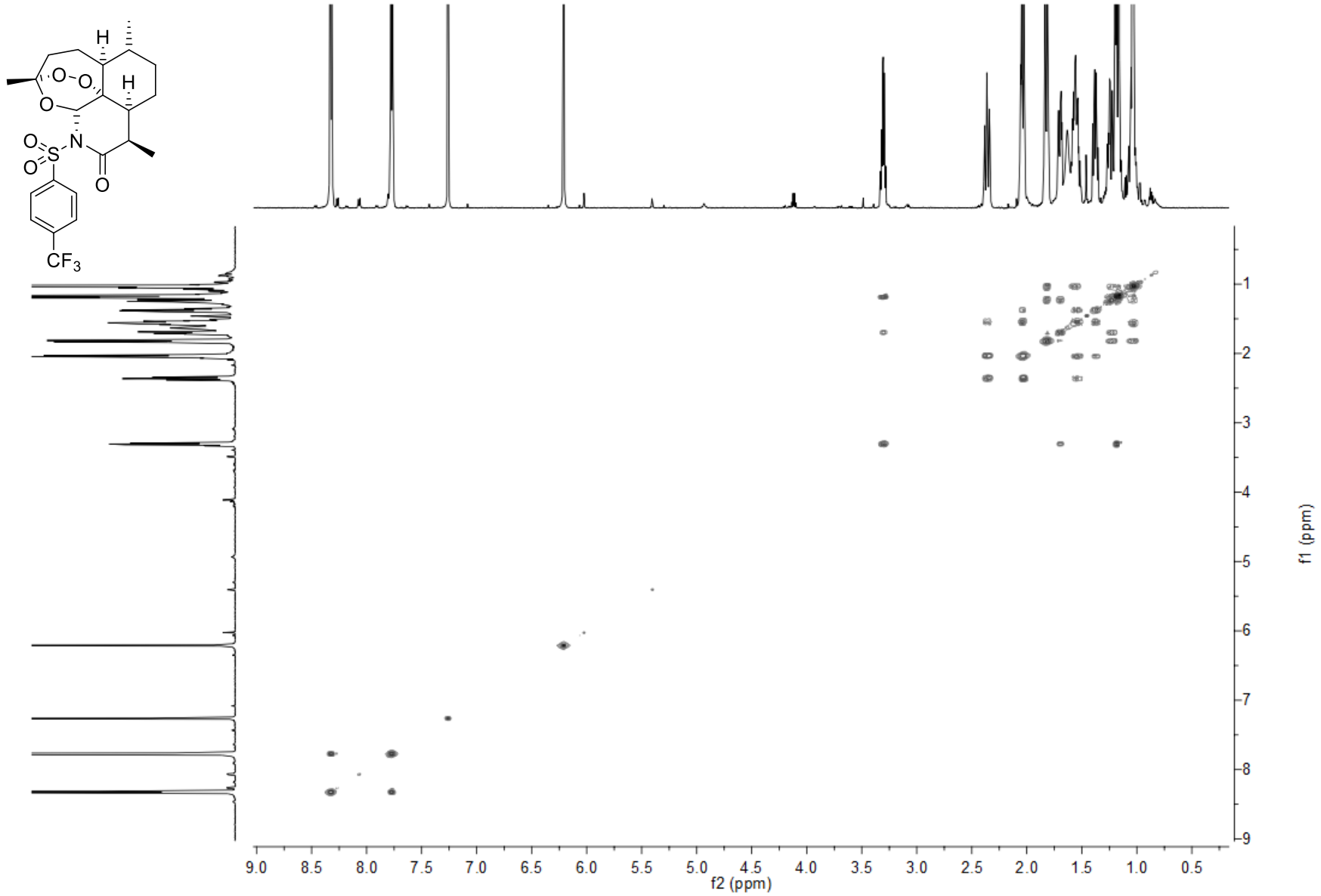
DEPT90 $CDCl_3$ 4-(Trifluoromethyl)benzenesulfonyl-azaartemisinin (**11**)



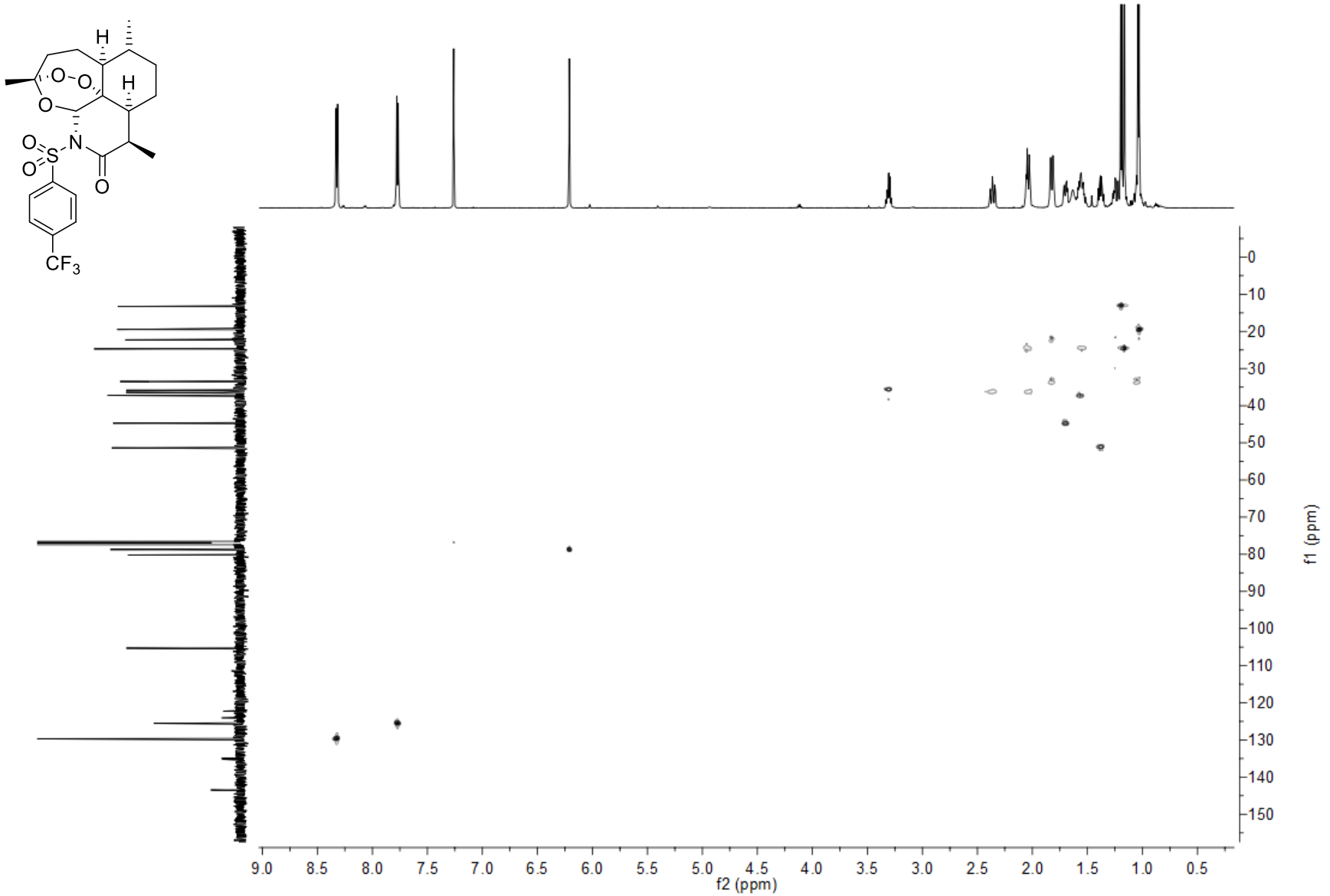
DEPT135 $CDCl_3$ 4-(Trifluoromethyl)benzenesulfonyl-azaartemisinin (**11**)



COSY $CDCl_3$ 4-(Trifluoromethyl)benzenesulfonyl-azaartemisinin (**11**)



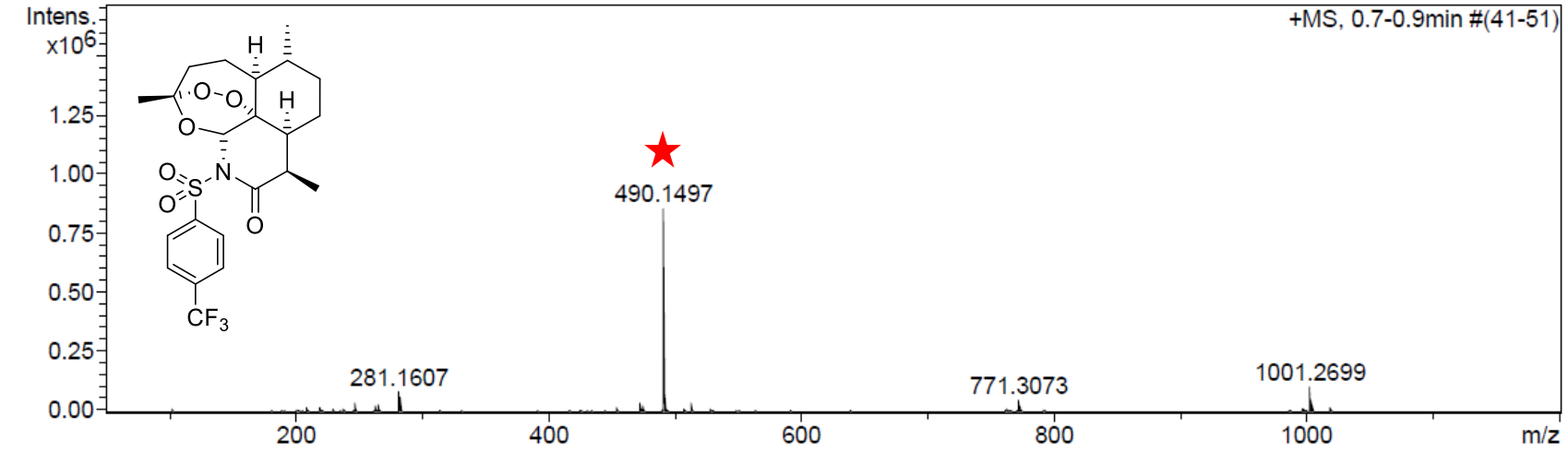
HSQC $CDCl_3$ 4-(Trifluoromethyl)benzenesulfonyl-azaartemisinin (**11**)



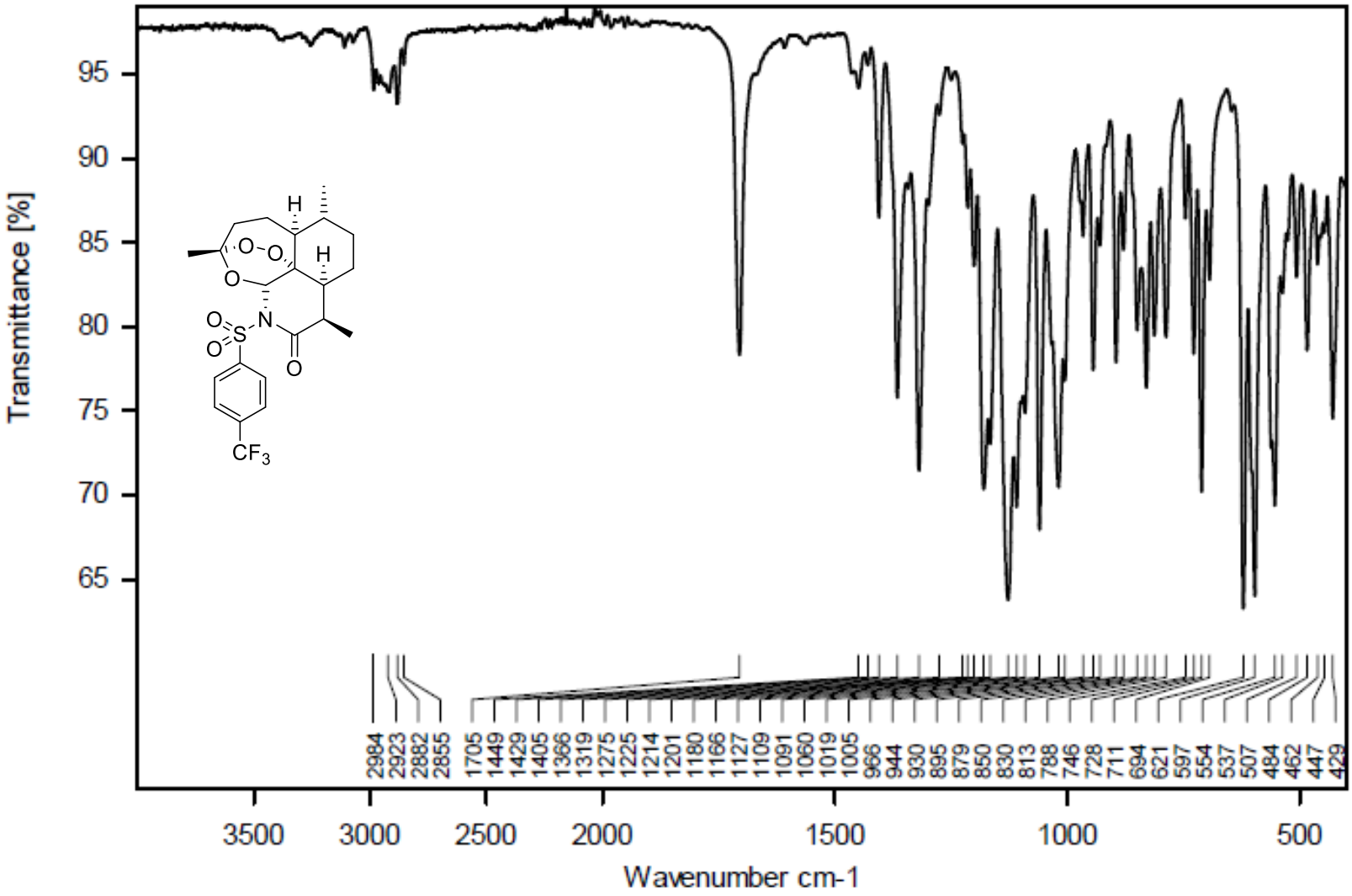
HRMS 4-(Trifluoromethyl)benzenesulfonyl-azaartemisinin (**11**)

Acquisition Parameter

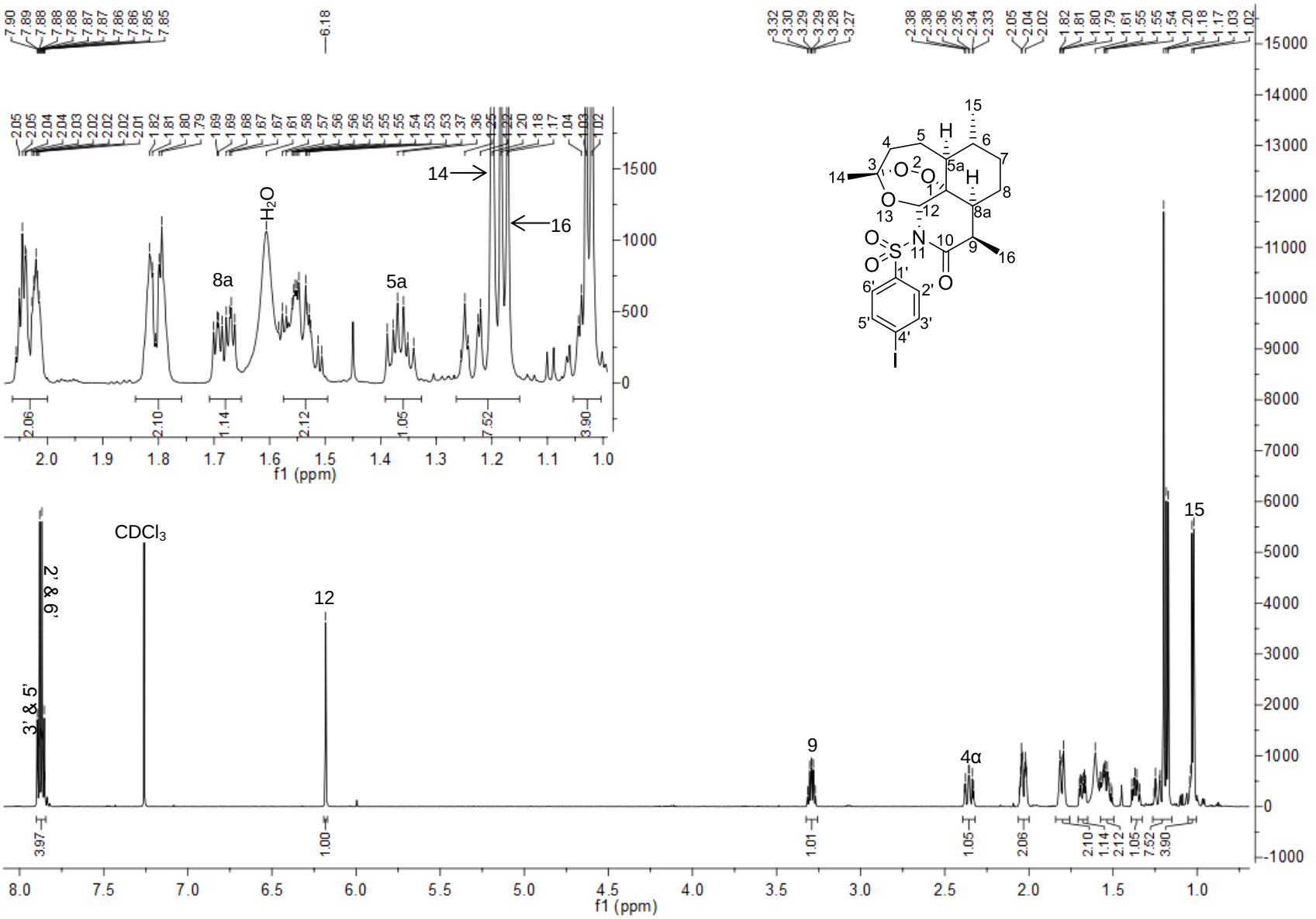
Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	0.4 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	180 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	4.0 l/min
Scan End	3000 m/z	Set Collision Cell RF	150.0 Vpp	Set Divert Valve	Waste



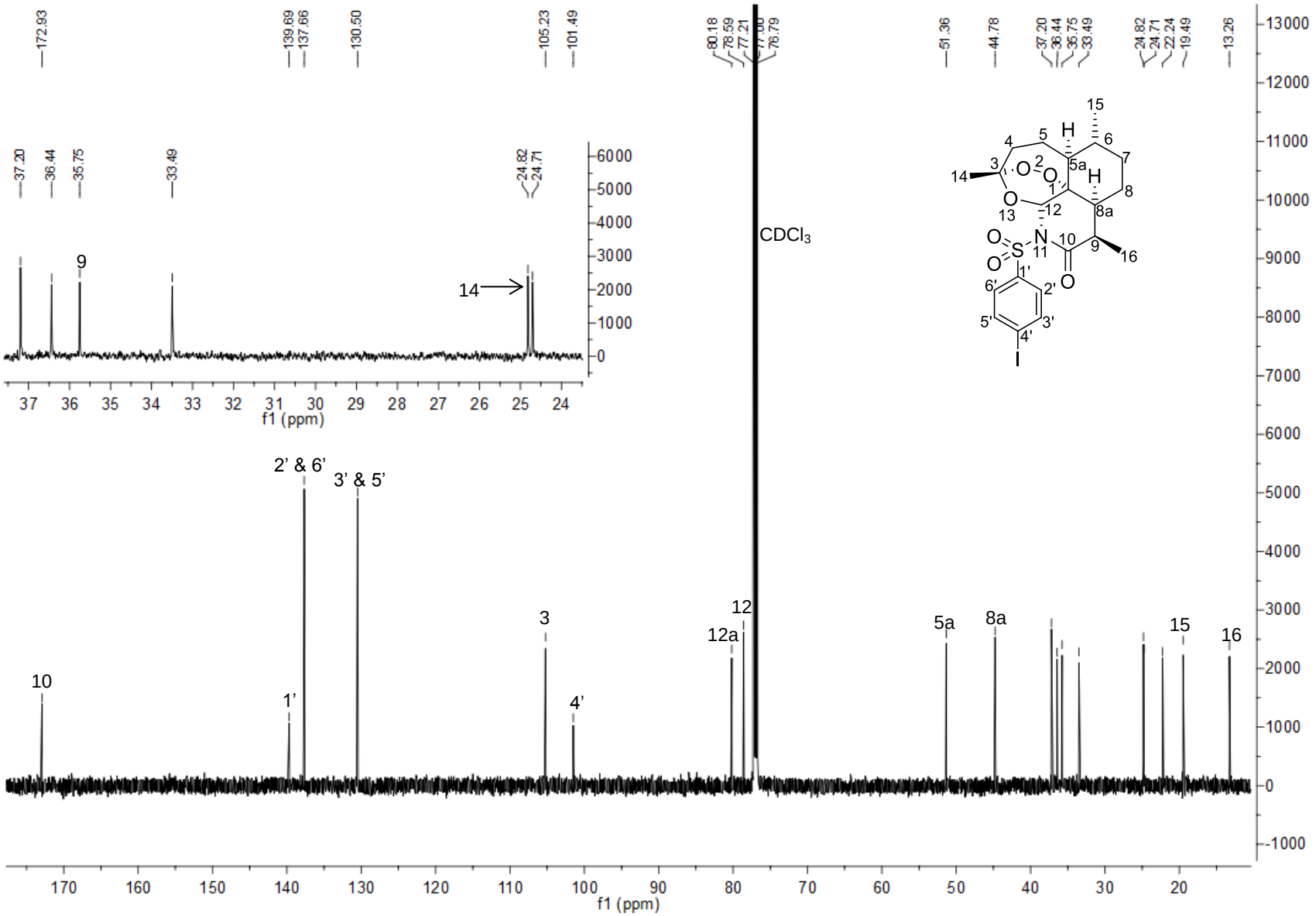
IR 4-(Trifluoromethyl)benzenesulfonyl-azaartemisinin (**11**)



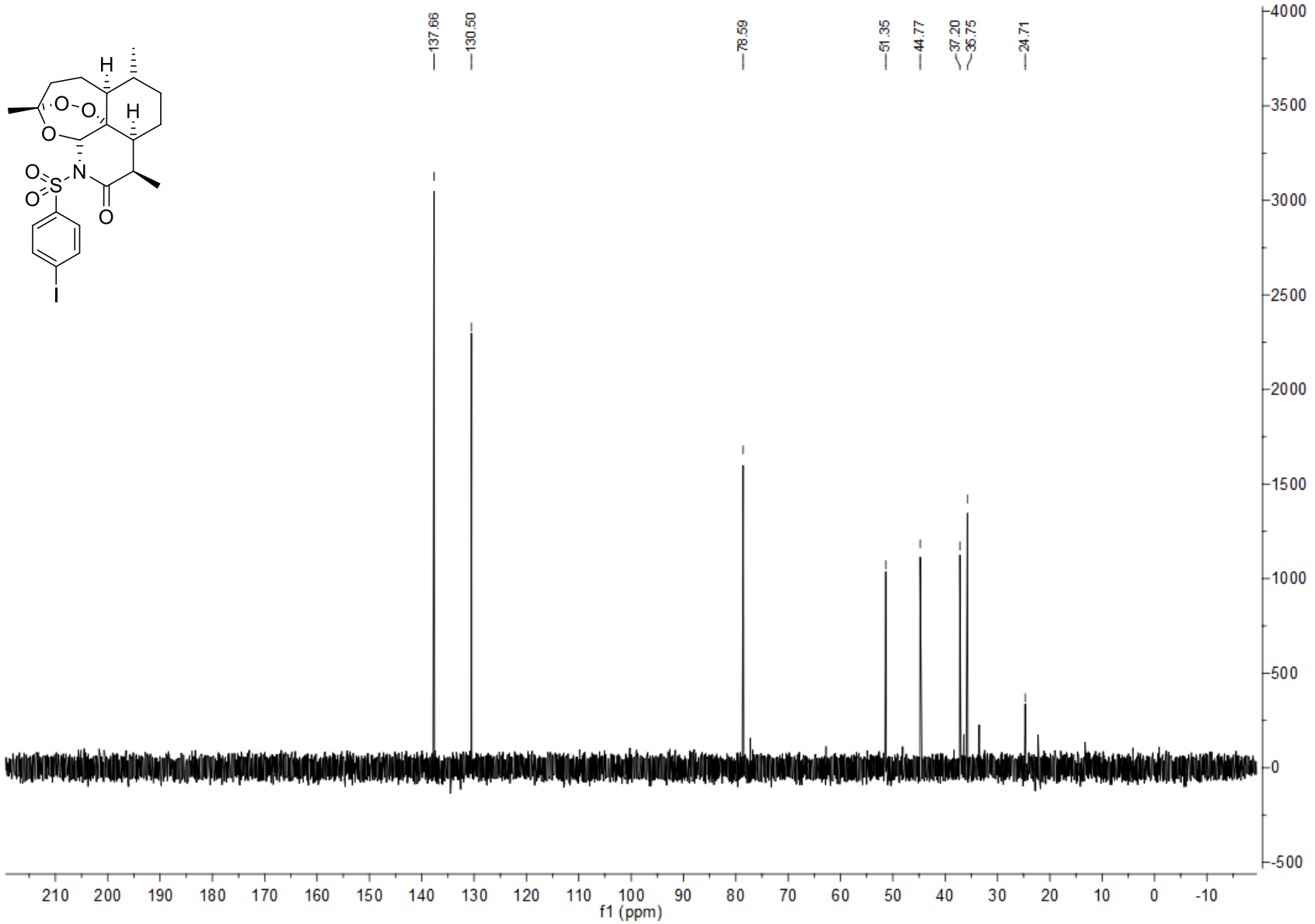
^{1}H NMR $CDCl_3$ 4-Iodobenzenesulfonyl-azaartemisinin (**12**)



