Dihydroartemisinin esters as prodrugs against resistant *P. falciparum* strains

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“"Depending on different criteria, [malaria] would bounce around between number one and number three. In terms of human misery, there's a pretty strong case it's number one."

- Bill Gates

ABSTRACT

Malaria is caused by the *Plasmodium sp*. parasite that infects the red blood cells. Of the four types of malaria, the most serious type is transmitted by *Plasmodium falciparum* species. It can be life threatening. The other types of malaria (*P. vivale, P. ovale* and *P. malaria*e) are generally less serious and are not life threatening. The existence of malaria as an enemy of humankind certainly predates written history. For thousands of years malaria has been a deadly scourge, and it remains one today. From American president John Adams who nearly succumbed to malaria in Amsterdam while on a diplomatic mission, back down to the timeline to the early Chinese, Indians, Greeks and Romans, malaria has not spared its victims, rich or poor.

It wasn't until the 19th Century that information about the true cause of malaria became known. Yet despite this knowledge, malaria still ravages Sub-Saharan Africa, South-East Asia and Latin America, taking as its victim's mainly young children and pregnant women. However, without certain discoveries leading to a better understanding of malaria, new groundbreaking work wouldn't be possible.

Artemisinin and its derivatives are developing into a very important new class of antimalarial and their usage is becoming more common in the fight against malaria. The most commonly used and applied of these derivatives are artesunate, artemether, arteether and dihydroartemisinin. The discovery of artemisinin as the pharmacological active ingredient in an age old Chinese herb, *Artemisia annua*, was a major breakthrough in malaria chemotherapy. Discovery of qinghaosu in the 1970s sparked a new age for chemotherapy of malaria, and greatly inspired further research on organic peroxides. This generated widespread interest and led to the design and synthesis of organic peroxides into a highly active area of organic chemistry.

The artemisinin derivatives act quickly and are eliminated quickly. Their rapid onset makes them especially effective against severe malaria. Their rapid disappearance may be a key reason why artemisinin resistance has been so slow to develop, and may be the reason why recrudences are so common when these drugs are used in monotherapy. Since their isolation, artemisinins have had a substantial impact on the treatment of malaria. Although very potent, the use of artemisinins as prophylactic antimalarials is not recommended.

The aim of this study was to synthesise ester derivatives of artemisinin, determine certain physicochemical properties such as aqueous solubility and partition coefficient, and to evaluate their antimalarial activity in comparison to dihydroartemisinin and chloroquine.

In this study eight esters of dihydroartemisinin (DHA) were synthesised by substitution at C-10. The structures of the prepared derivatives were confirmed by nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS).

The new artemisinin esters were tested *in vitro* against the chloroquine sensitive strain of *Plasmodium falciparum* (D10). All the compounds tested showed activity against the D10 strain. All of the esters showed potency significantly better than chloroquine, except the octyl and decyl esters which were less active. The reason for the low activity could be ascribed to the fact that these two esters are both water immiscible oils, leading to solubility problems. The ethyl, butyl, phenyl and *p*-nitrophenyl esters all had similar IC_{50} values making their activity similar. The lowest IC_{50} value was displayed by the butyl ester with a value of 3.2×10^{-3} μM.

The poorest activity was recorded by the two oils, the octyl and decyl esters, with IC_{50} values of 38×10^{-3} μM and 90.2×10^{-3} μM respectively. All other compounds showed less antimalarial potency against the D10 strain compared with the other reference drug dihydroartemisinin, except the butyl ester. The butyl ester **12** displayed activity comparable to that of DHA (IC_{50}; 3.2×10^{-3} μM versus 3.8×10^{-3} μM), and is thus worthwhile being further investigated in terms of pharmacokinetics in order to determine its half-life. Statistically it is impossible to make structure-activity relationship (SAR) deductions from the data received as the number of compounds in the series is too small.

The butyl (**12**) (IC_{50} = 3.2 μM), 4-nitrobenzyl (**16**) (IC_{50} =15 μM), 2-(acetyloxy) acetyl (**17**) (IC_{50} = 8.6 μM), and 2-phenylacetyl (**18**) (IC_{50} = 12.4 μM) esters showed on a 0.05 level statistically significantly better activity against the chloroquine sensitive D10 strain of *Plasmodium falciparum* than chloroquine itself while the decyl ester (**14**) (IC_{50} = 90.2 μM) was statistically significantly less potent. The activity of the octyl (**13**) (IC_{50} = 38.0 μM) and benzyl (**15**) (IC_{50} = 25.7 μM) esters did not differ from that of chloroquine. In comparison to dihydroartemisinin the propyl (**11**) (IC_{50} = 24.1 μM), octyl (**13**) (IC_{50} = 38.0 μM), decyl (**14**) (IC_{50} = 90.0 μM), and benzyl (**15**) (IC_{50} = 25.7 μM) esters proved to be statistically significantly less potent than DHA while the activity of the butyl (**12**) (IC_{50} = 3.2 μM), 4-nitrobenzyl (**16**) (IC_{50} =15.3 μM), 2-(acetyloxy) acetyl (**17**) (IC_{50} = 8.6 μM), and 2-phenylacetyl (**18**) (IC_{50} = 12.4 μM) esters did not differ from that of DHA.