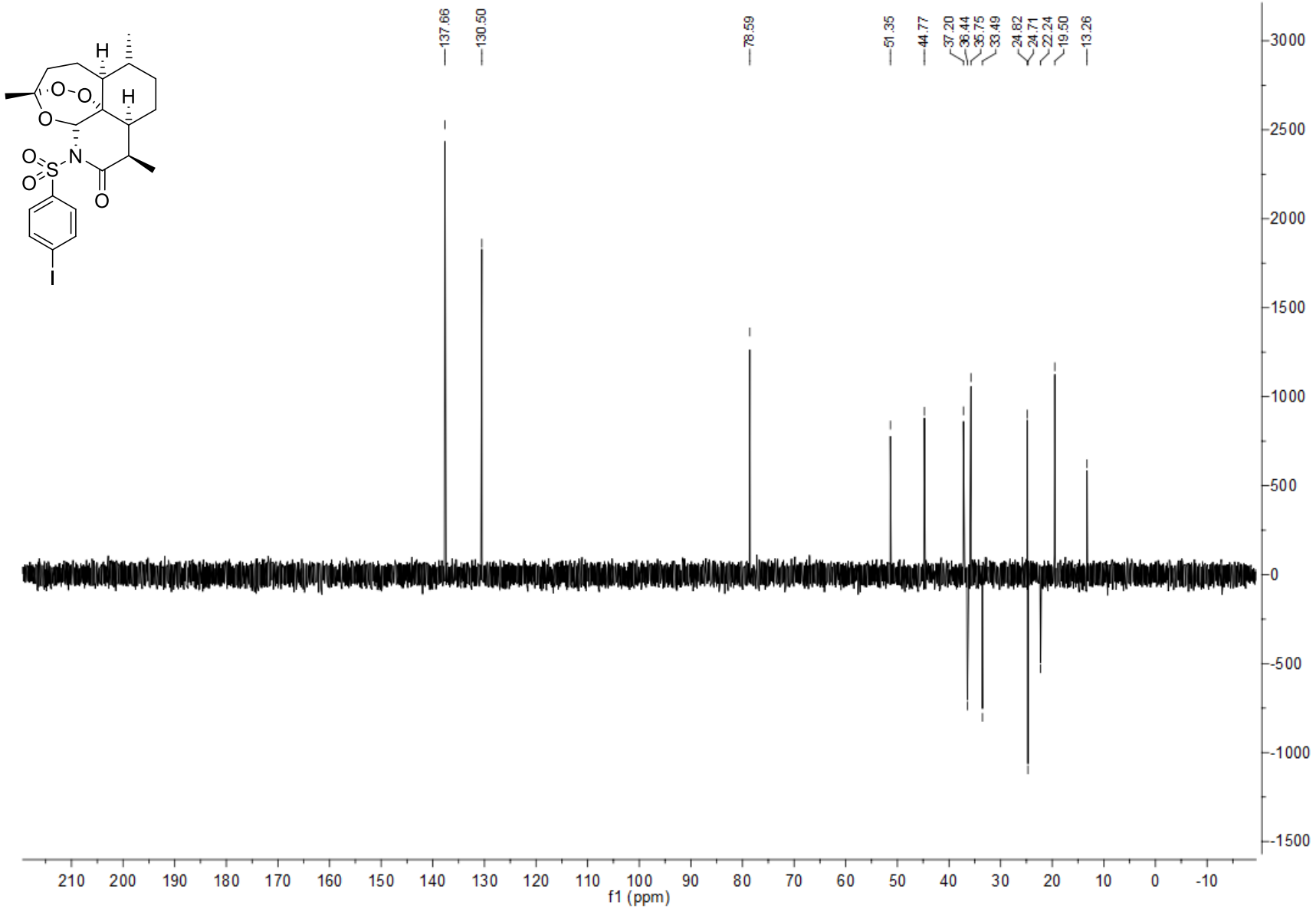
^{13}C NMR $CDCl_3$ 4-Iodobenzenesulfonyl-azaartemisinin (**12**)



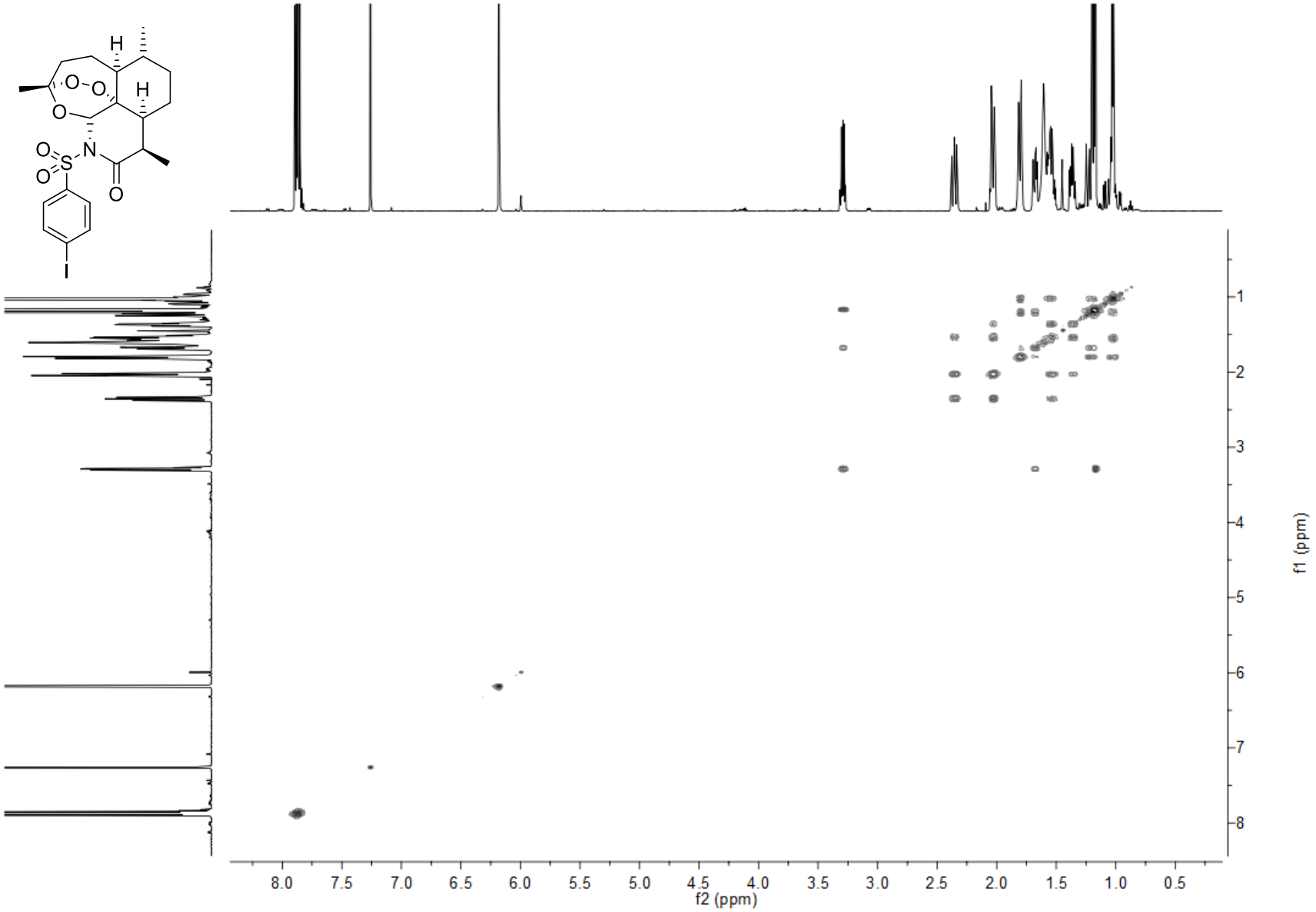
DEPT90 $CDCl_3$ 4-Iodobenzenesulfonyl-azaartemisinin (**12**)



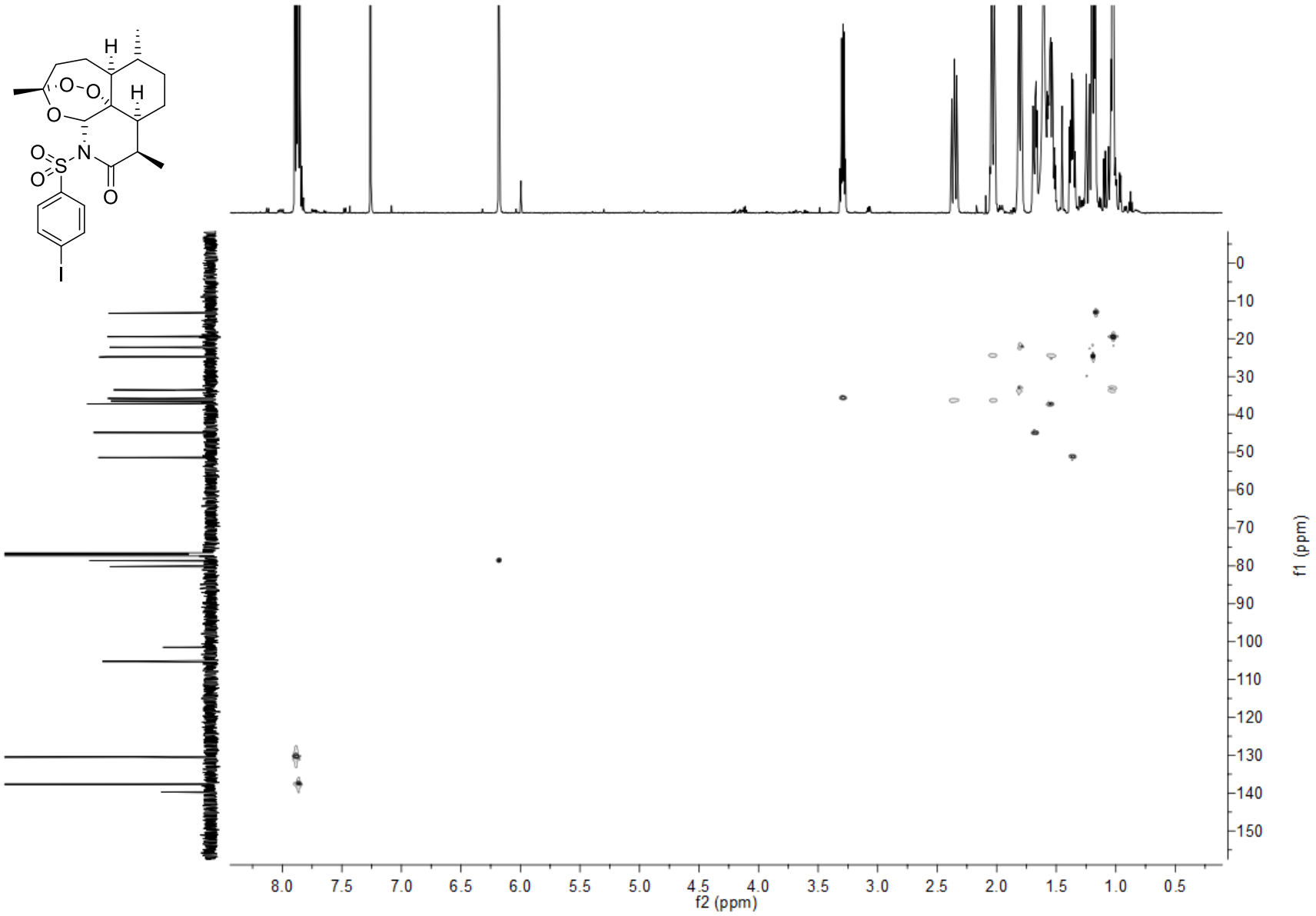
DEPT135 $CDCl_3$ 4-Iodobenzenesulfonyl-azaartemisinin (**12**)



COSY $CDCl_3$ 4-Iodobenzenesulfonyl-azaartemisinin (**12**)



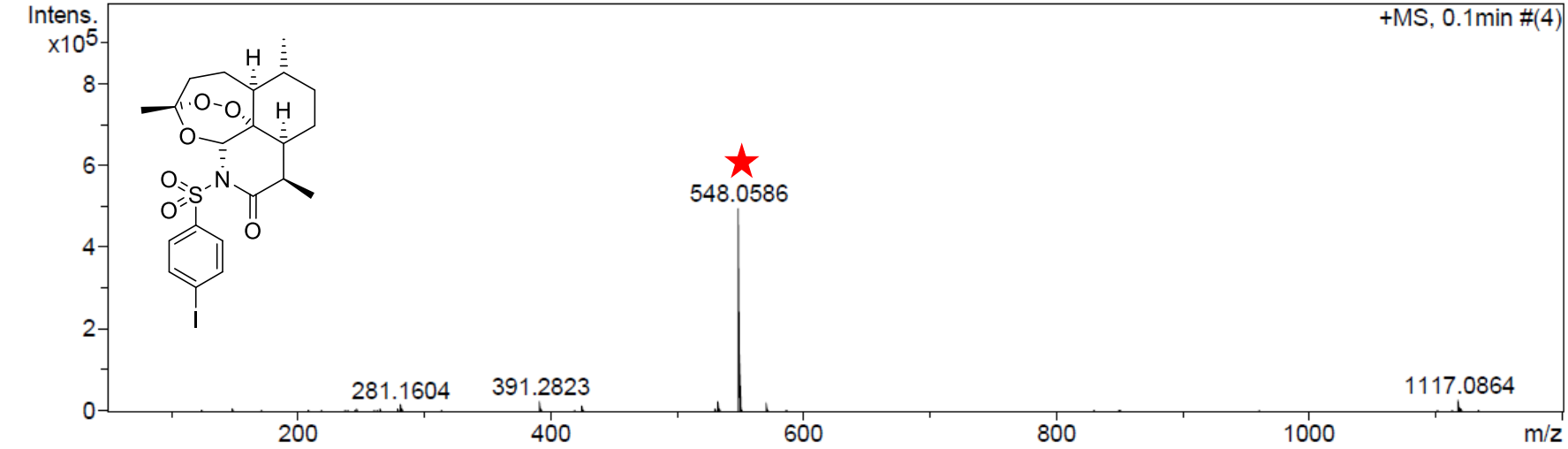
HSQC $CDCl_3$ 4-Iodobenzenesulfonyl-azaartemisinin (**12**)



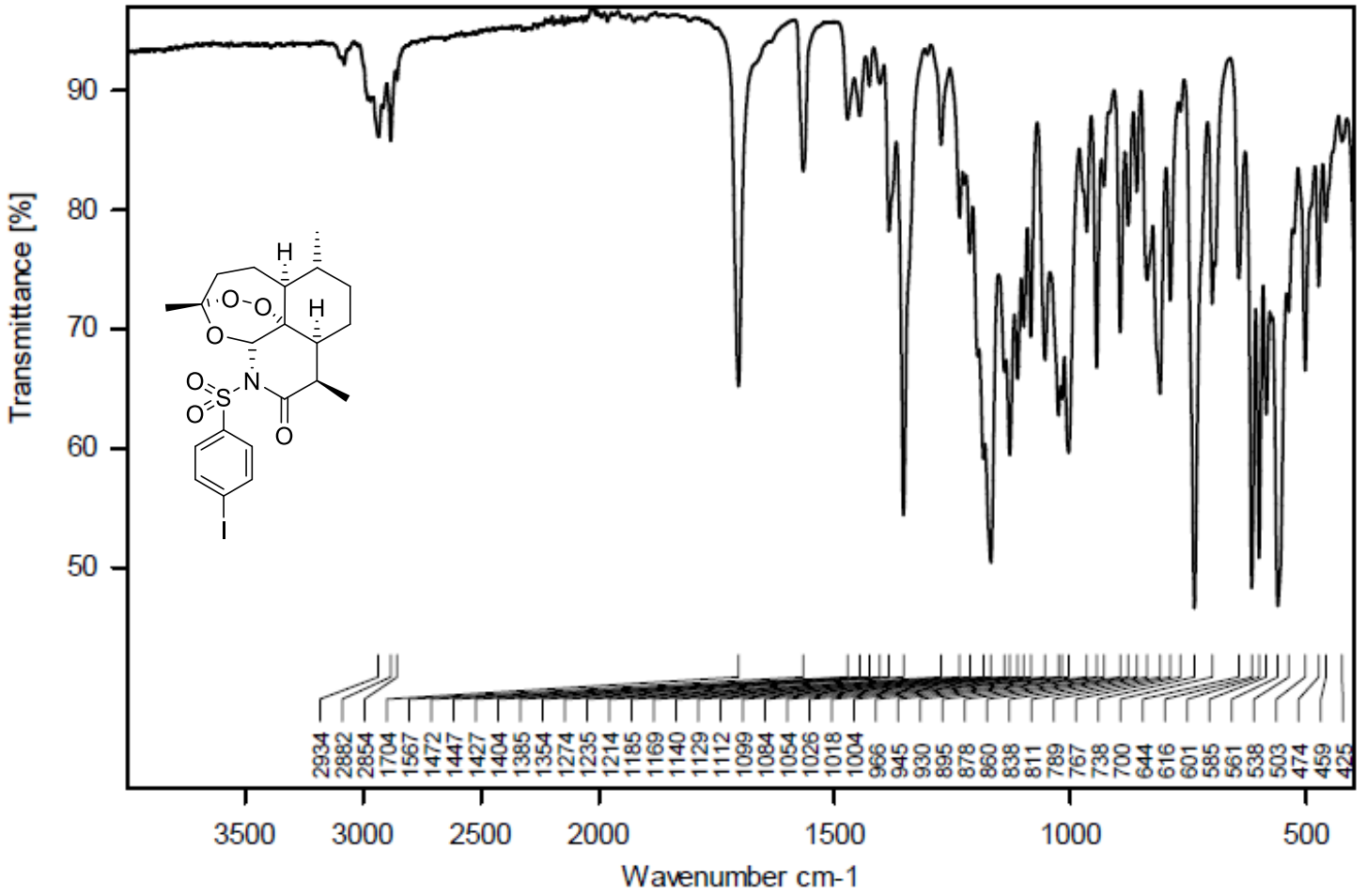
HRMS 4-Iodobenzenesulfonyl-azaartemisinin (**12**)

Acquisition Parameter

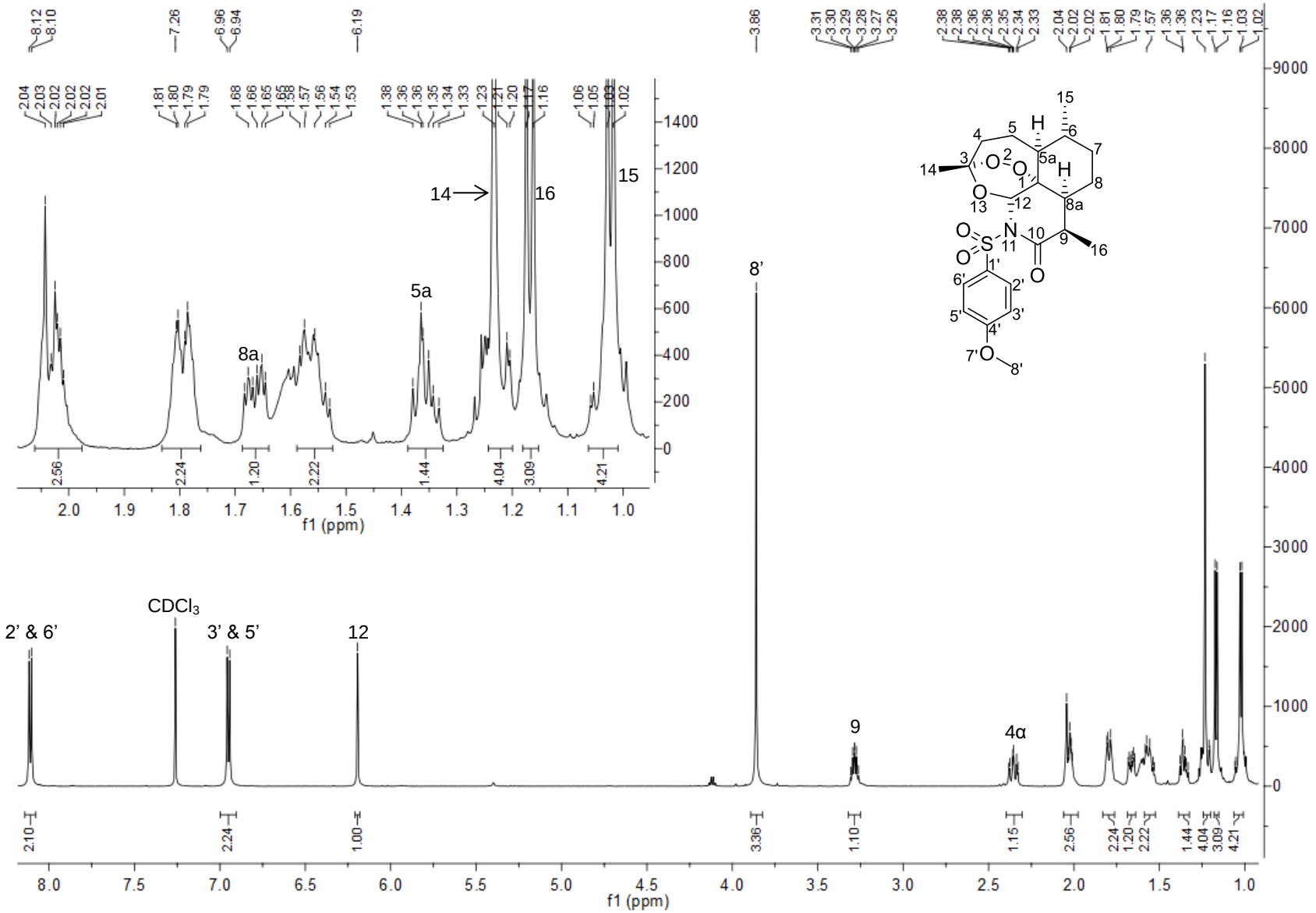
Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	0.4 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	180 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	4.0 l/min
Scan End	3000 m/z	Set Collision Cell RF	150.0 Vpp	Set Divert Valve	Waste



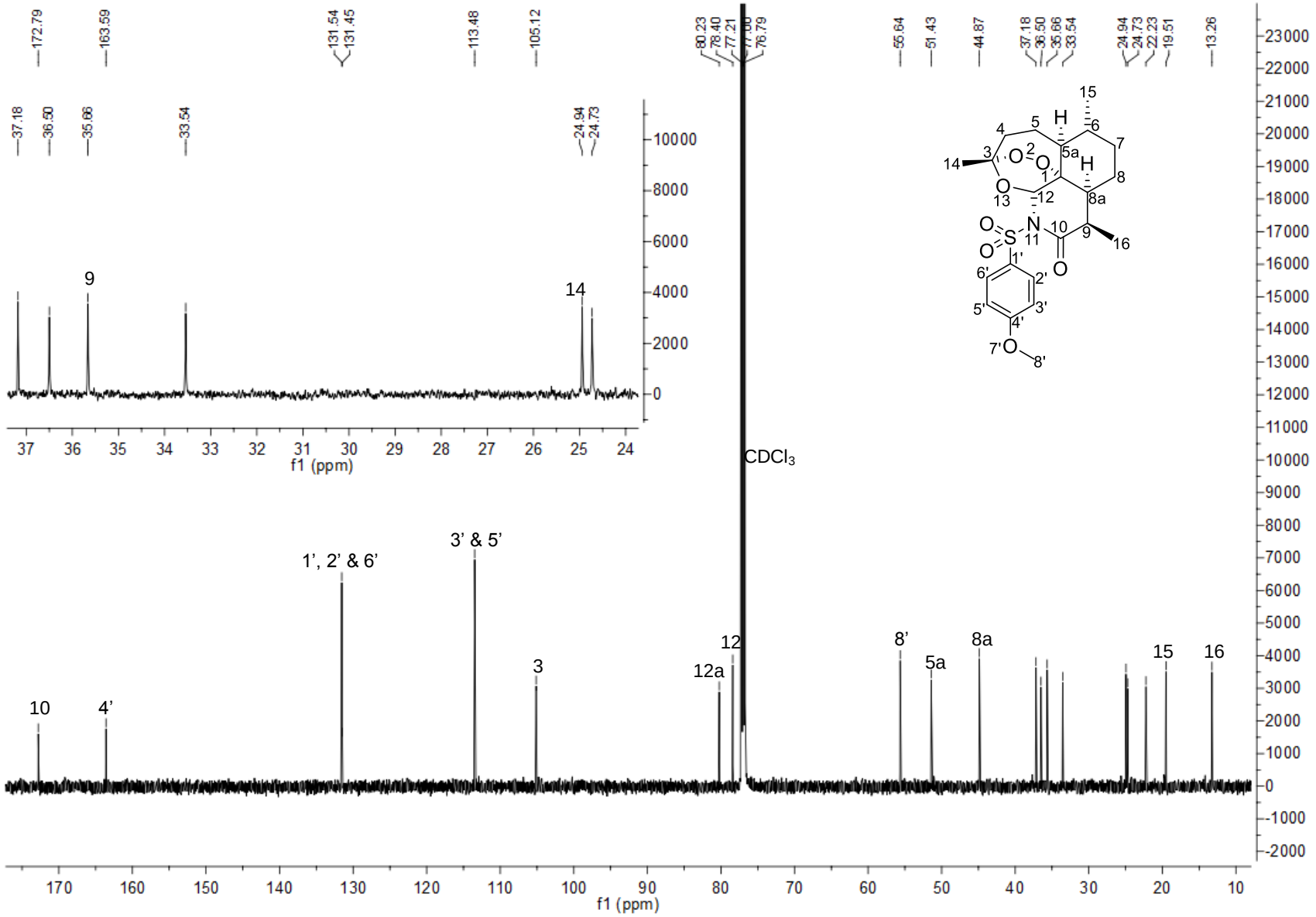
IR 4-Iodobenzenesulfonyl-azaartemisinin (**12**)



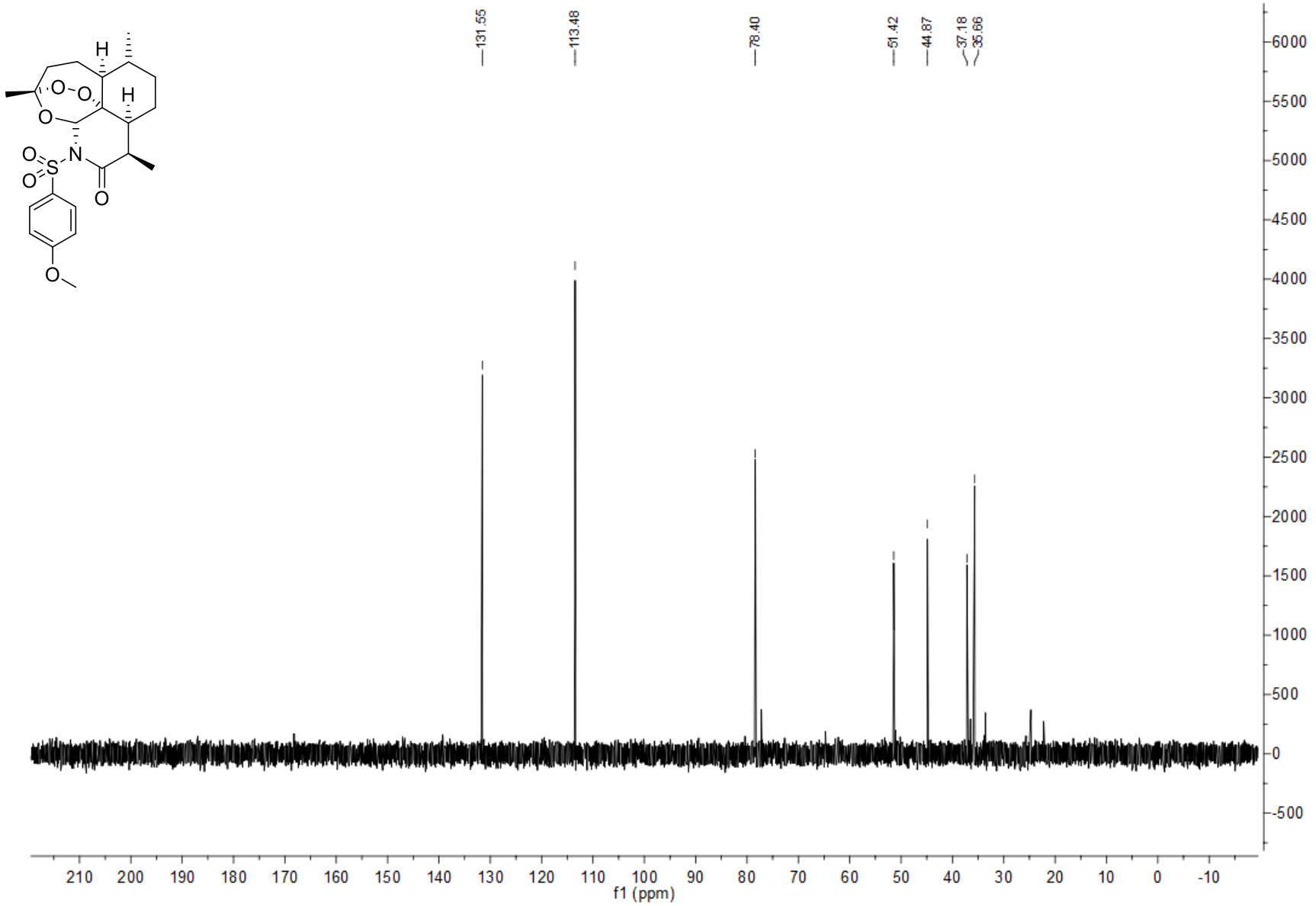
^{1}H NMR $CDCl_3$ 4-Methoxybenzenesulfonyl-azaartemisinin (**13**)



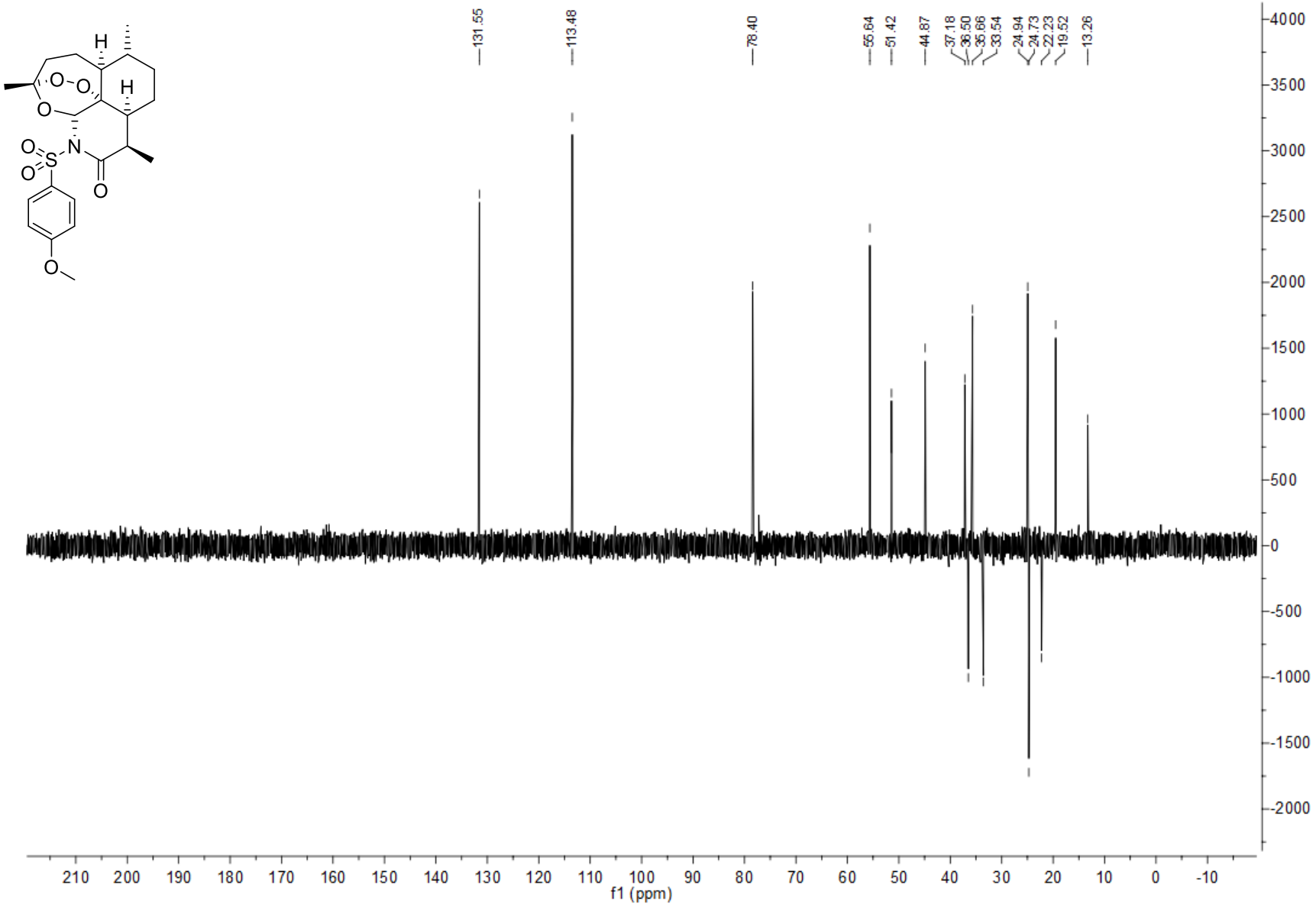
^{13}C NMR $CDCl_3$ 4-Methoxybenzenesulfonyl-azaartemisinin (**13**)



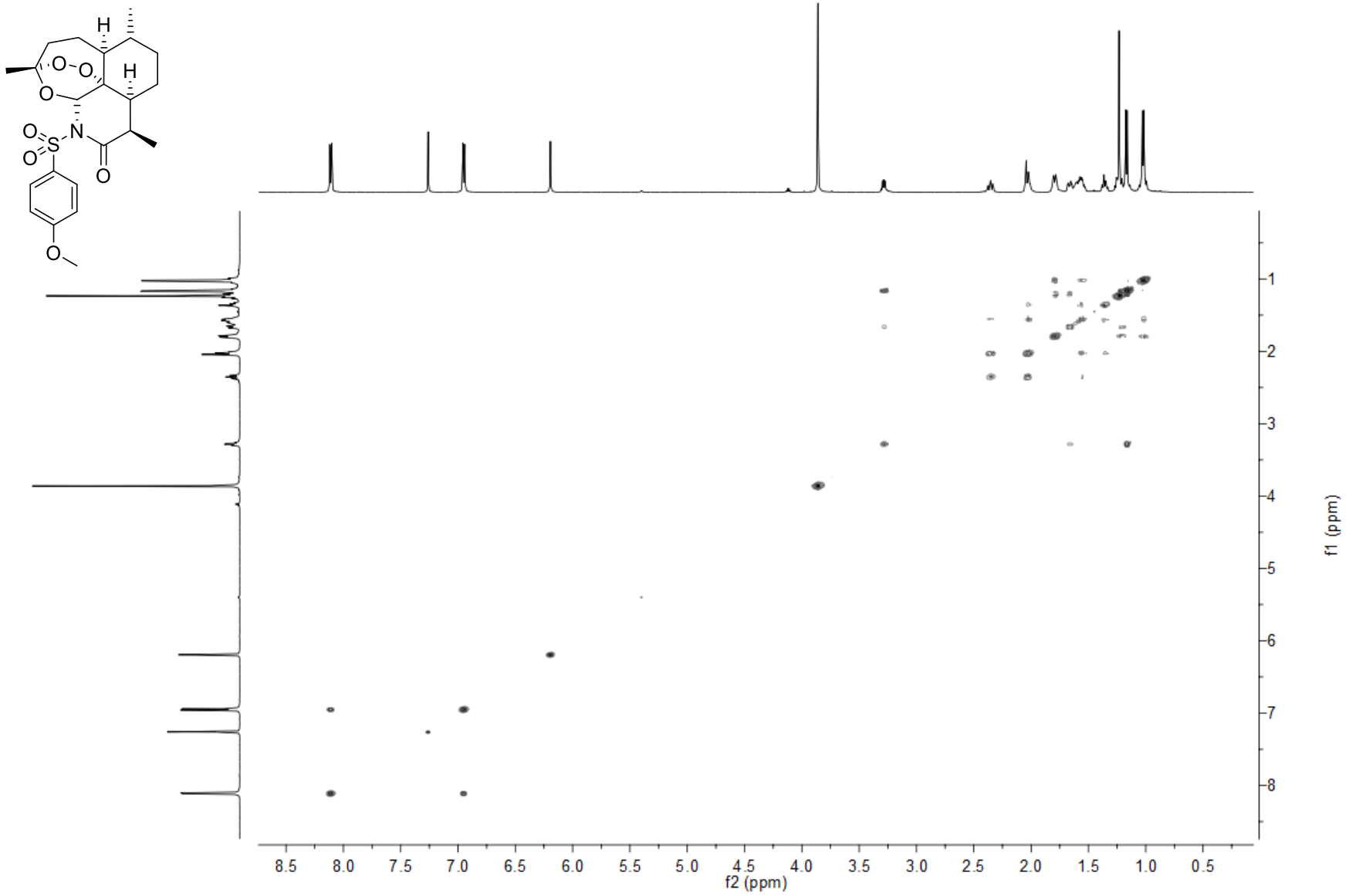
DEPT90 $CDCl_3$ 4-Methoxybenzenesulfonyl-azaartemisinin (**13**)



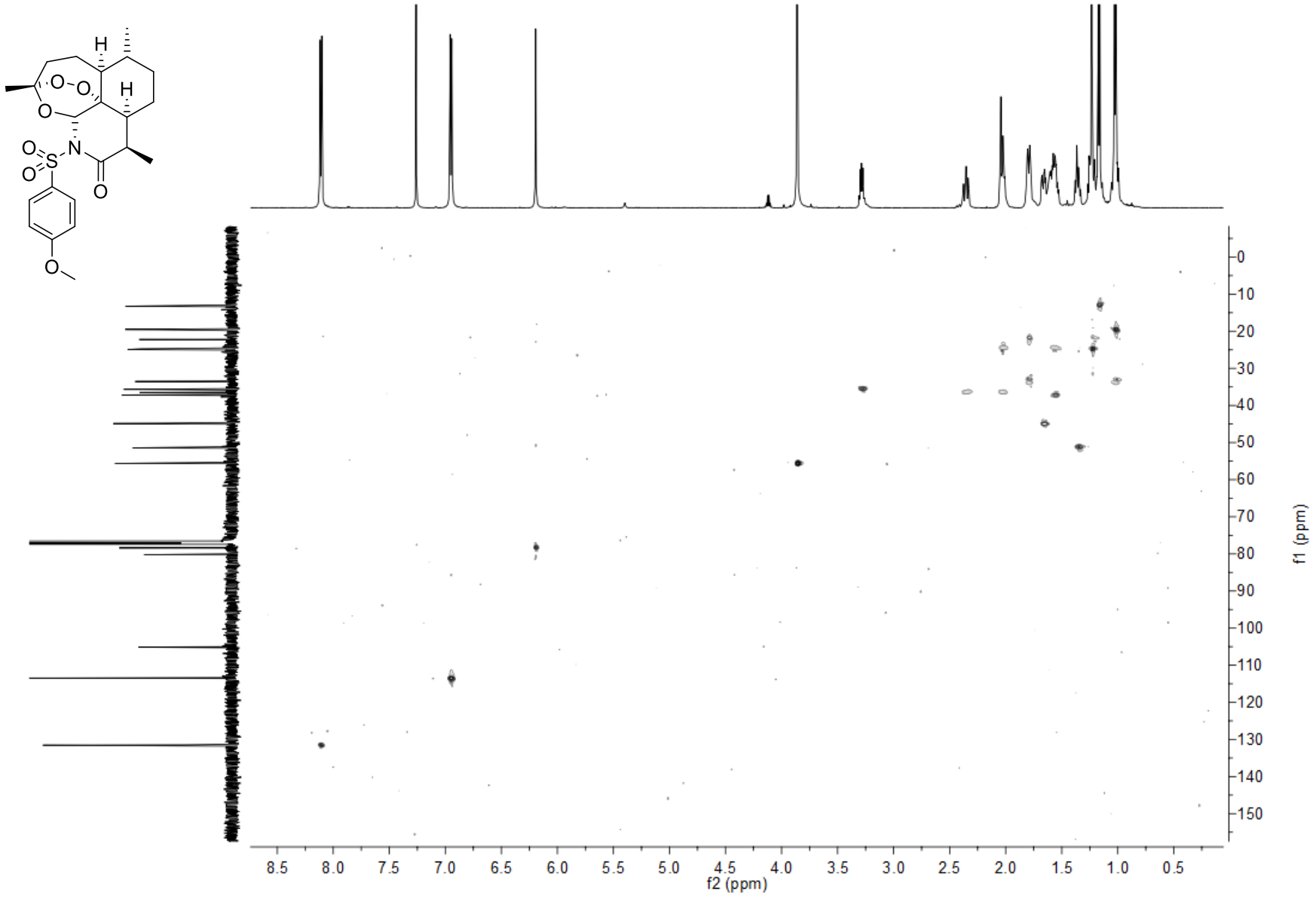
DEPT135 $CDCl_3$ 4-Methoxybenzenesulfonyl-azaartemisinin (**13**)



COSY $CDCl_3$ 4-Methoxybenzenesulfonyl-azaartemisinin (**13**)



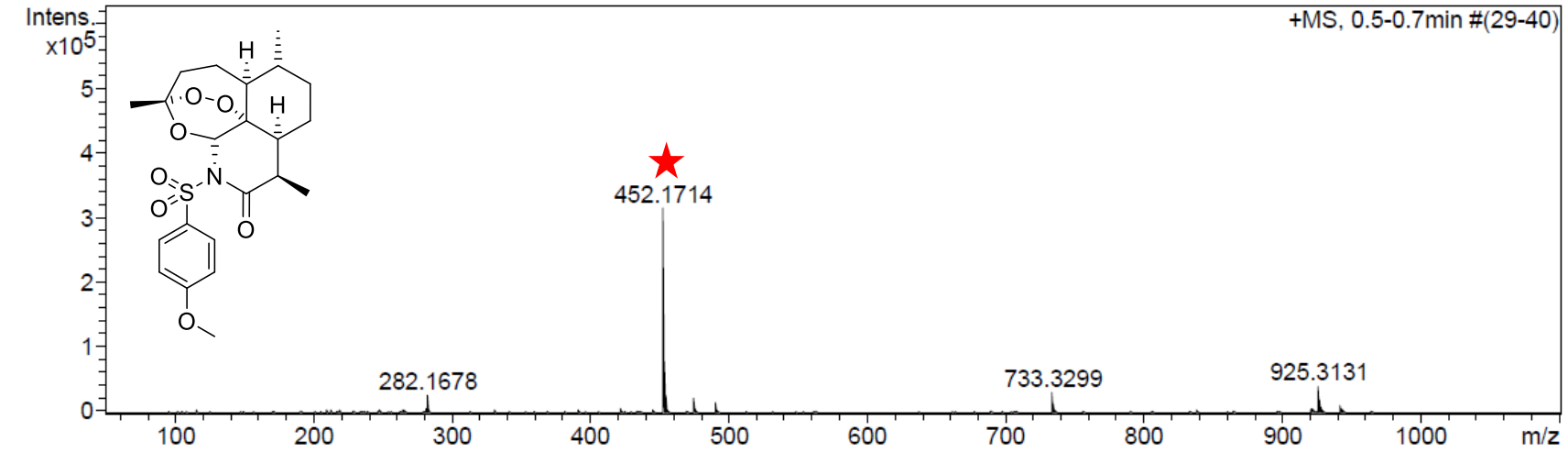
HSQC $CDCl_3$ 4-Methoxybenzenesulfonyl-azaartemisinin (**13**)



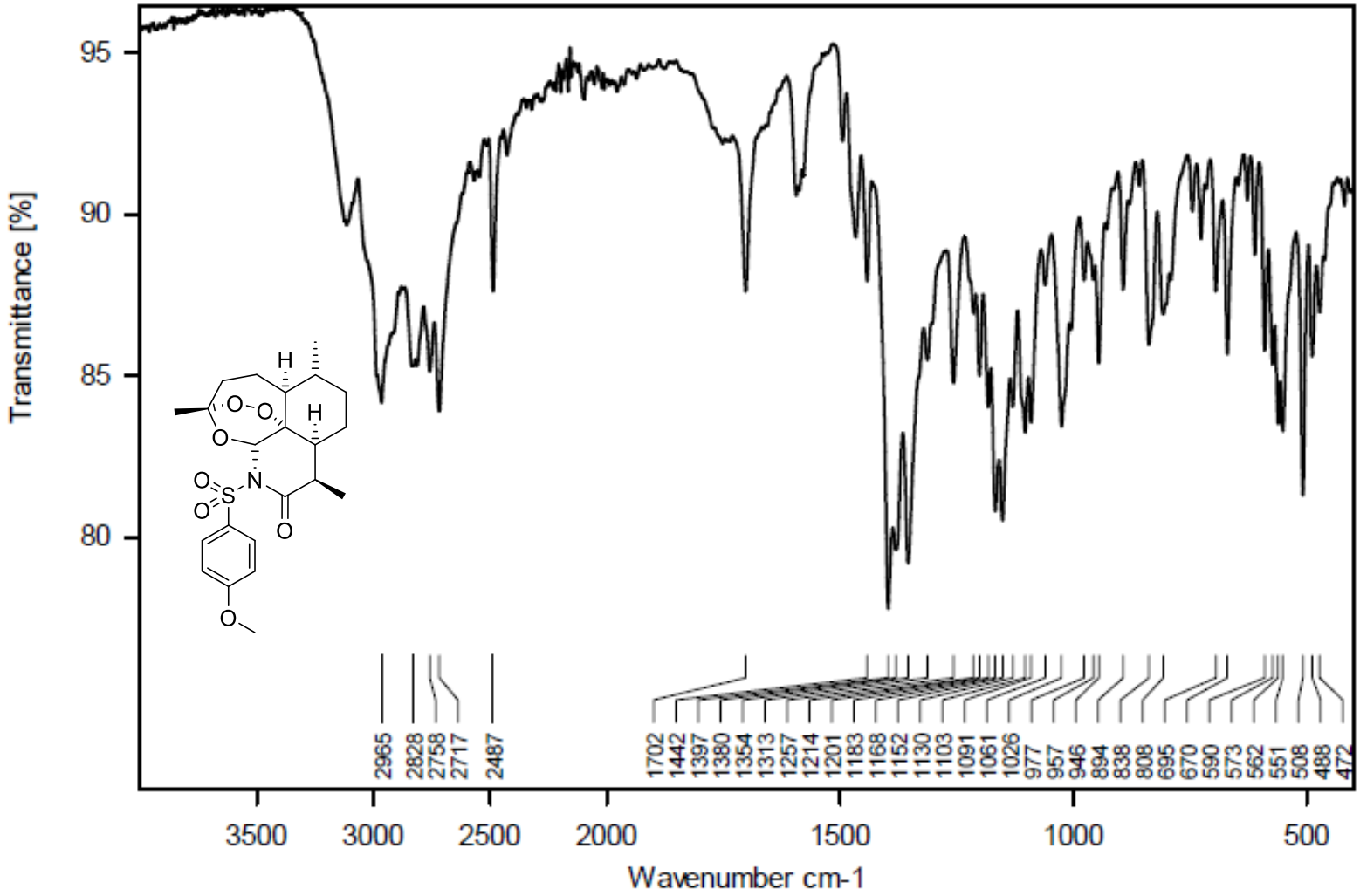
HRMS 4-Methoxybenzenesulfonyl-azaartemisinin (**13**)

Acquisition Parameter

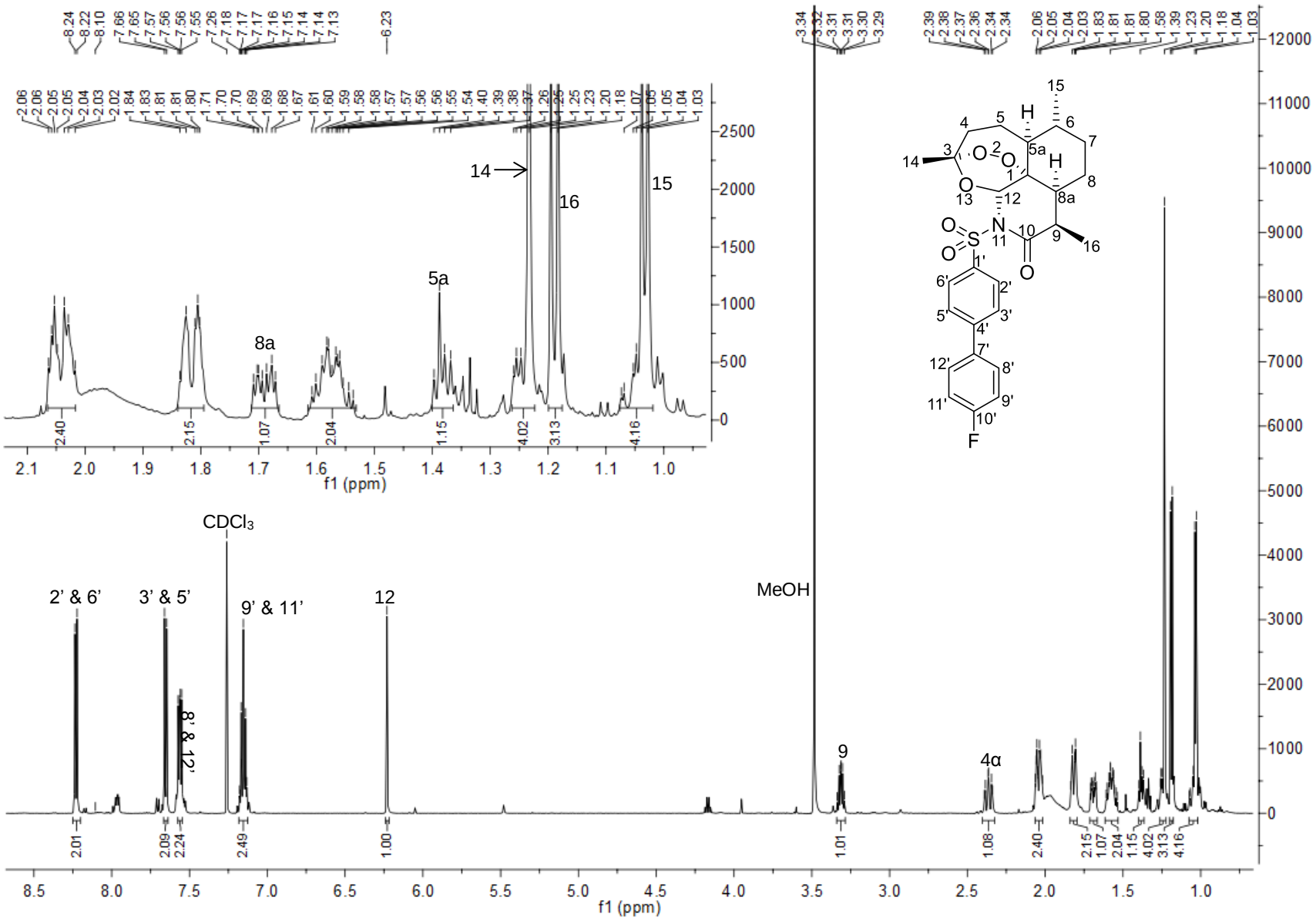
Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	0.4 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	180 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	4.0 l/min
Scan End	3000 m/z	Set Collision Cell RF	150.0 Vpp	Set Divert Valve	Waste



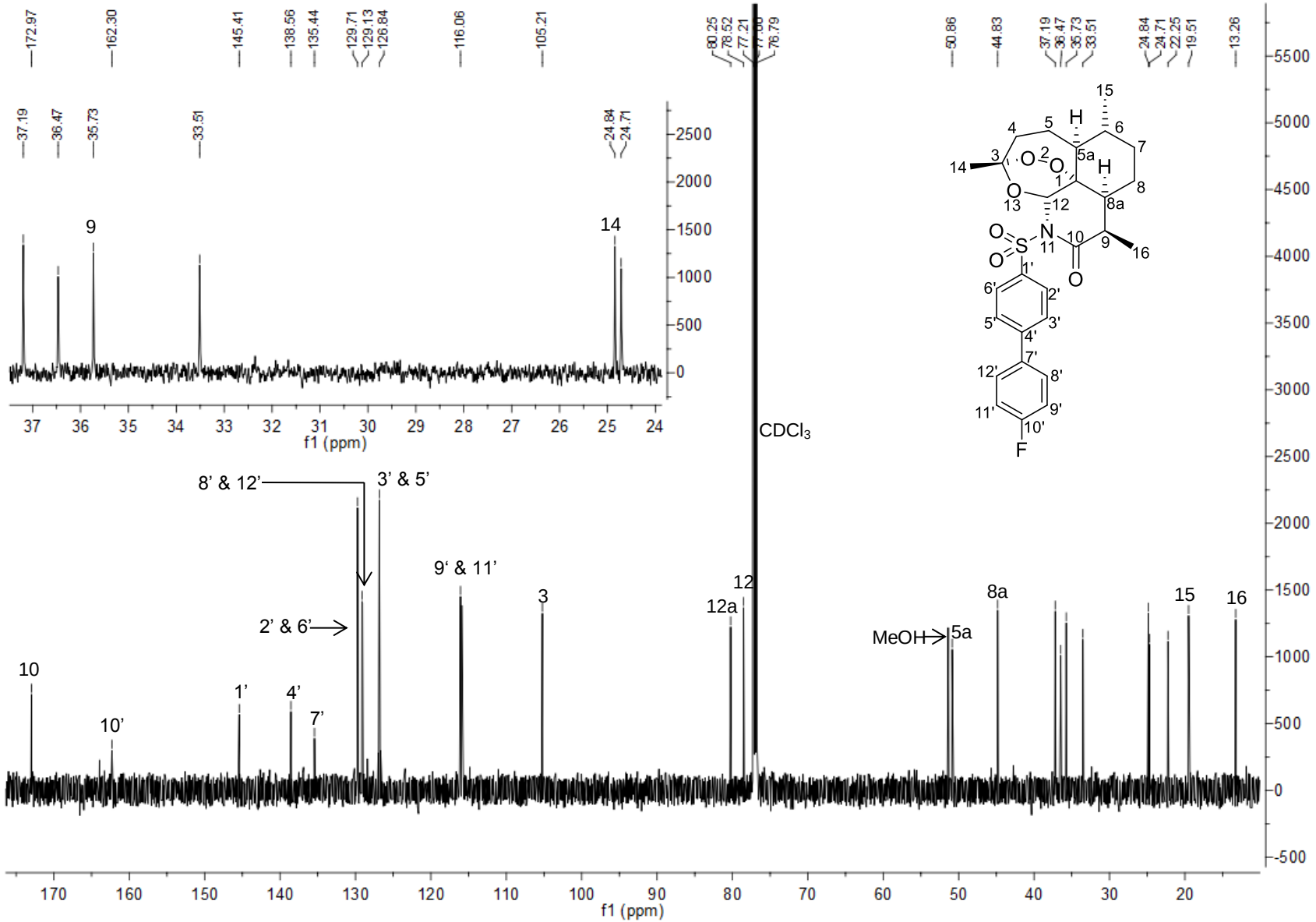
HRMS 4-Methoxybenzenesulfonyl-azaartemisinin (**13**)



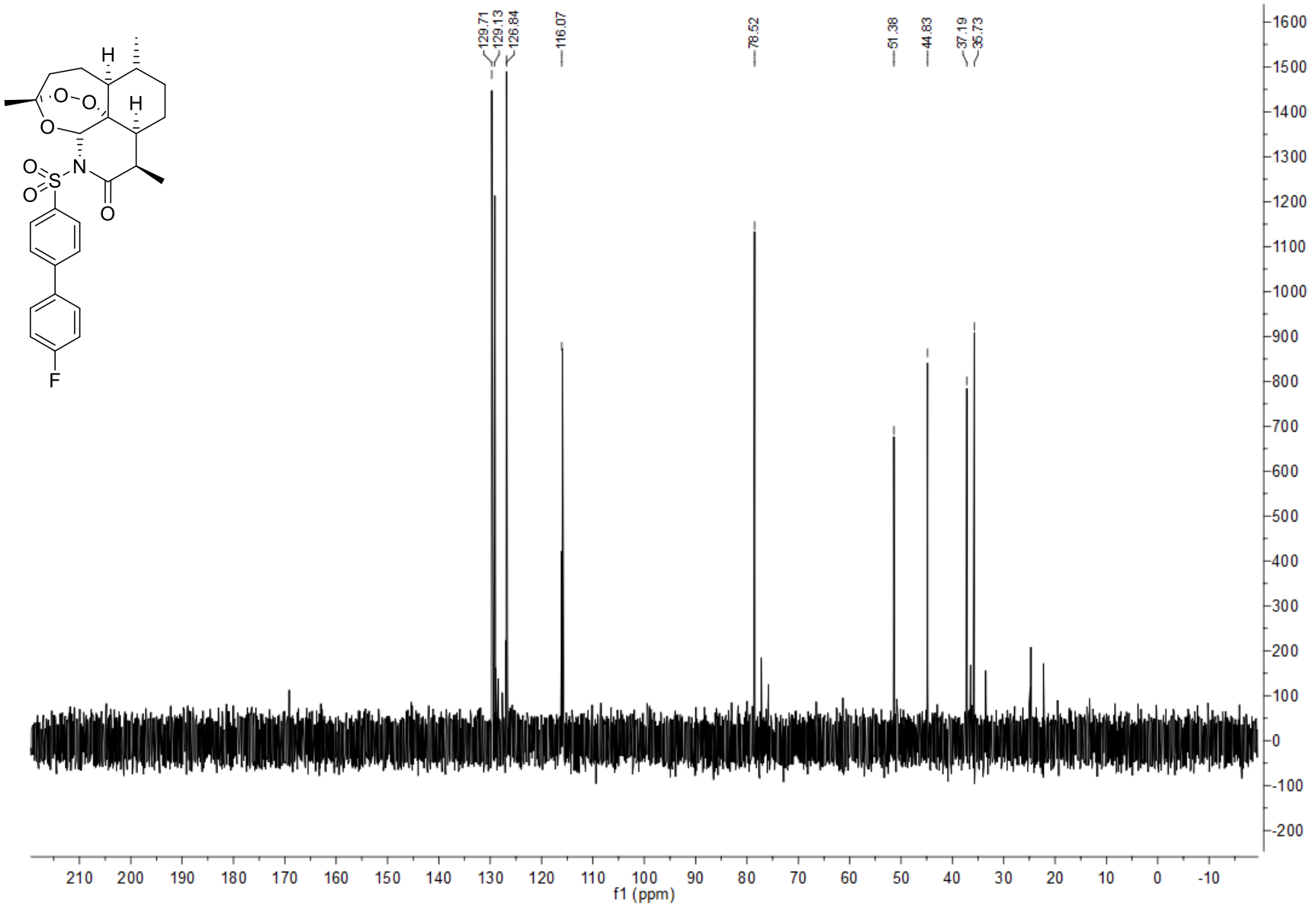
^{1}H NMR $CDCl_3$ 4-Fluorobiphenyl-4-sulfonyl-azaartemisinin (**14**)



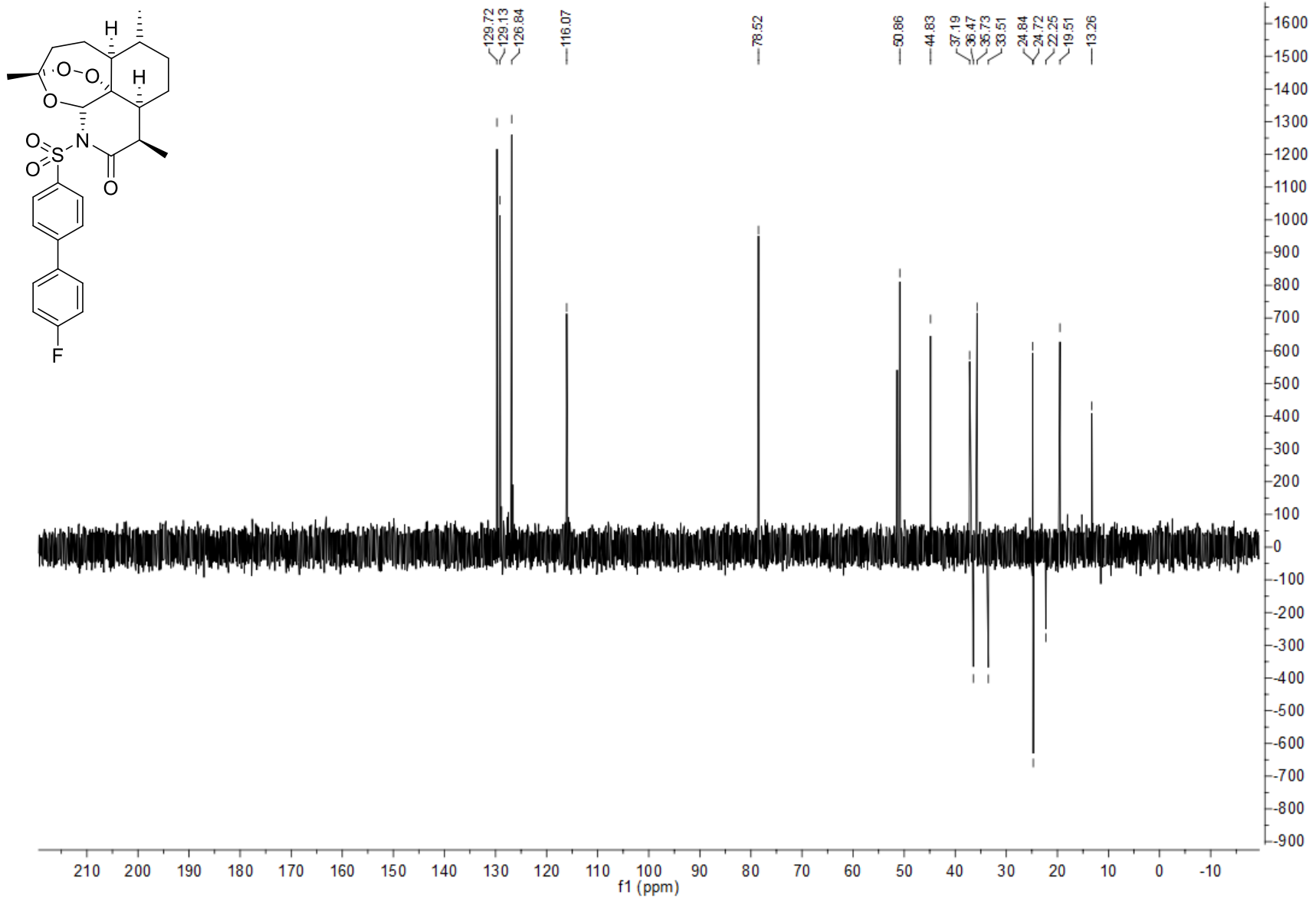
^{13}C NMR $CDCl_3$ 4-Fluorobiphenyl-4-sulfonyl-azaartemisinin (**14**)



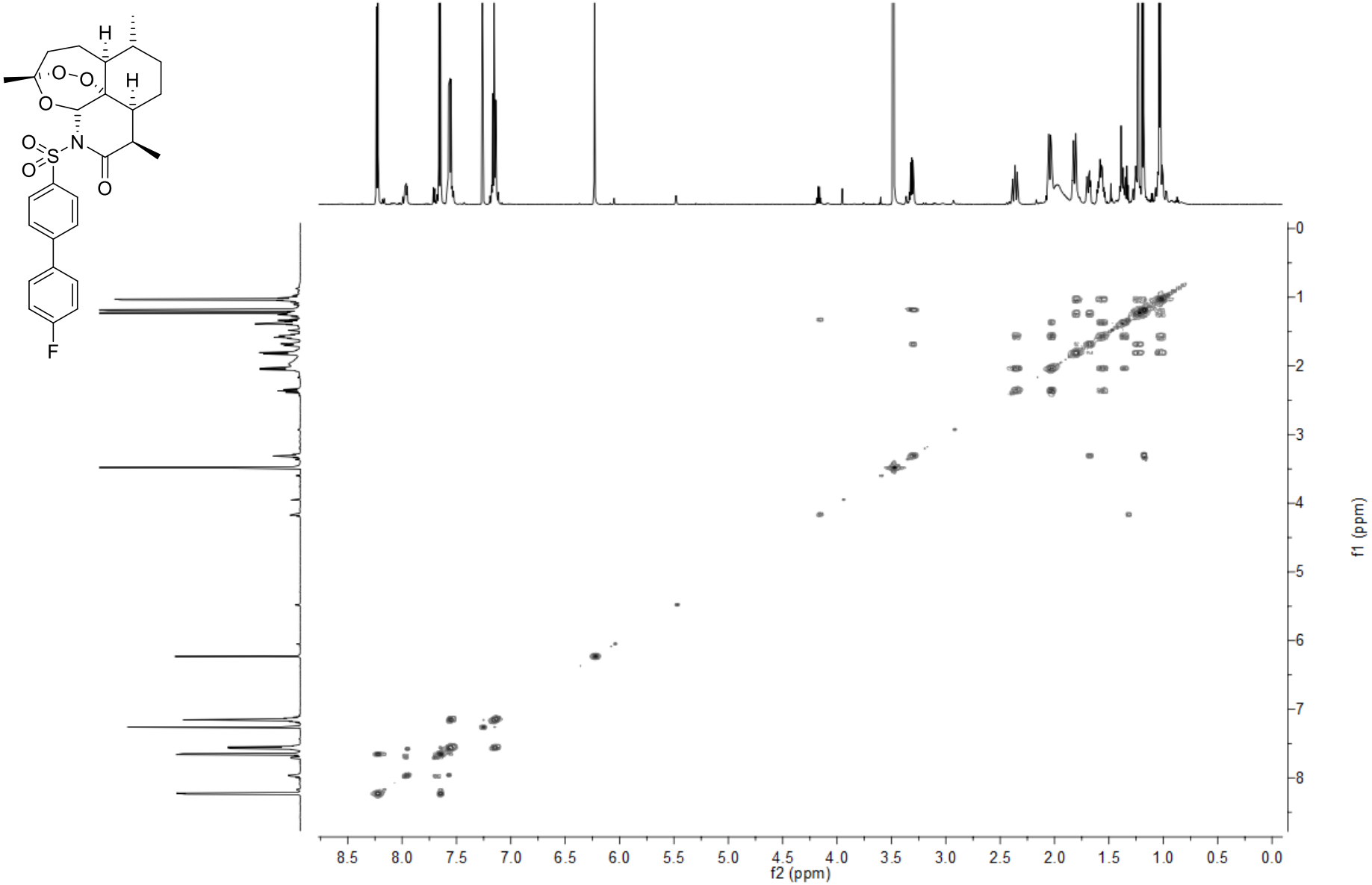
DEPT90 $CDCl_3$ 4-Fluorobiphenyl-4-sulfonyl-azaartemisinin (**14**)



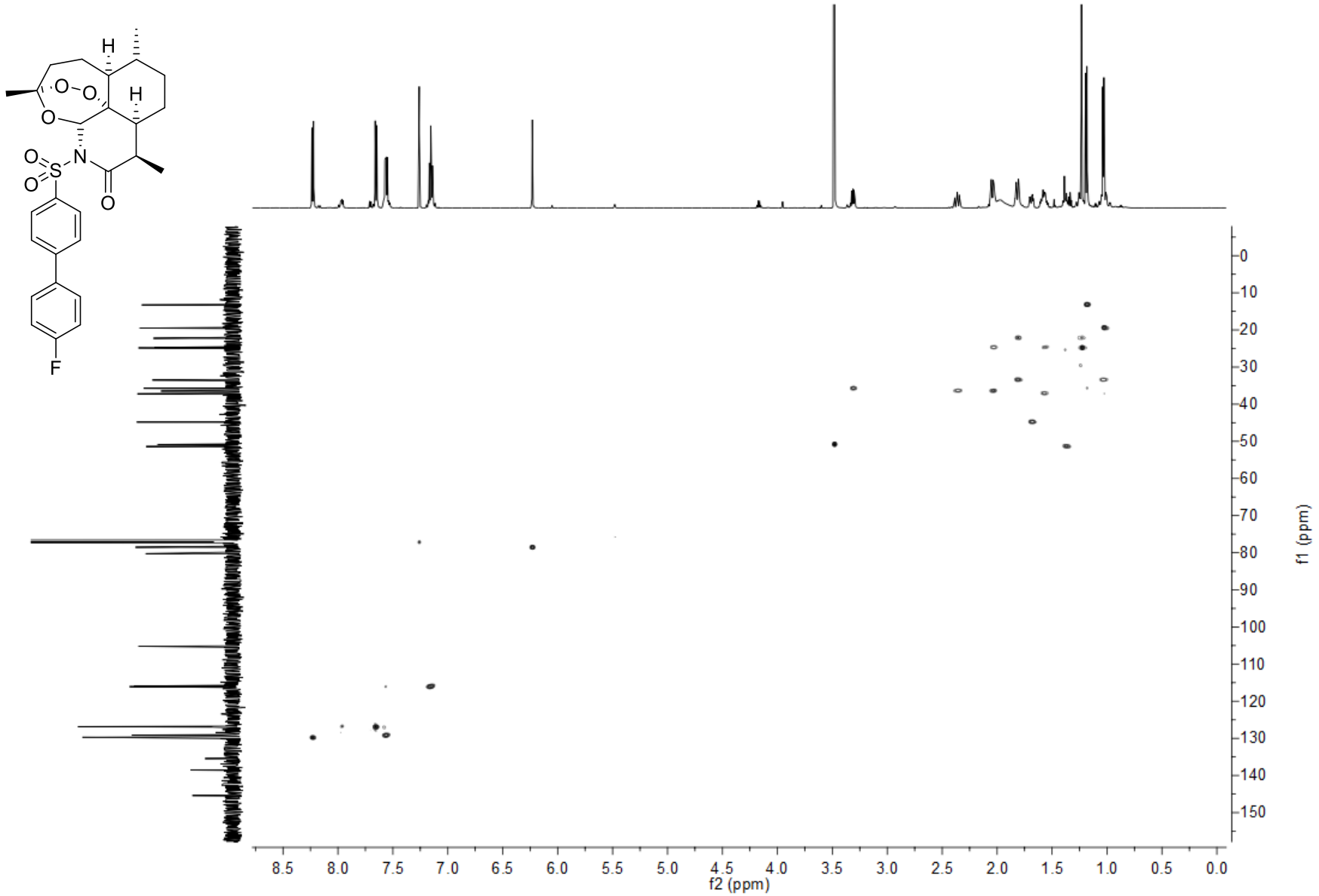
DEPT135 $CDCl_3$ 4-Fluorobiphenyl-4-sulfonyl-azaartemisinin (**14**)



COSY $CDCl_3$ 4-Fluorobiphenyl-4-sulfonyl-azaartemisinin (**14**)



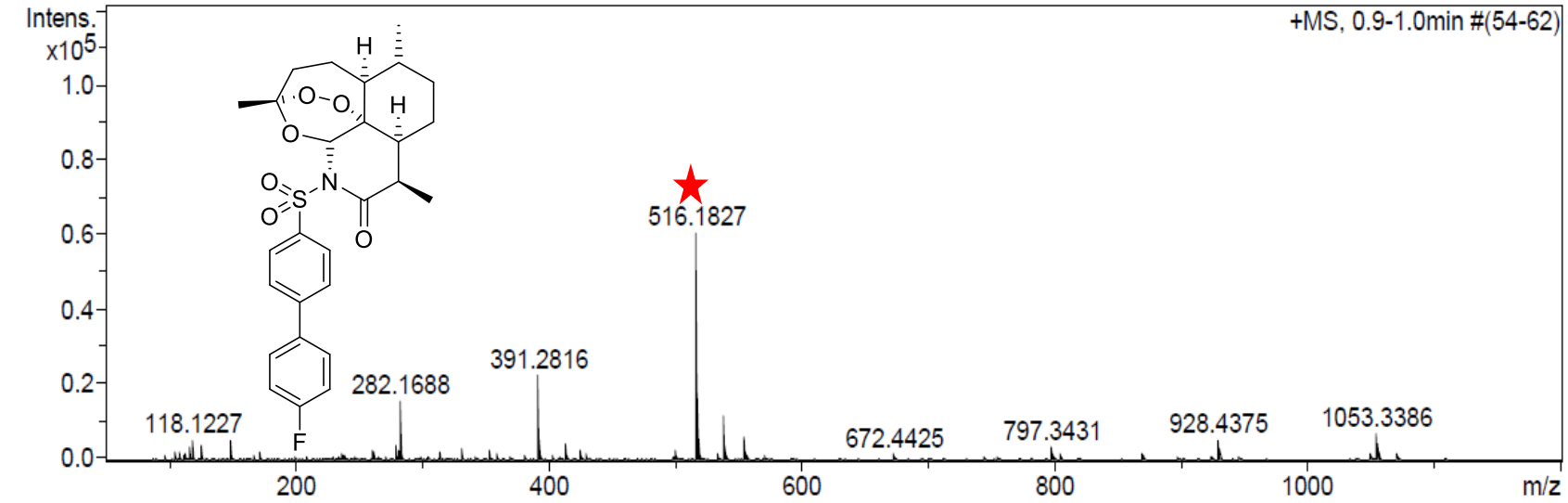
HSQC $CDCl_3$ 4-Fluorobiphenyl-4-sulfonyl-azaartemisinin (**14**)



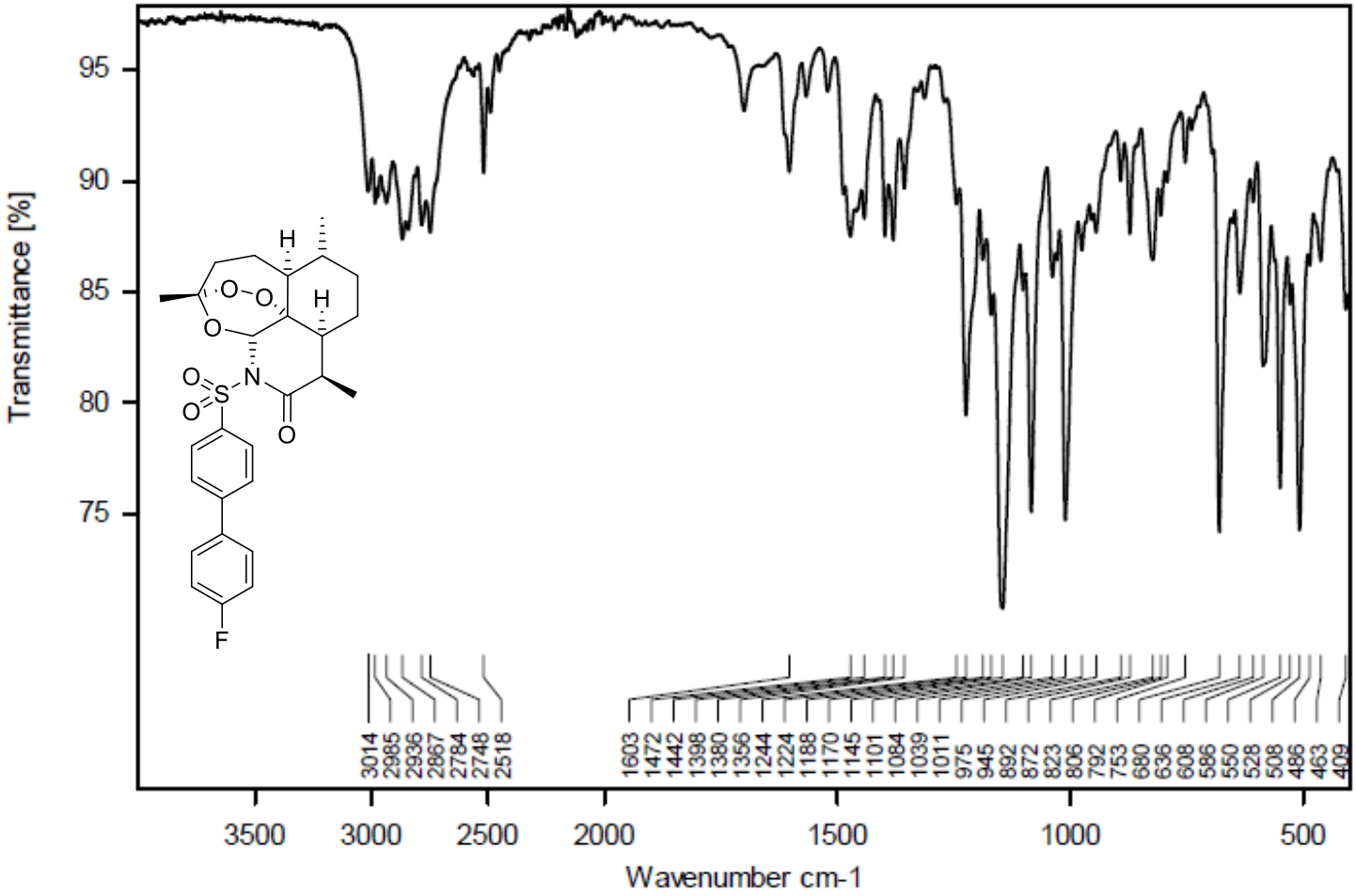
HRMS 4-Fluorobiphenyl-4-sulfonyl-azaartemisinin (**14**)

Acquisition Parameter

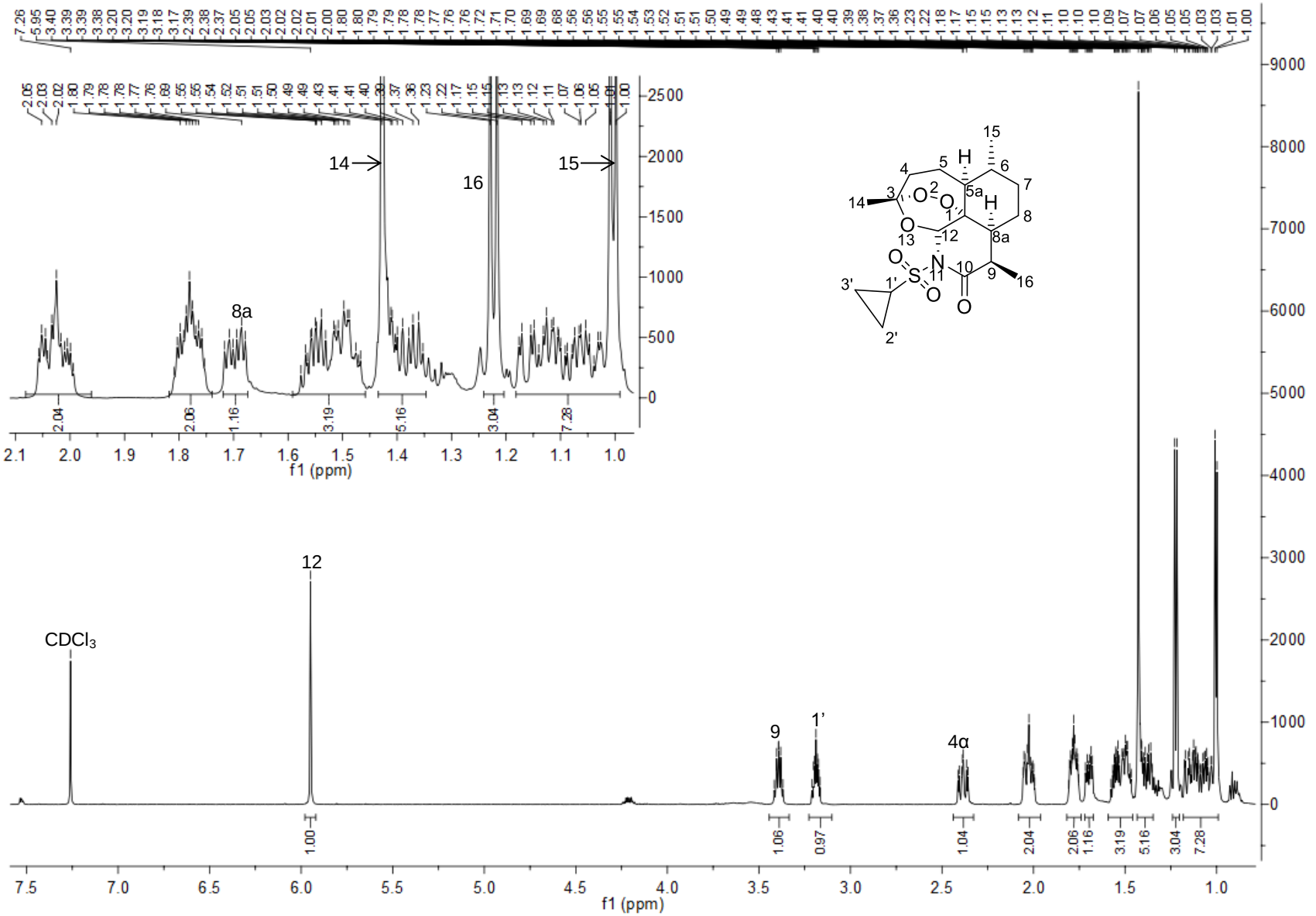
Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	0.4 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	180 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	4.0 l/min
Scan End	3000 m/z	Set Collision Cell RF	150.0 Vpp	Set Divert Valve	Waste



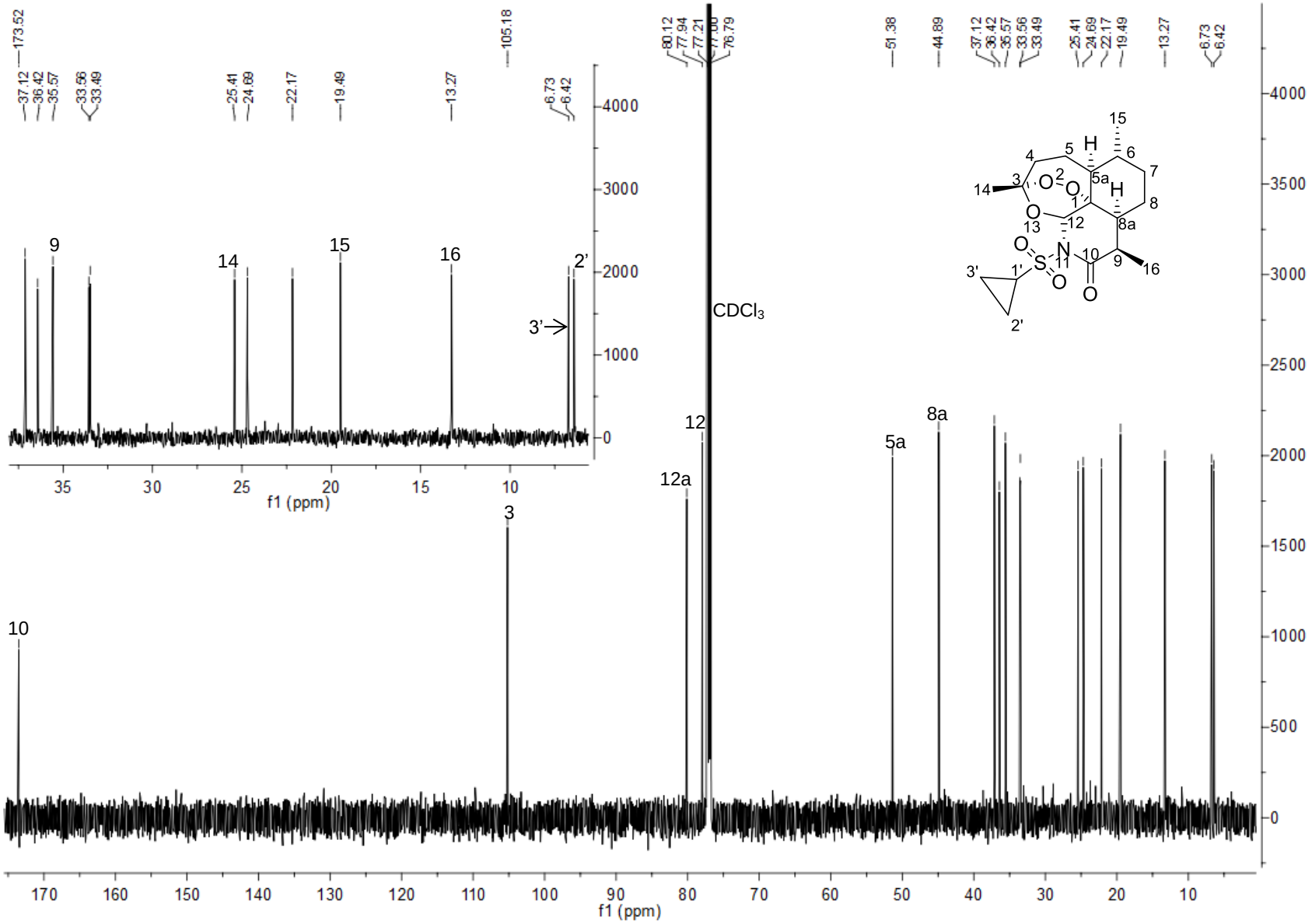
IR 4-Fluorobiphenyl-4-sulfonyl-azaartemisinin (**14**)



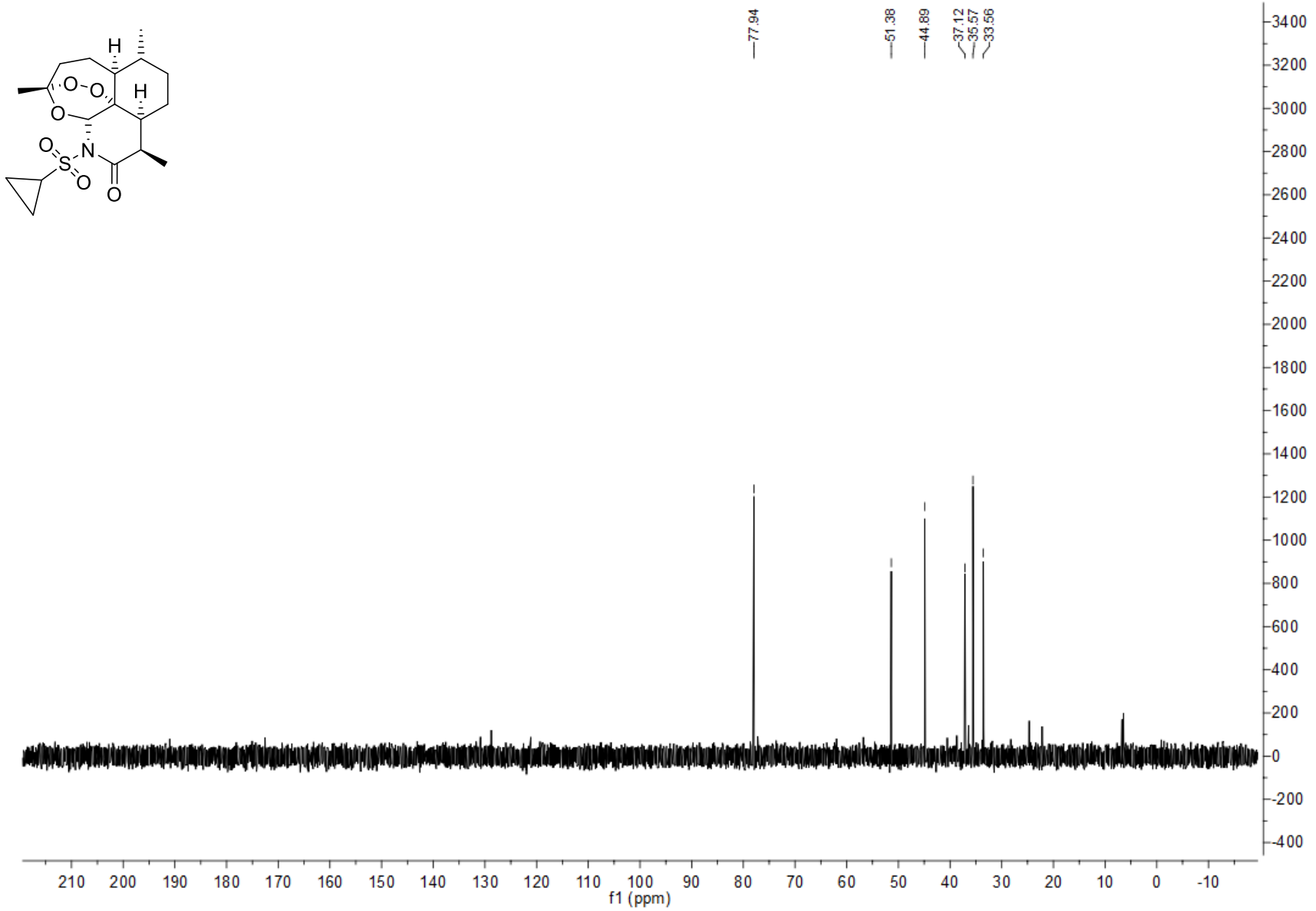
^{1}H NMR $CDCl_3$ Cyclopropanesulfonyl-azaartemisinin (**15**)



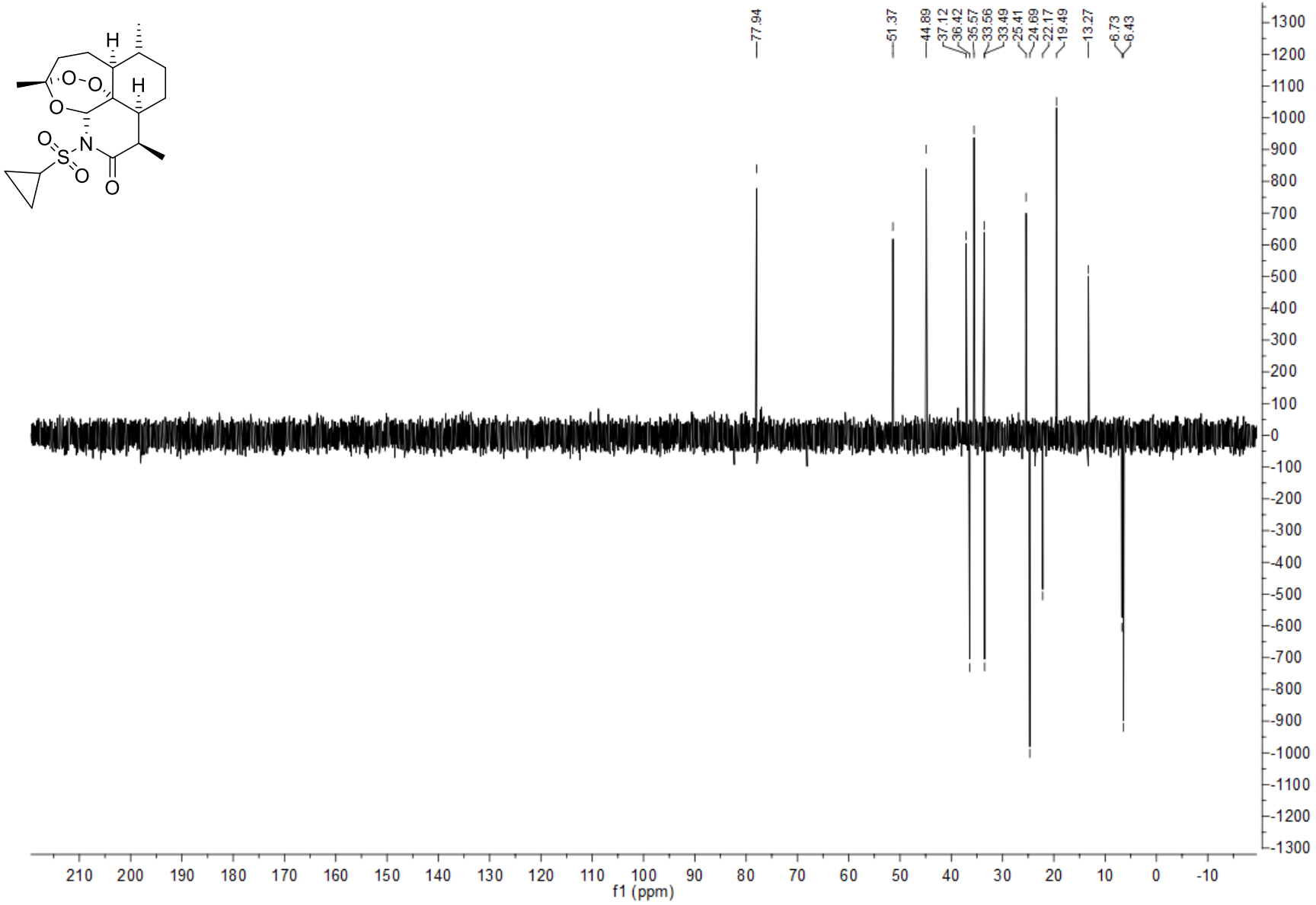
^{13}C NMR $CDCl_3$ Cyclopropanesulfonyl-azaartemisinin (**15**)



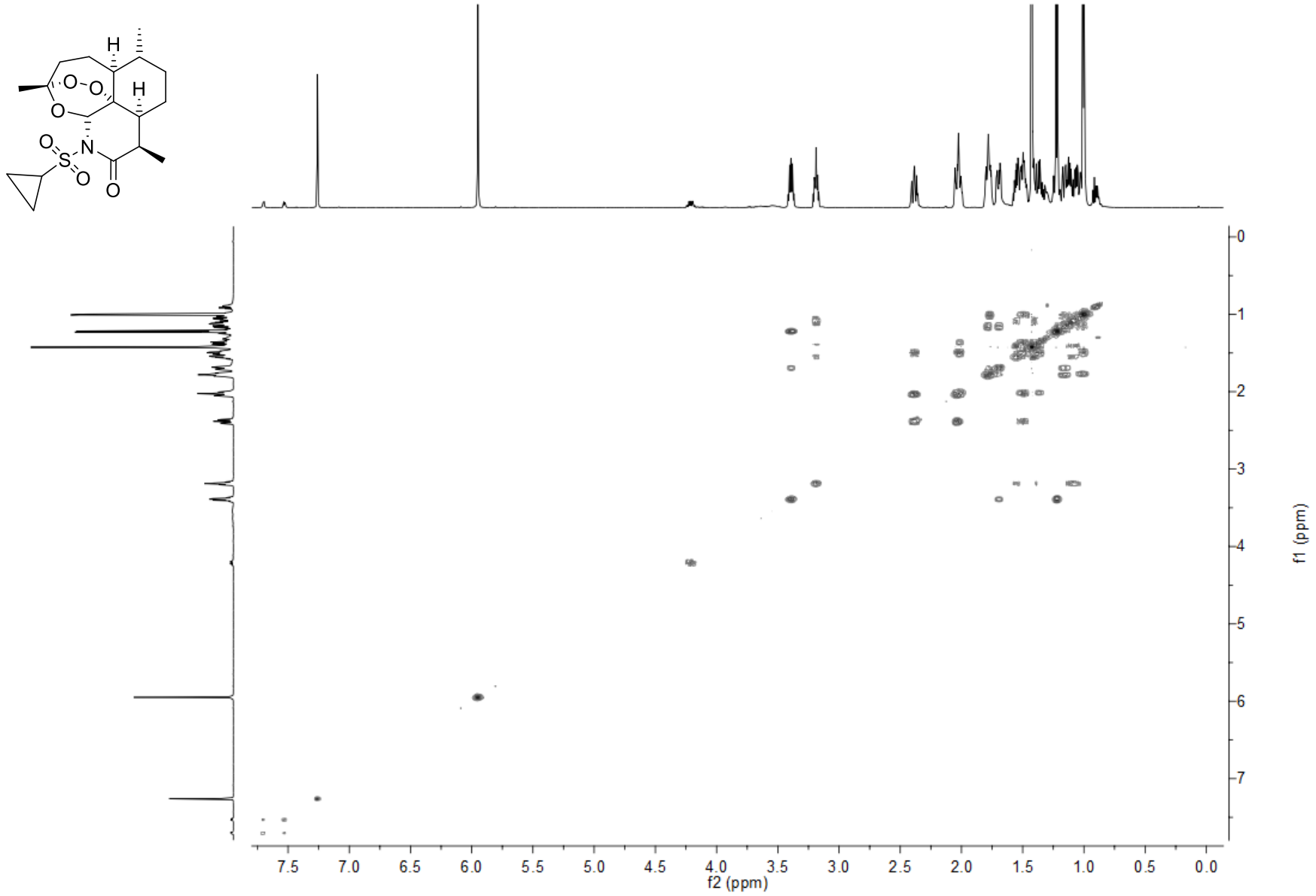
DEPT90 $CDCl_3$ Cyclopropanesulfonyl-azaartemisinin (**15**)



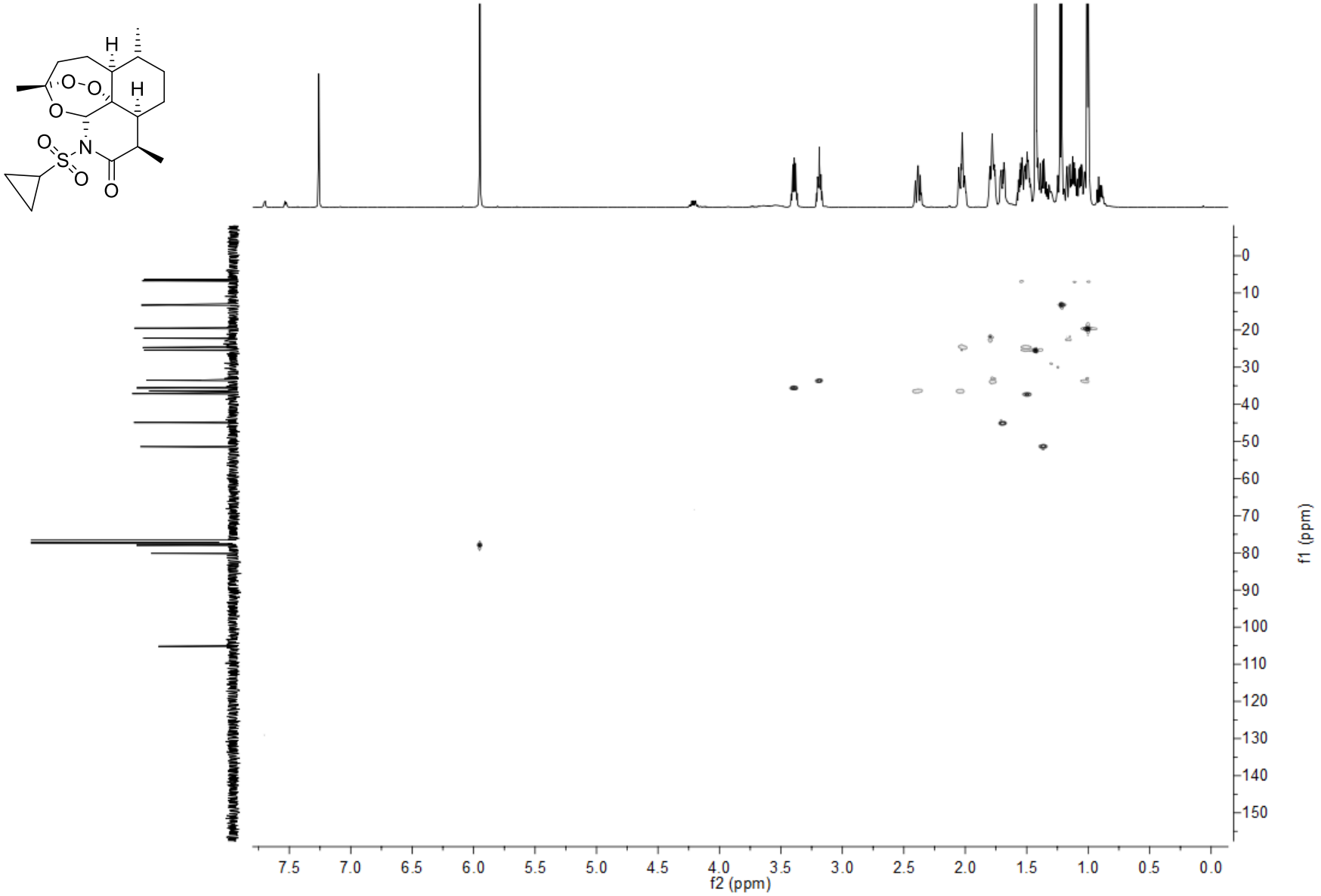
DEPT135 $CDCl_3$ Cyclopropanesulfonyl-azaartemisinin (**15**)



COSY $CDCl_3$ Cyclopropanesulfonyl-azaartemisinin (**15**)



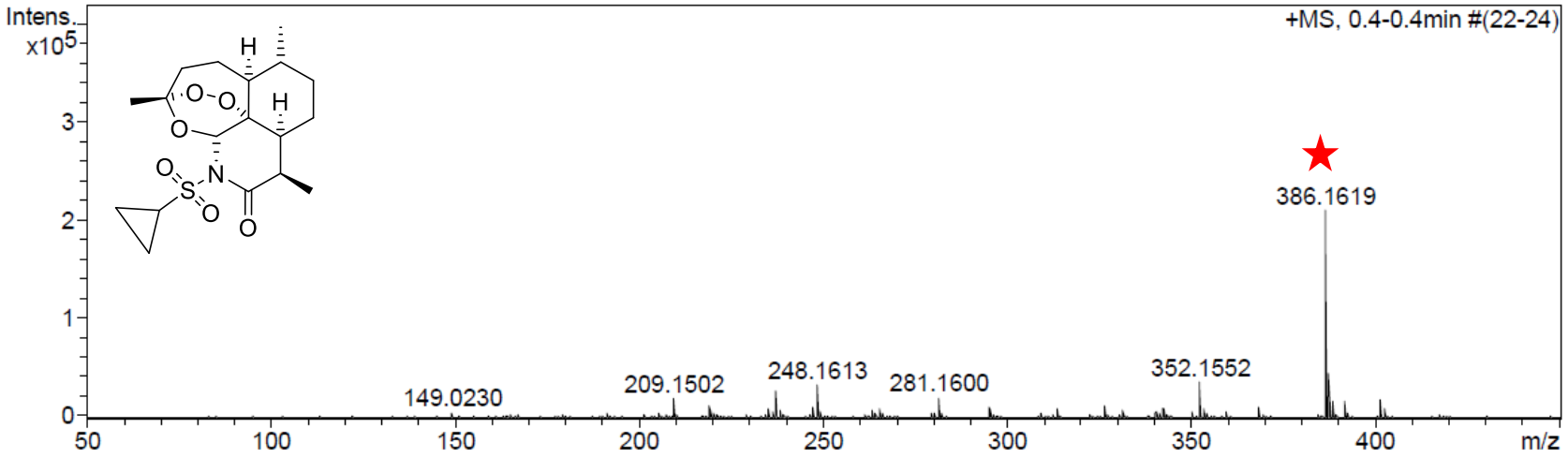
HSQC $CDCl_3$ Cyclopropanesulfonyl-azaartemisinin (**15**)



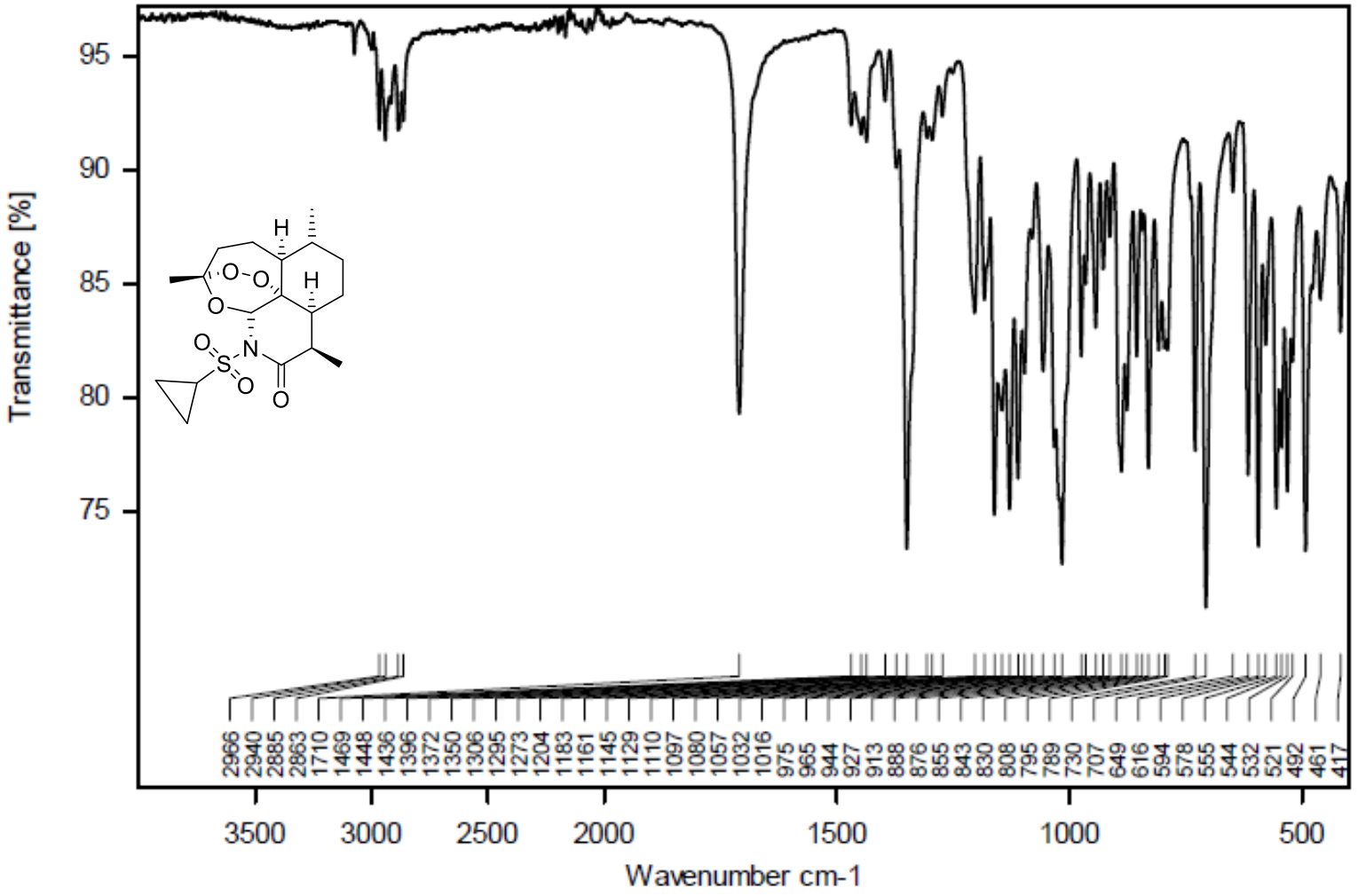
HRMS Cyclopropanesulfonyl-azaartemisinin (**15**)

Acquisition Parameter

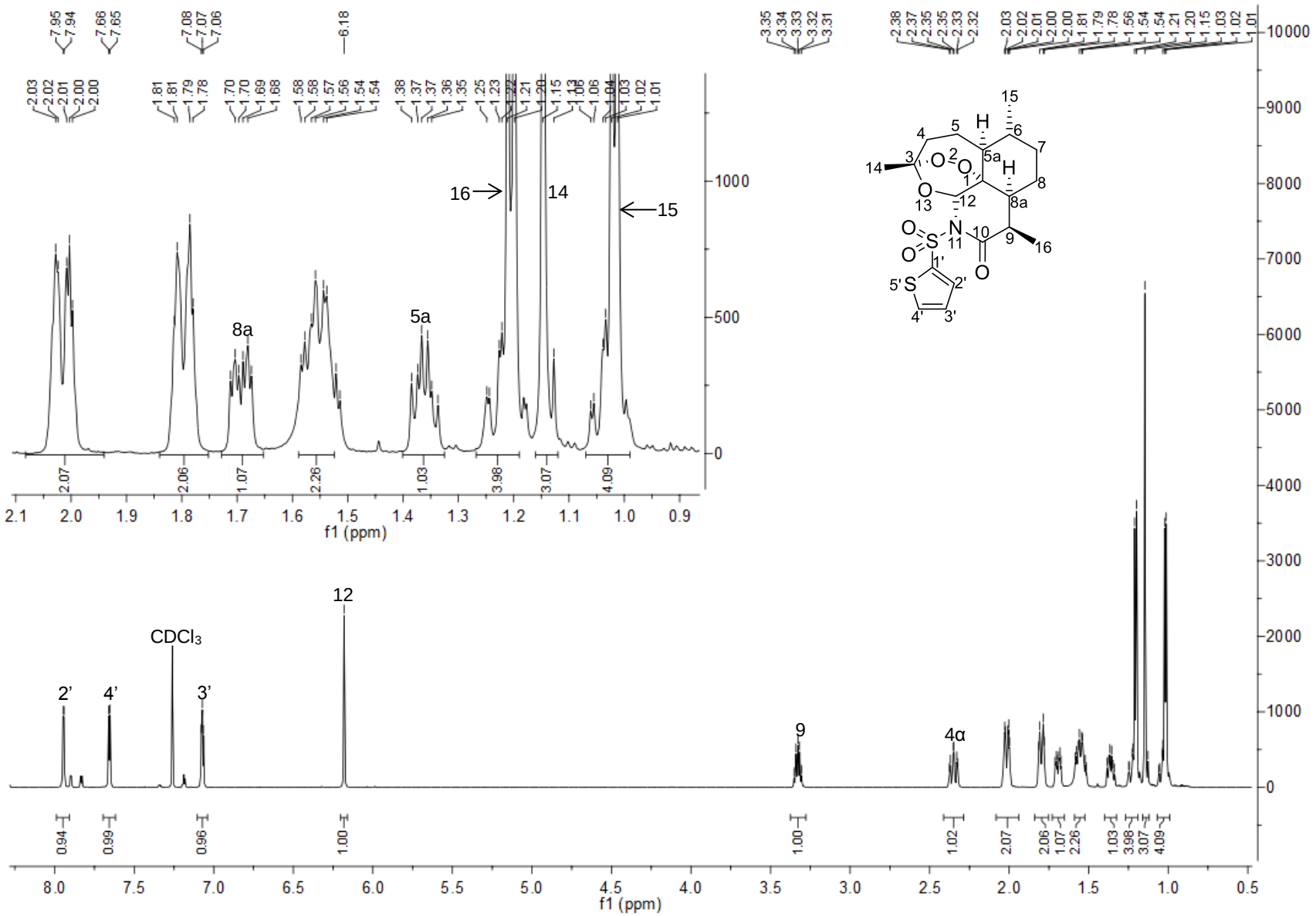
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	3000 m/z	Set Collision Cell RF	150.0 Vpp	Set Divert Valve	Waste



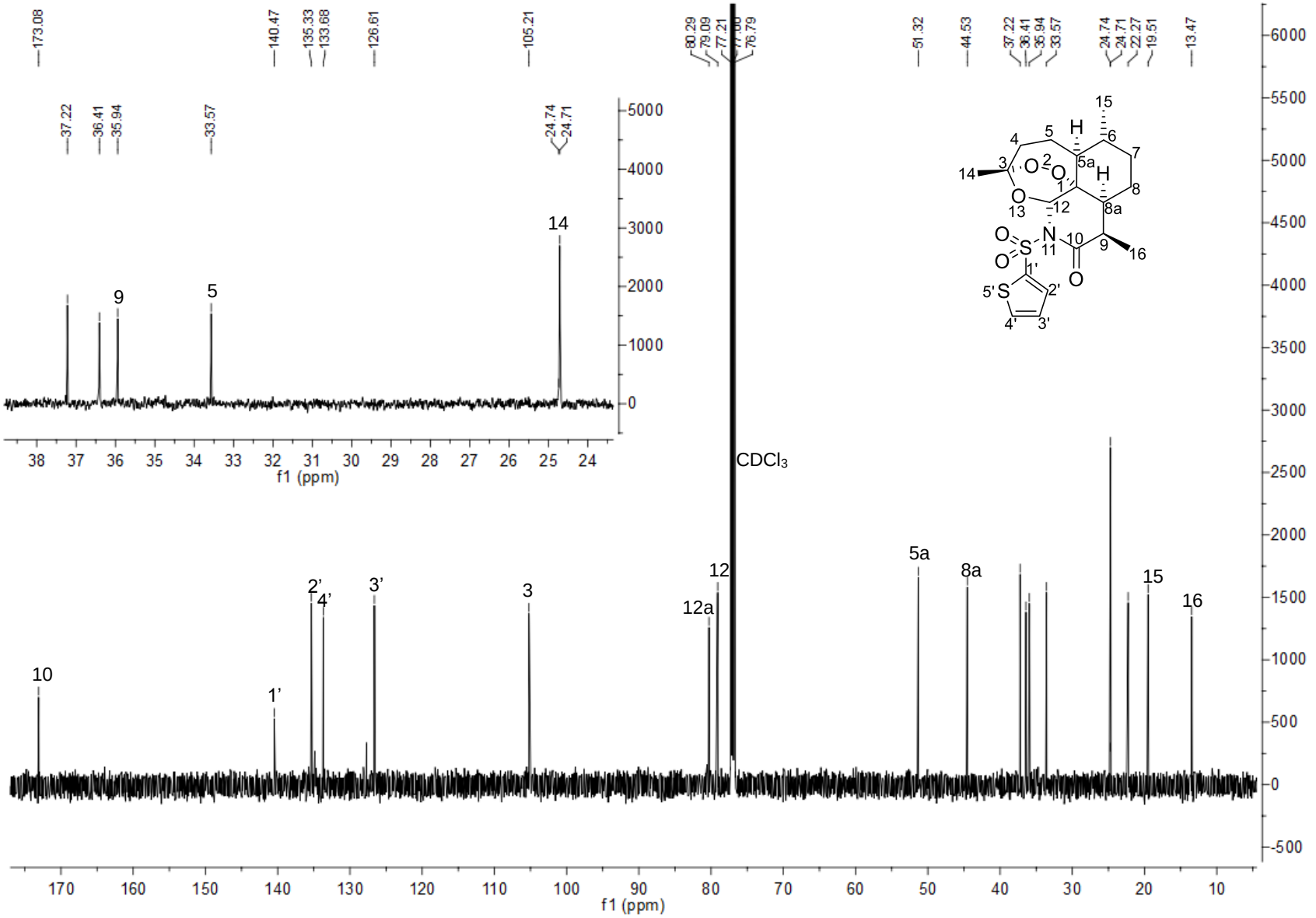
IR Cyclopropanesulfonyl-azaartemisinin (**15**)



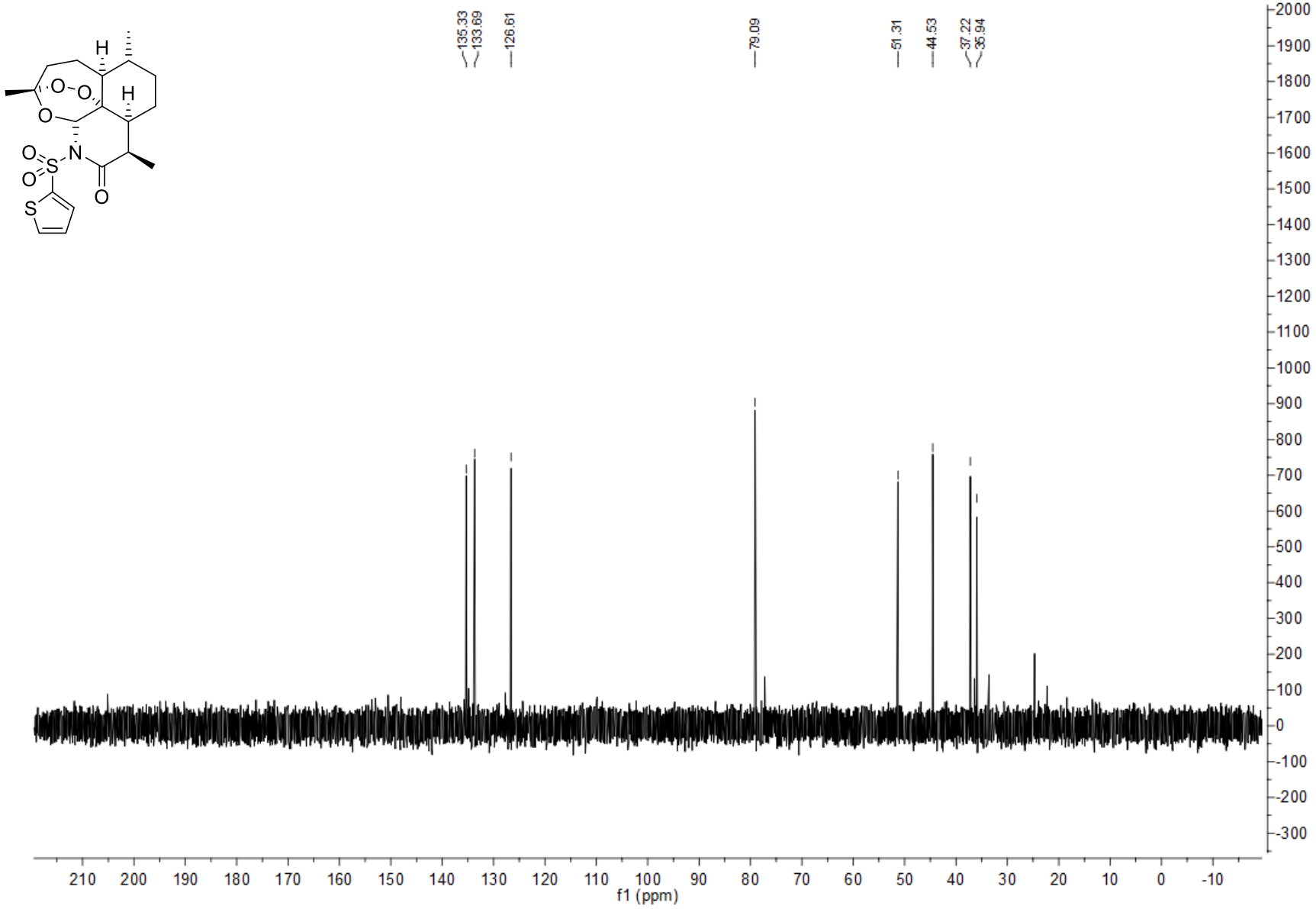
^{1}H NMR $CDCl_3$ 2'-Thiophenesulfonylazaartemisinin (**16**)



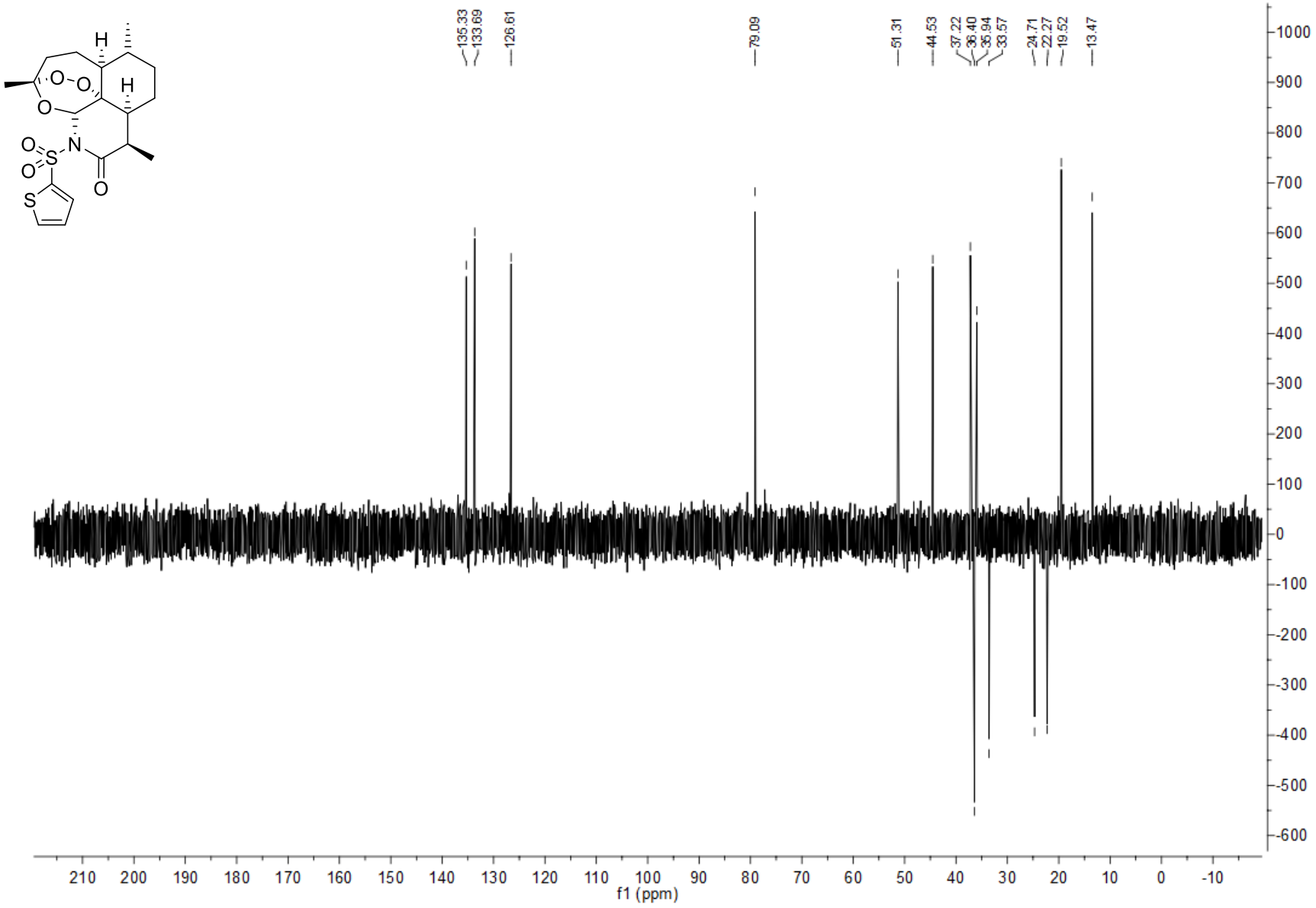
^{13}C NMR $CDCl_3$ 2'-Thiophenesulfonylazaartemisinin (**16**)



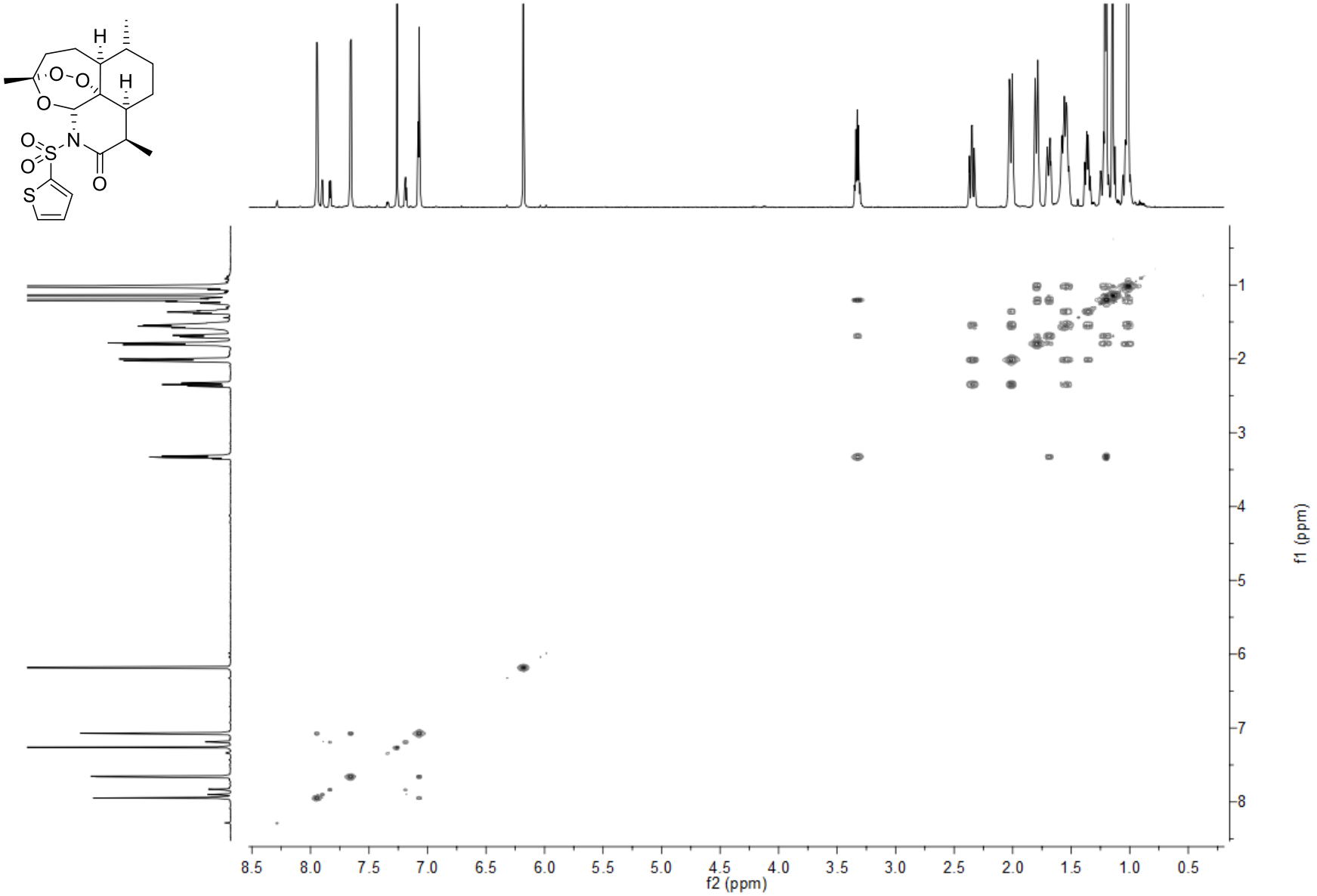
DEPT90 $CDCl_3$ 2'-Thiophenesulfonylazaartemisinin (**16**)



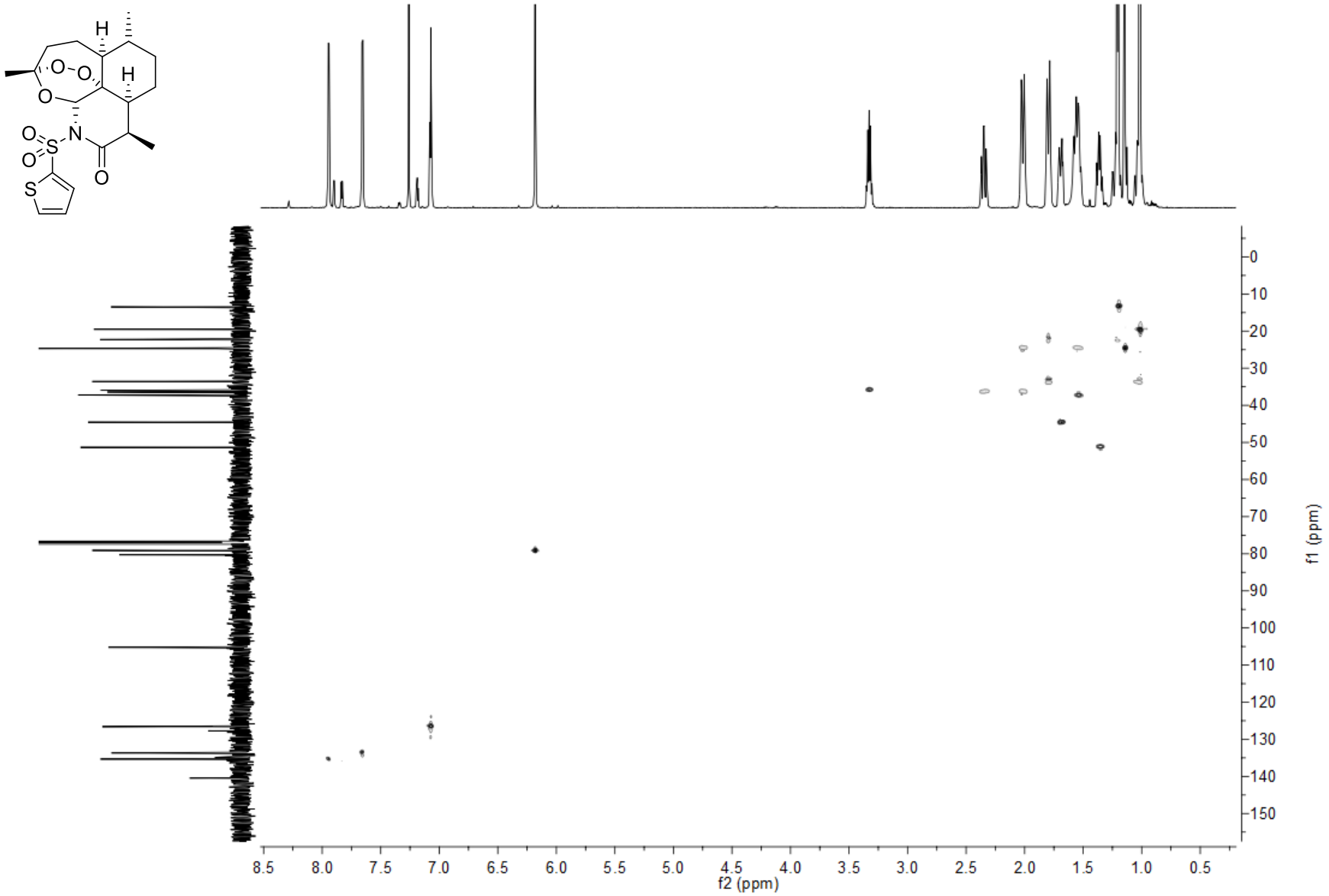
DEPT135 $CDCl_3$ 2'-Thiophenesulfonylazaartemisinin (**16**)



COSY $CDCl_3$ 2'-Thiophenesulfonylazaartemisinin (**16**)



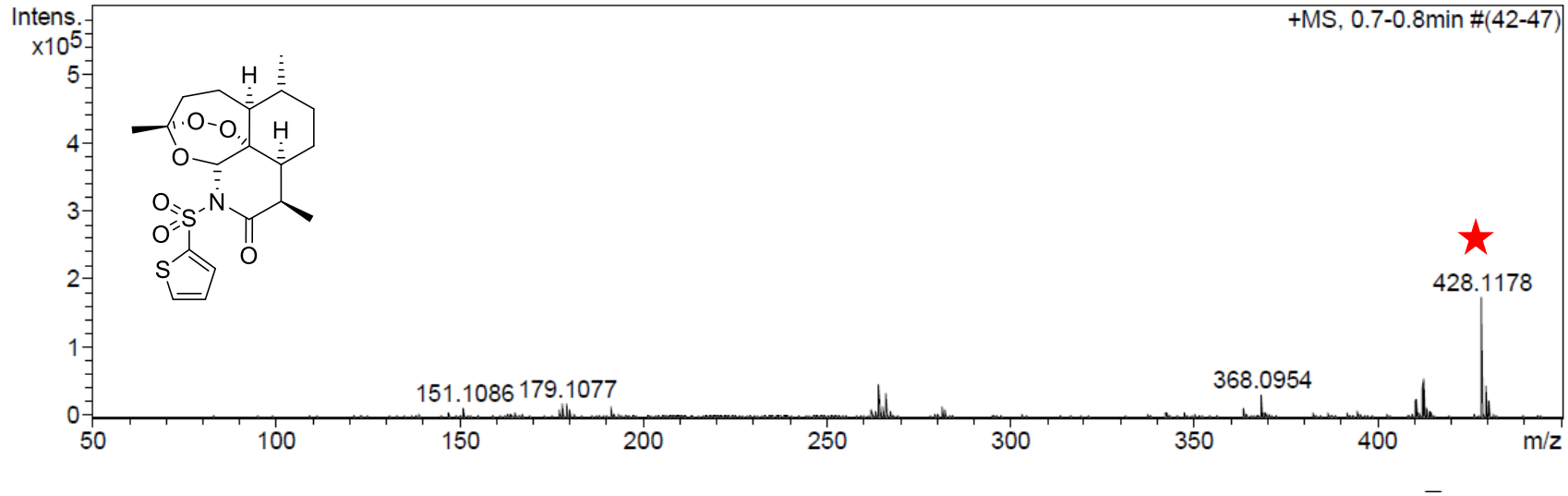
HSQC $CDCl_3$ 2'-Thiophenesulfonylazaartemisinin (**16**)



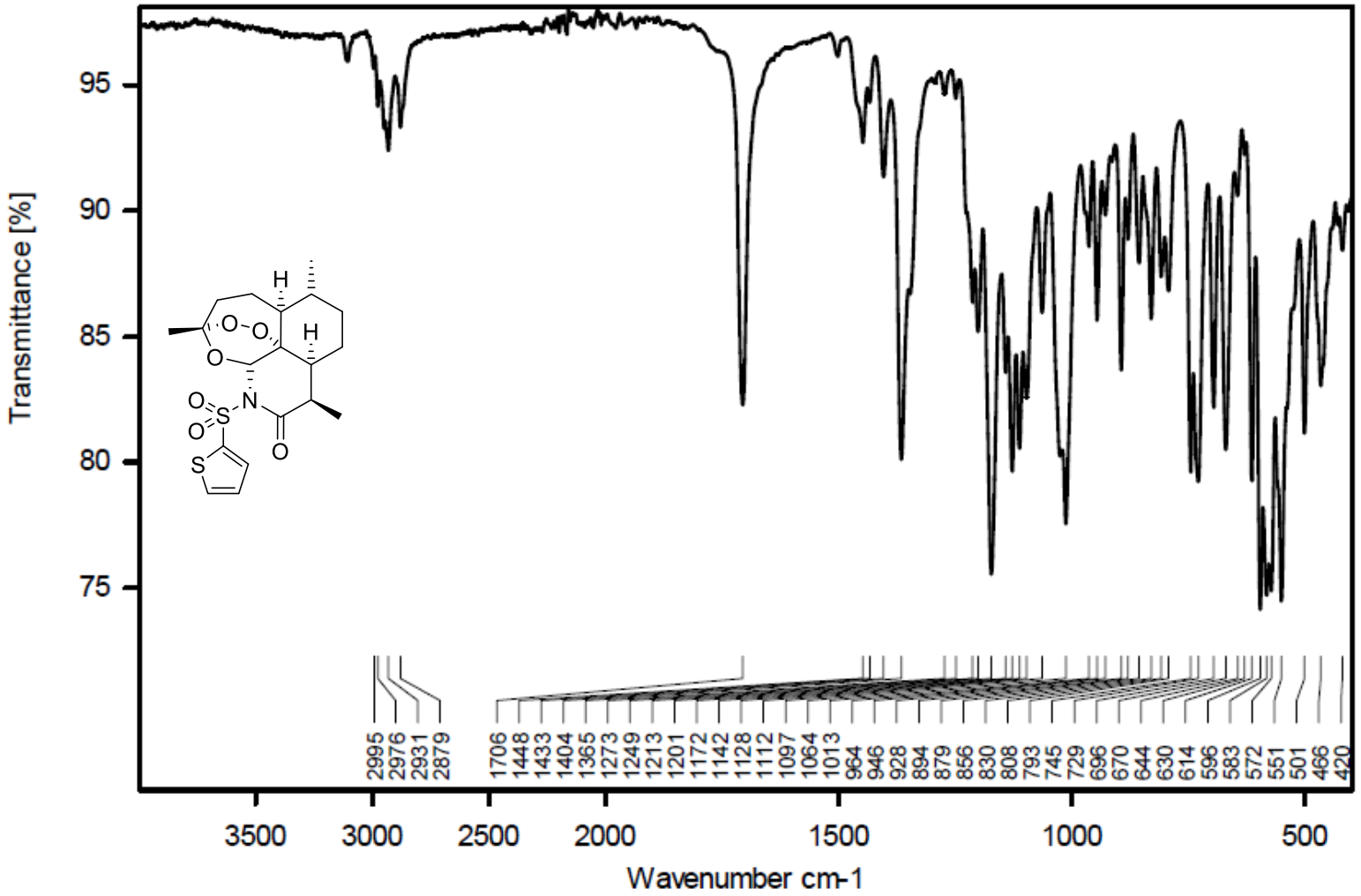
HRMS 2'-Thiophenesulfonylazaartemisinin (**16**)

Acquisition Parameter

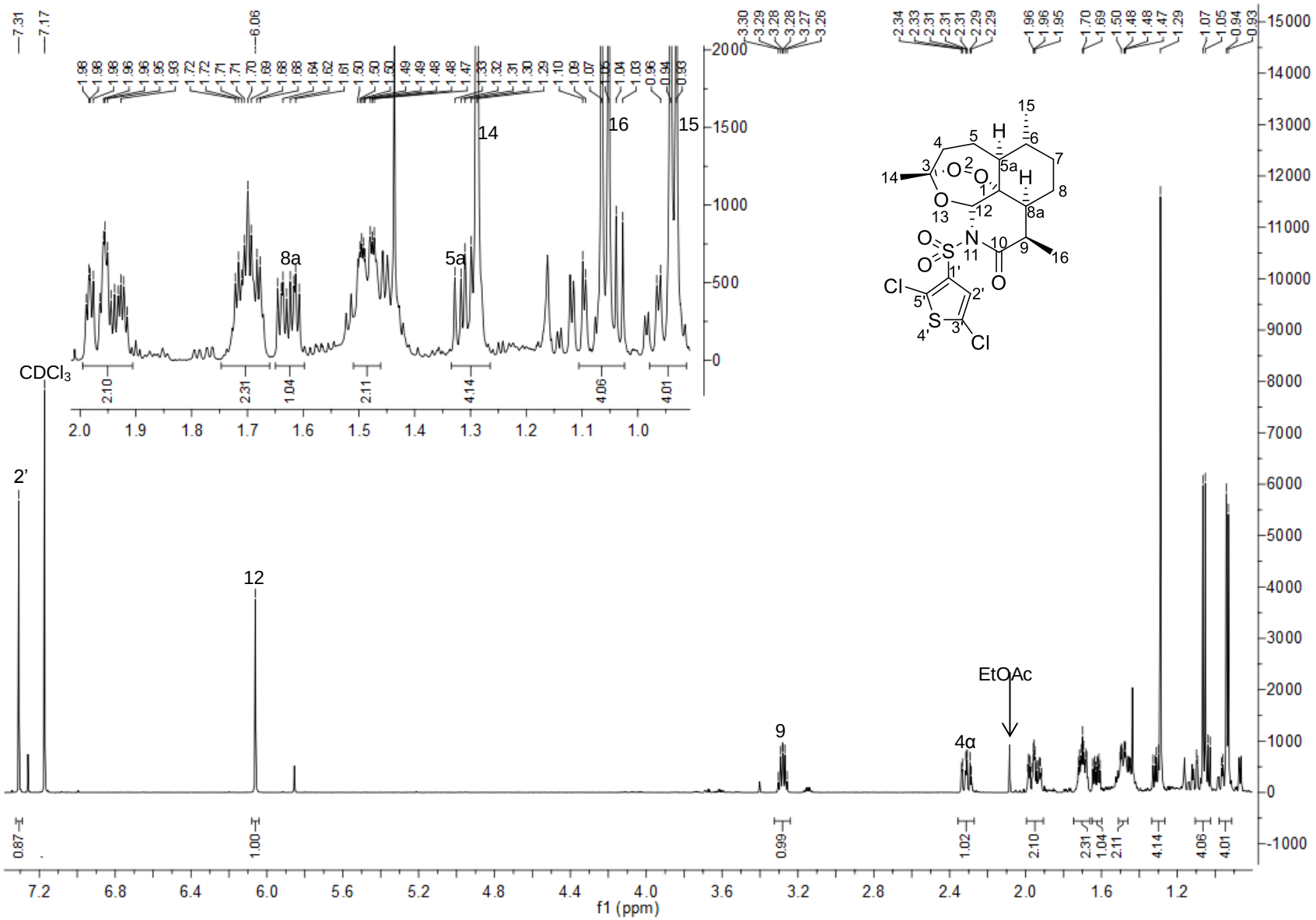
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	3000 m/z	Set Collision Cell RF	150.0 Vpp	Set Divert Valve	Waste



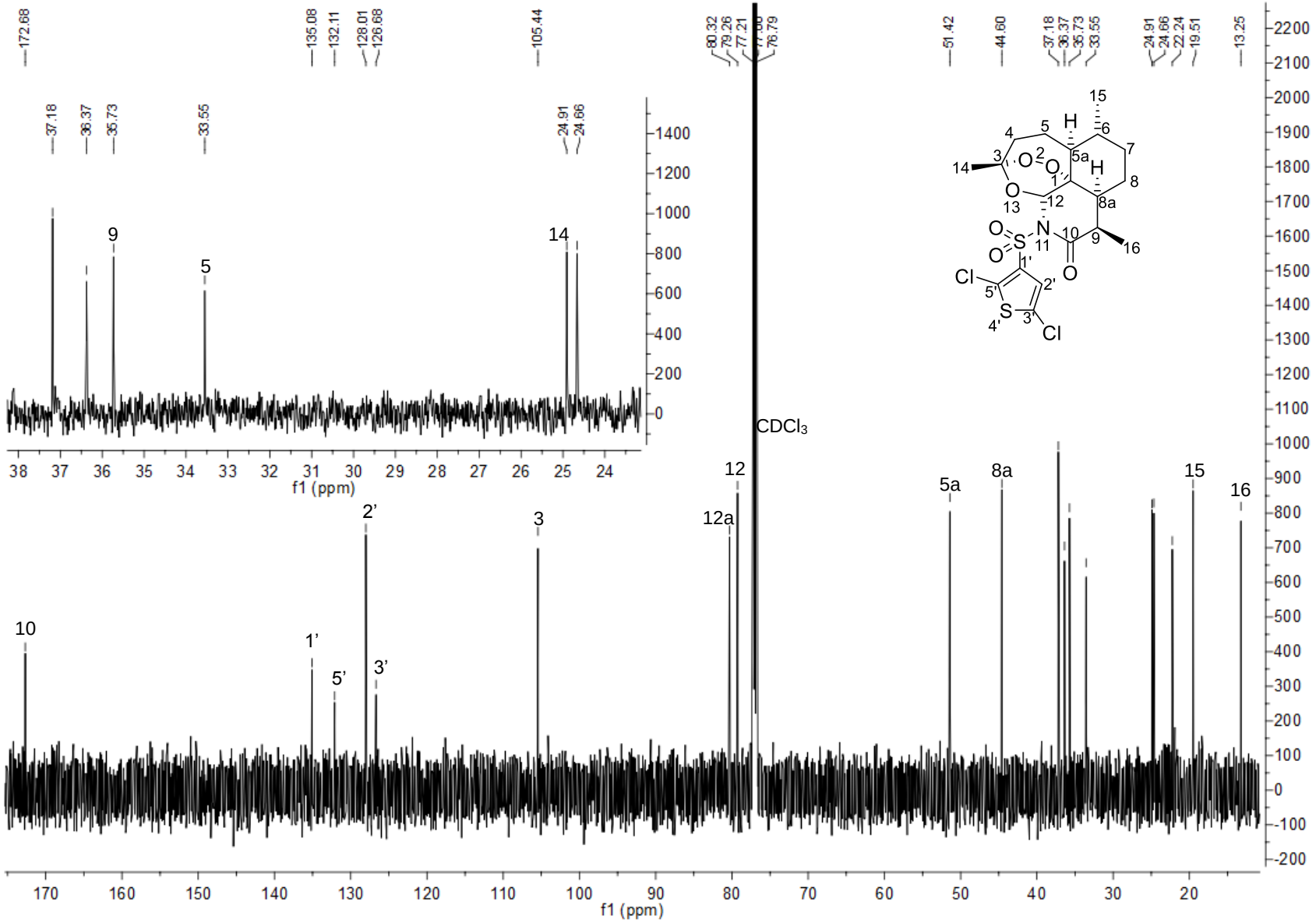
IR 2'-Thiophenesulfonylazaartemisinin (**16**)



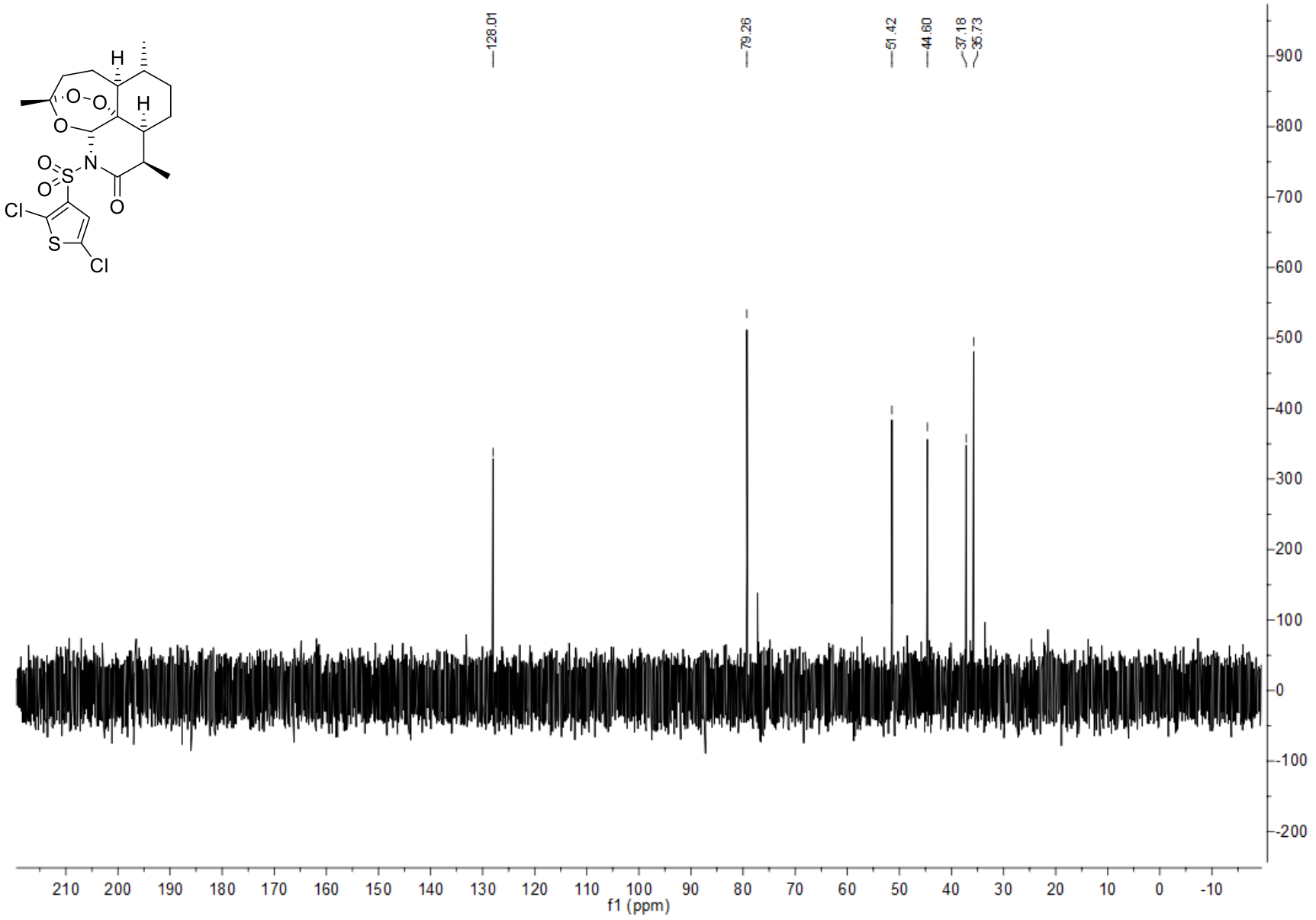
^{1}H NMR $CDCl_3$ 2,5-Dichlorothiophenesulfonyl-azaartemisinin (**17**)



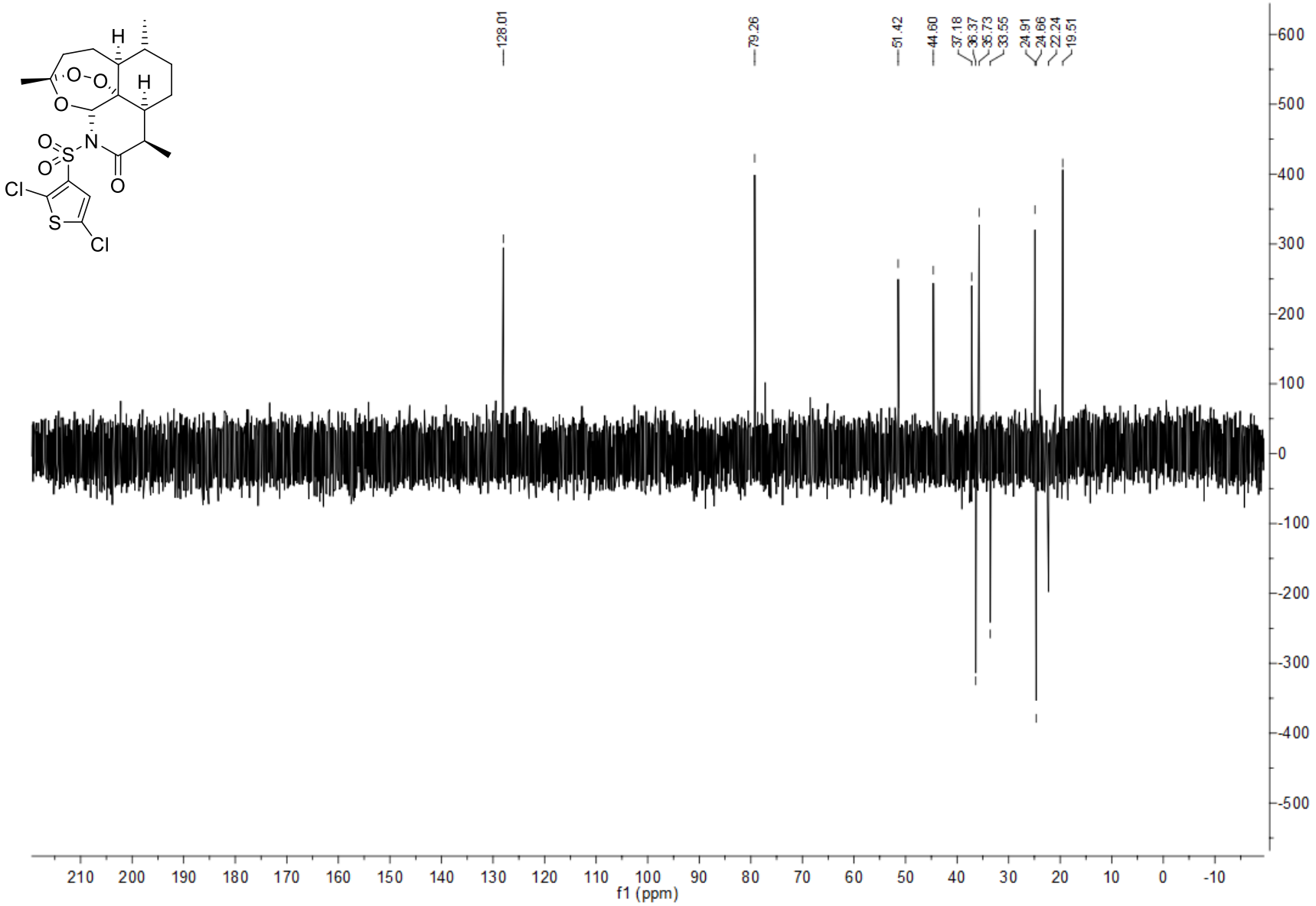
^{13}C NMR $CDCl_3$ 2,5-Dichlorothiophenesulfonyl-azaartemisinin (**17**)



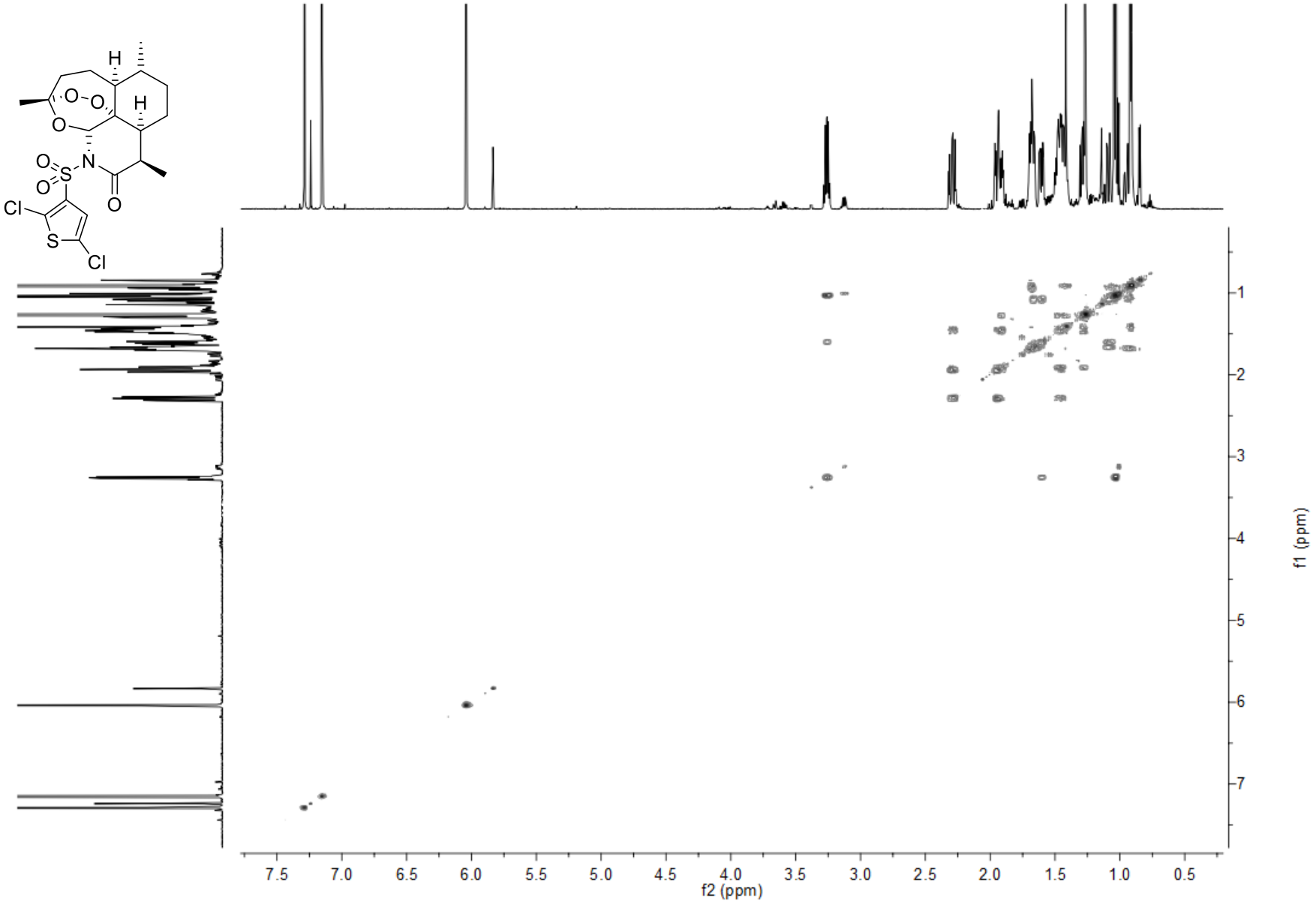
DEPT90 $CDCl_3$ 2,5-Dichlorothiophenesulfonyl-azaartemisinin (**17**)



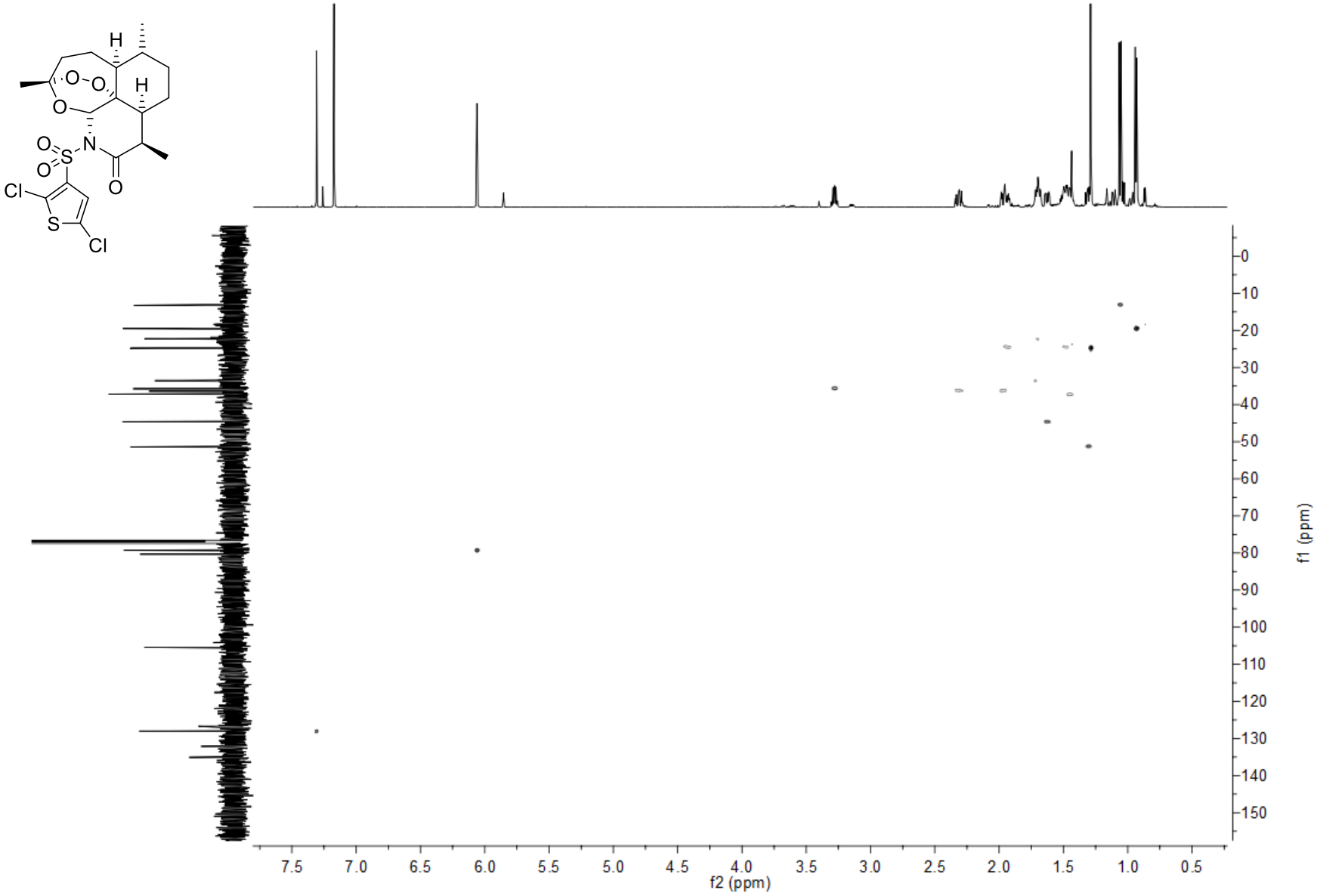
DEPT135 $CDCl_3$ 2,5-Dichlorothiophenesulfonyl-azaartemisinin (**17**)



COSY $CDCl_3$ 2,5-Dichlorothiophenesulfonyl-azaartemisinin (**17**)



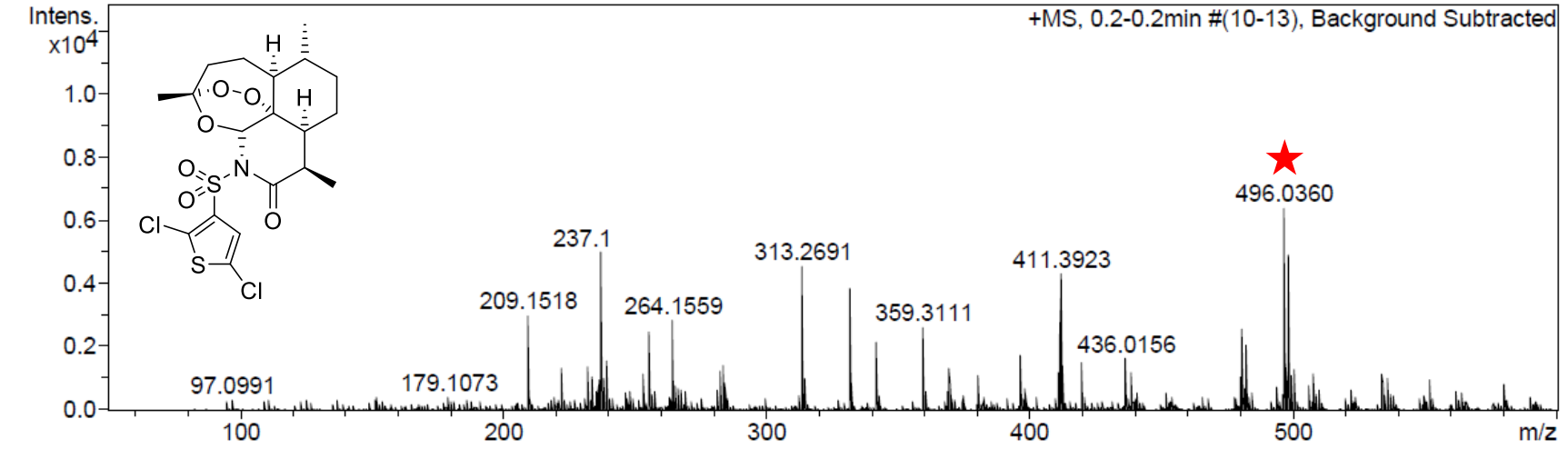
HSQC $CDCl_3$ 2,5-Dichlorothiophenesulfonyl-azaartemisinin (**17**)



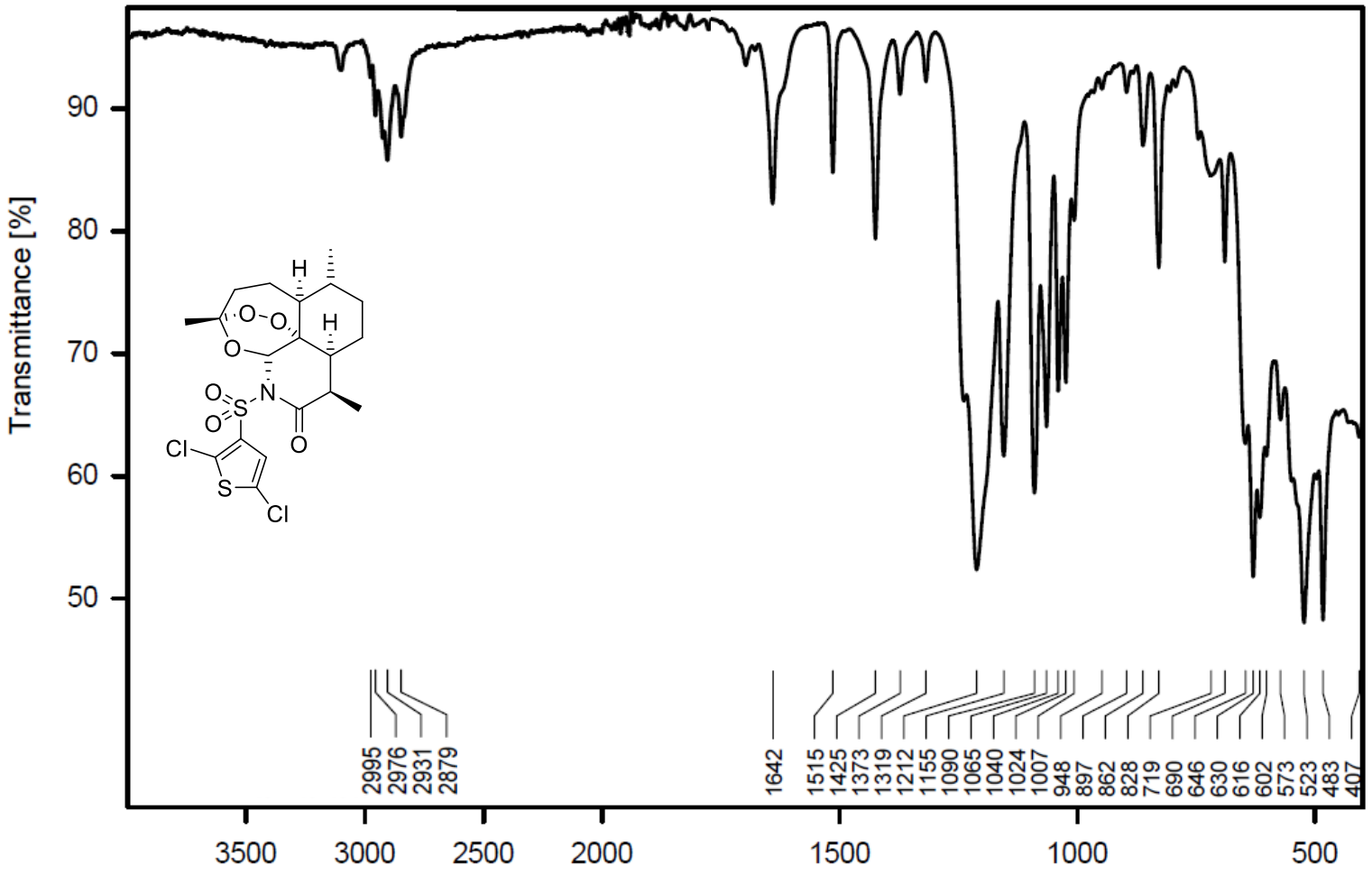
HRMS 2,5-Dichlorothiophenesulfonyl-azaartemisinin (**17**)

Acquisition Parameter

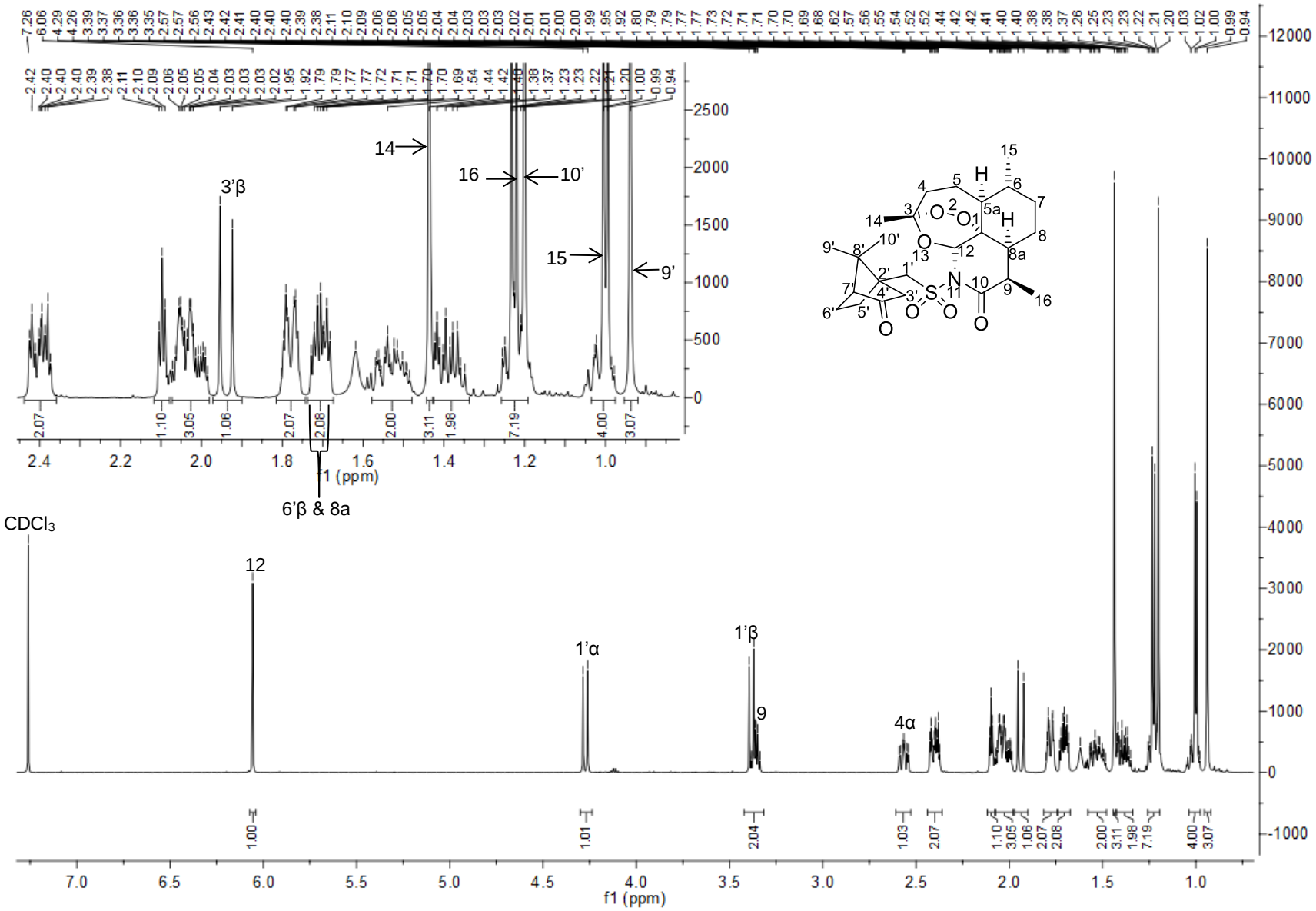
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	3000 m/z	Set Collision Cell RF	150.0 Vpp	Set Divert Valve	Waste



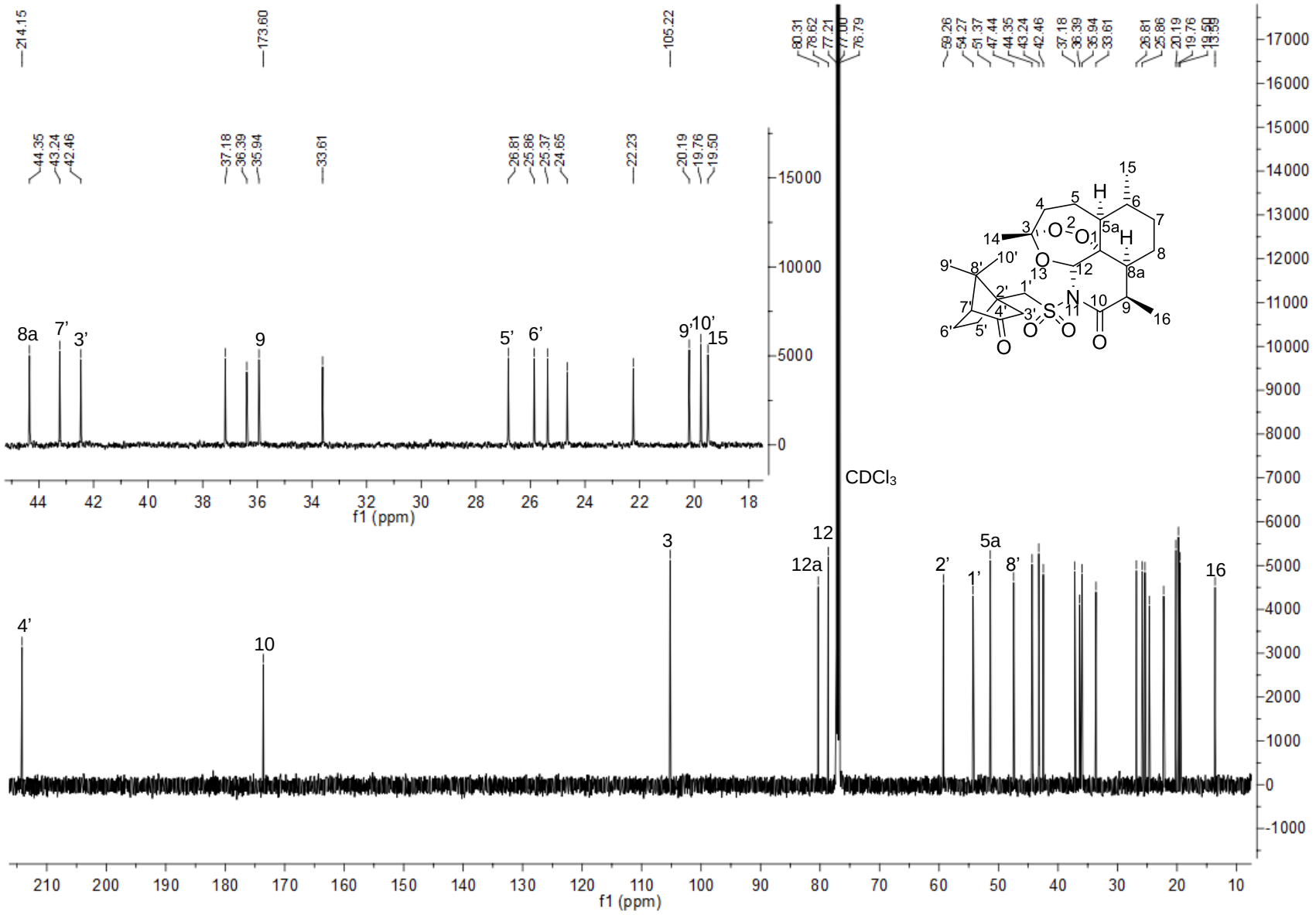
IR 2,5-Dichlorothiophenesulfonyl-azaartemisinin (**17**)



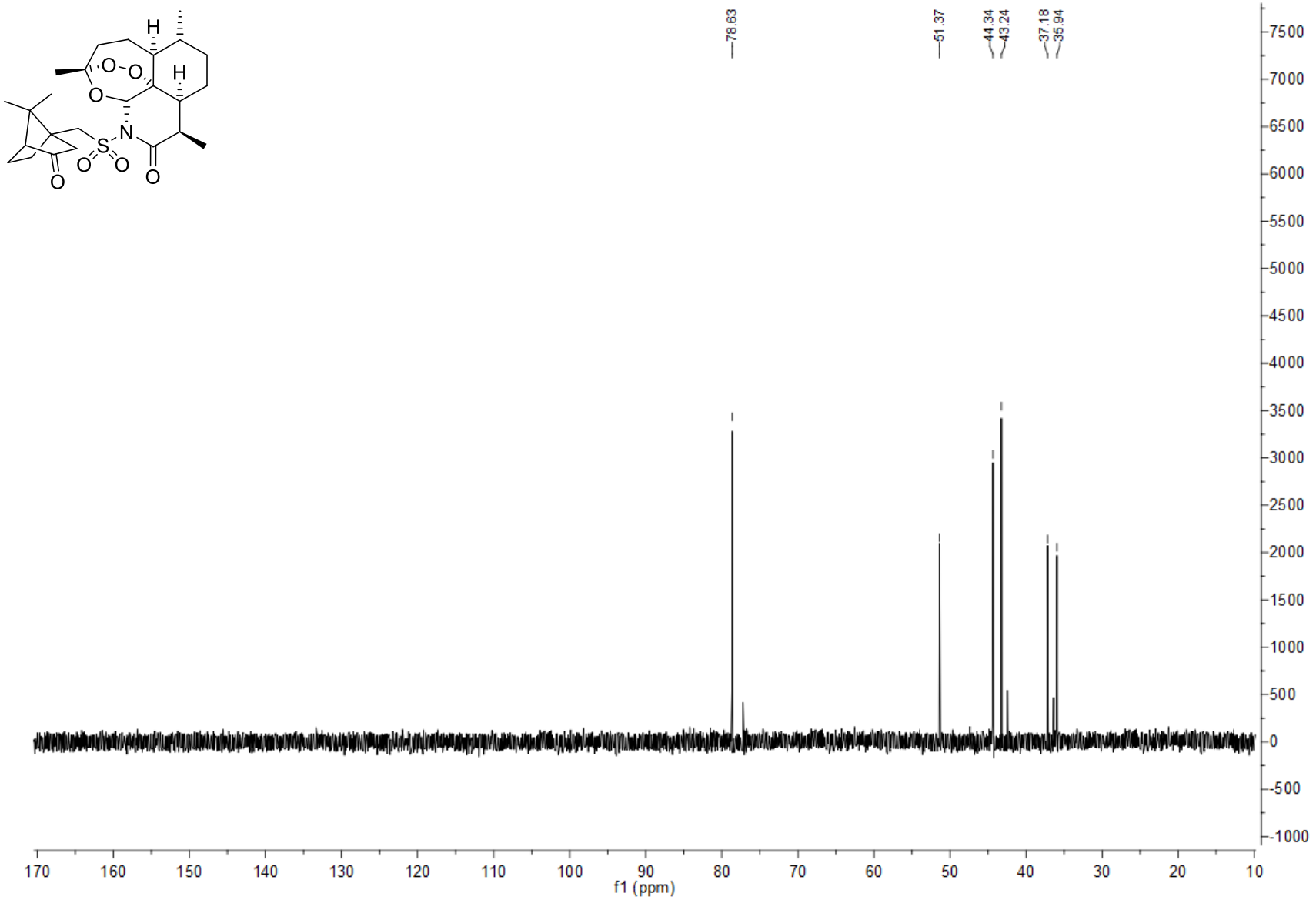
^{1}H NMR $CDCl_3$ 10-Camphoryl-azaartemisinin (**18**)



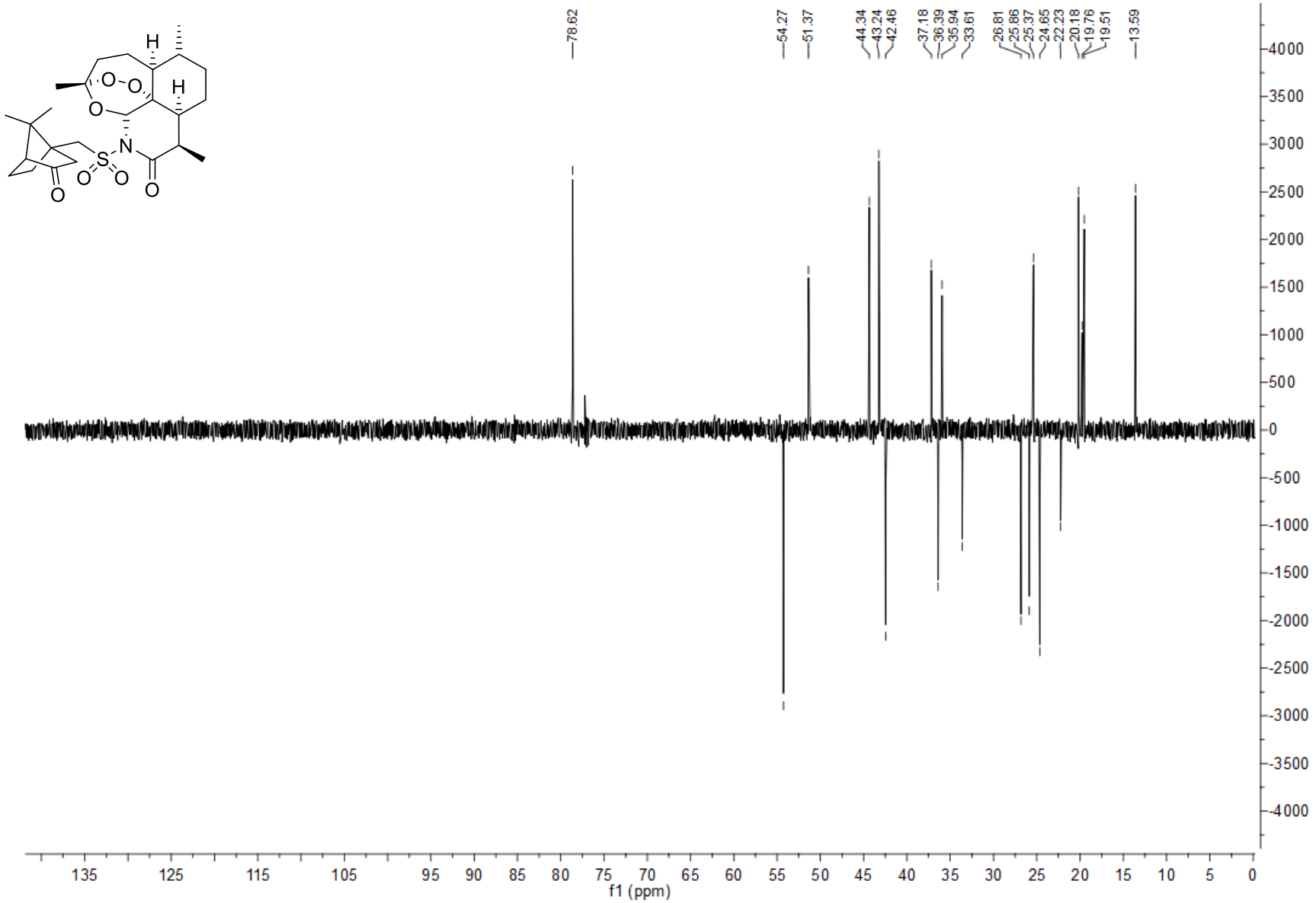
^{13}C NMR $CDCl_3$ 10-Camphoryl-azaartemisinin (**18**)



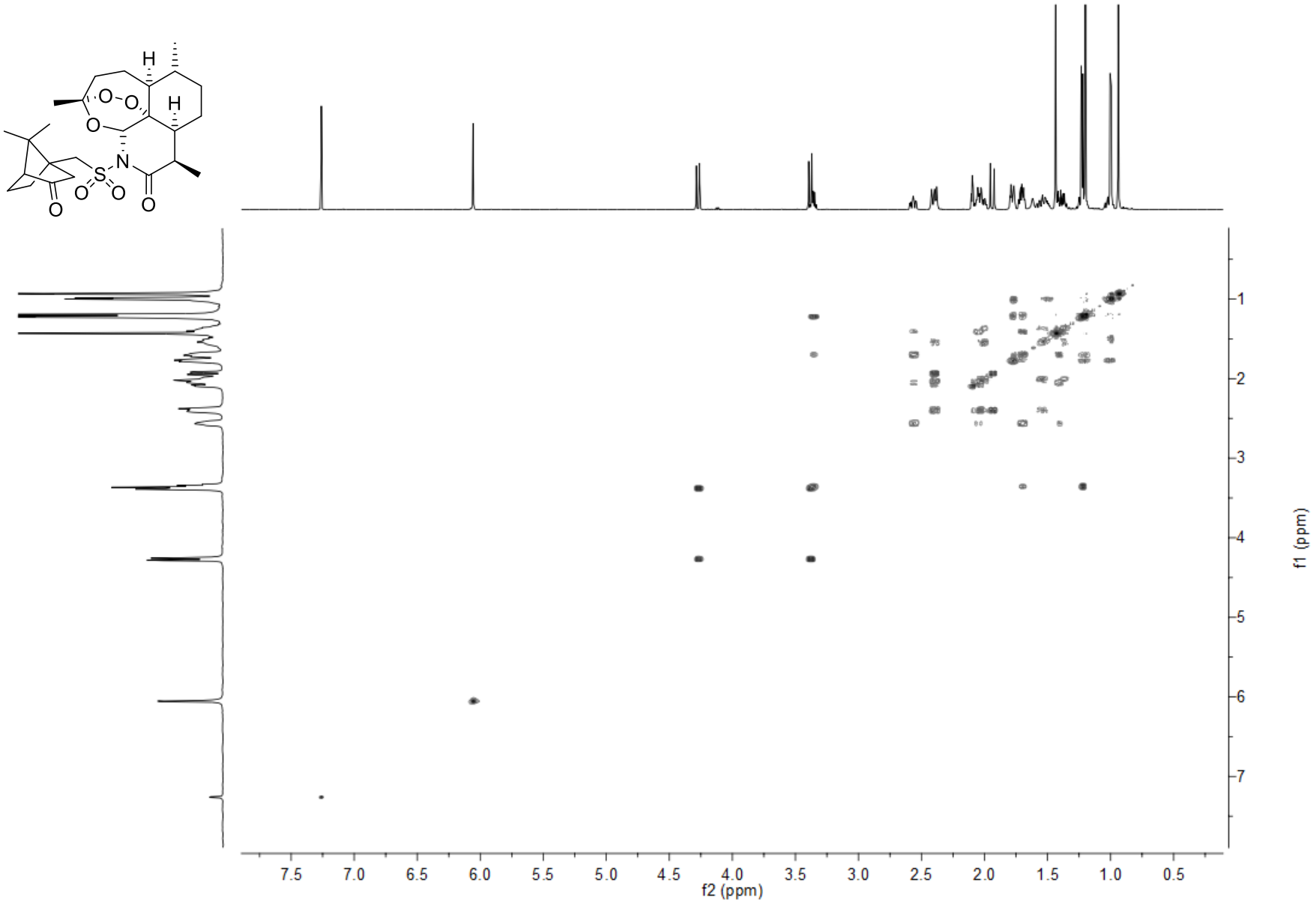
DEPT90 $CDCl_3$ 10-Camphoryl-azaartemisinin (**18**)



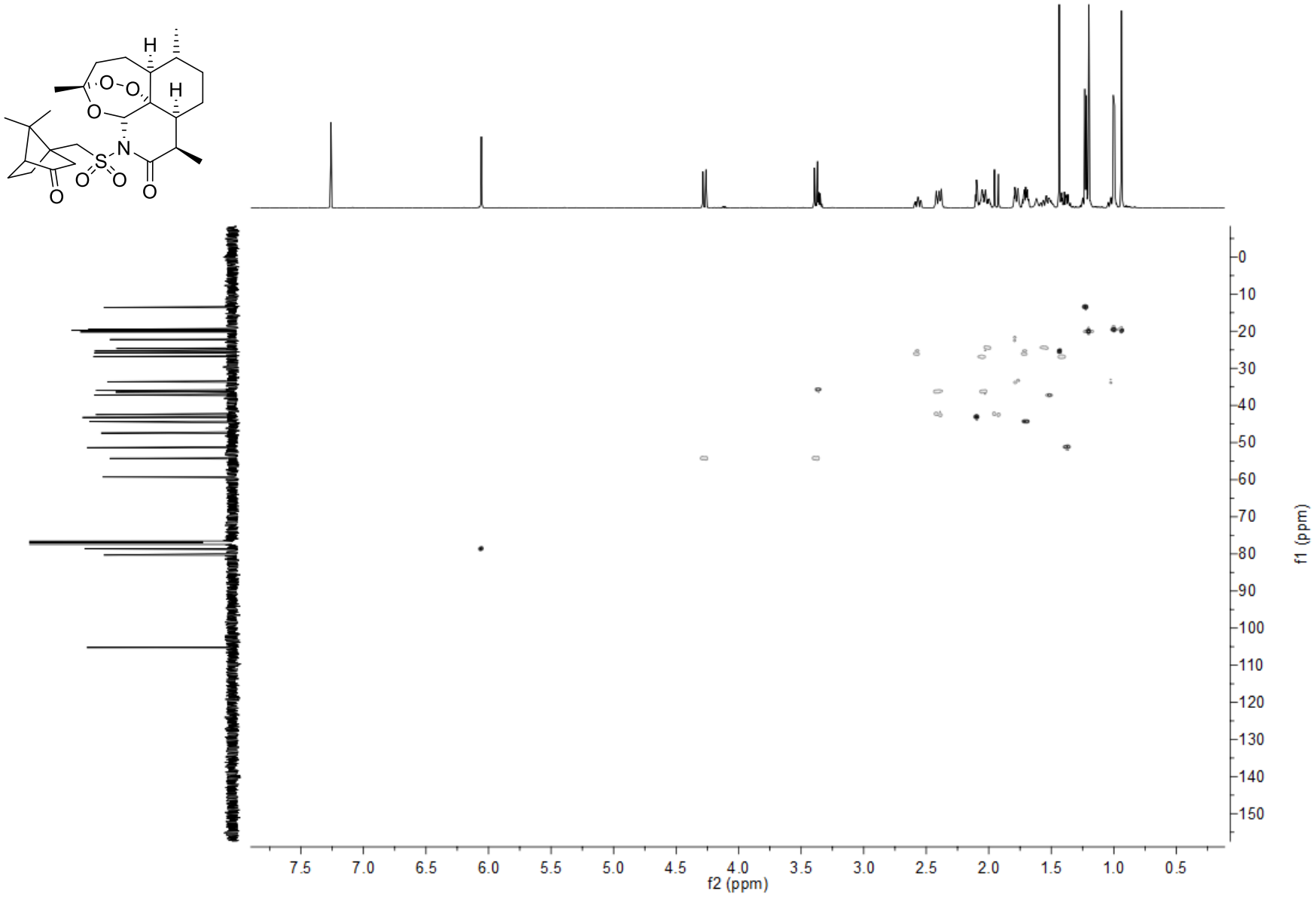
DEPT135 $CDCl_3$ 10-Camphoryl-azaartemisinin (**18**)



COSY $CDCl_3$ 10-Camphoryl-azaartemisinin (**18**)



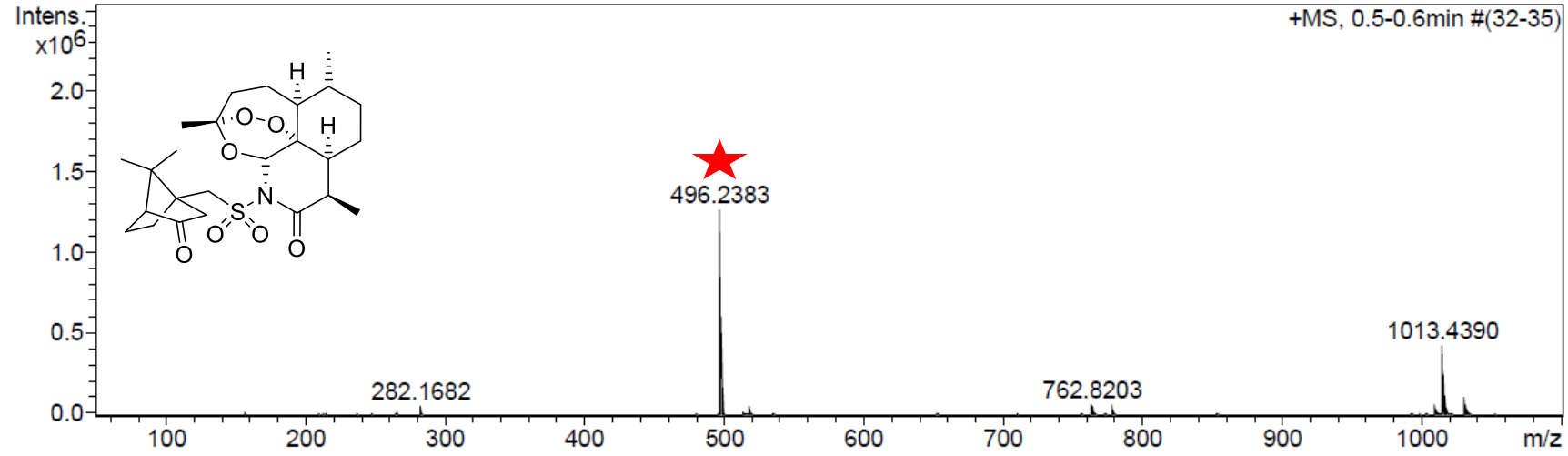
HSQC $CDCl_3$ 10-Camphoryl-azaartemisinin (**18**)



HRMS 10-Camphoryl-azaartemisinin (**18**)

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	3000 m/z	Set Collision Cell RF	150.0 Vpp	Set Divert Valve	Waste



IR 10-Camphoryl-azaartemisinin (**18**)