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CHAPTER 1

INTRODUCTION AND PROBLEM STATEMENT

1.1 Introduction

One of the deadliest diseases known to man from the beginning of time, and still is, malaria. In conjunction with Aids and TB, it remains one of three major worldwide communicable diseases (Lewinson & Strivastava. 2008). It is a protozoan disease that is transmitted to humans via the bite of the female Anopheles mosquito. P.falciparum is the most prevalent, and deadliest of the malaria species. The others *include P. vivax, P. malariae and P. ovale* (Gkrania-Klotsas & Lever. 2007).

Previously widespread even in Europe, where it has been eradicated, the biggest infectious areas are in Africa, South-East Asia and South America. It is estimated that 10% of the almost 1 million child deaths in Sub-Saharan Africa can be contributed to malaria alone. Poor infrastructure, struggling healthcare and general poverty seem to compound the problem (Lewinson & Strivastava. 2008).The toll that it has on Africa especially, is of major concern. The exact incidence and prevalence is difficult to gauge, but the general consensus is that approximately 270 million people suffer from malaria every year. Although human interventions like vector control, treatment and mosquito nets have shown some promise, antimalarials still remain the first choice of malaria control (Ashley *et al*. 2006).

The most critical problem faced in the treatment of malaria, is the widespread and emergence of parasitic resistance to antimalarials (Woodrow *et al*. 2005). The impact is worldwide and of great concern. Especially in Africa chloroquine (CQ) resistance has long been underestimated. The impact of resistance is vast, as CQ is the only drug recommended for first line treatment in most countries (Snow *et al*. 2002).

The discovery of new and effective antimalarial treatment is thus of the utmost importance in the struggle to conquer this disease. Nearly all currently used antimalarials were developed over 30 years ago, although the spread and mortality of the disease has increased (Stratton *et al*. 2008). There is now however, a drug that shows promise in addressing this problem. Artemisinin (or qinghaosu) is the API (active pharmacological ingredient) in *Artemisia annua*, or sweet wormwood as it is also known. Its structure was isolated in 1972, and is completely

unique compared to other antimalarials. Artemisinin is a sesquiterpene lactone, with an endoperoxide bridge, which is essential for its activity. Artemisinins exhibit a quick onset of action, and is highly effective against the blood stages of malaria infection. It also shows significant efficacy against the various other stages of *P. falciparum* (Golenser *et al*. 2006). However, the use of such endoperoxides is restricted by their poor oral bioavailability, a short plasma half-life (30 minutes) and the high rate of recrudescent infections when used as monotherapy in short-course treatments, even though these drugs have a rapid onset of action and low reported toxicity (Gupta *et al*. 2002).

1.2 Aim and objectives of the study

The aim of this study was to synthesise a series of esters of the antimalarial drug artemisinin, to determine their physicochemical properties and to evaluate their antiplasmodial activity against strains of *Plasmodium falciparum*.

In order to achieve this goal, the following objectives were set:

- Synthesise ester derivatives of artemisinin and confirm their structures using various analytical techniques.
- Experimentally determine and evaluate the relevant physicochemical properties such as the aqueous solubility and the partition coefficient for the synthesised artemisinin ester derivatives and to compare the experimental aqueous solubilities and the partition coefficients of the synthesised esters to that of the known antimalarial dihydroartemisinin.
- Evaluate the *in vitro* antimalarial activity of the artemisinin esters against the chloroquine sensitive D10 strain of *Plasmodium falciparum* in comparison with that of the reference drug dihydroartemisinin.

2.1 Malaria and antimalarial compounds

2.1.1 Introduction

Malaria is a major health problem, and the most important vector borne disease in the world. The prevalence and spread of malaria is so great, that 2,4 billion, or at least 40% of the worlds entire population is exposed to malaria in some degree (Crawley & Nahlen. 2009). There seems to be some discrepancy between figures on malaria infection, depending on the type of research methods used, as well as the scope of the area. Generally, it is accepted that 350-500 million people worldwide will be affected with malaria, causing mortality in over 1 million of these cases (WHO. 2006). Human intervention to eradicate the disease has failed, and it is thought that malaria is endemic in 88 countries. That translates to over 25% of the entire land mass of the globe (Gkrania-Klotsas & Lever. 2007*). P. falciparum* dominates Africa, mostly south of the Sahara, and Haiti. On the other hand *P. vivax* is seen more commonly in Latin America, South East Asia and Northern Africa. *P. malariae* is found in small numbers in most endemic areas, and *P. ovale* is rarely found outside of the African continent. *P. falciparum* has by far the highest mortality rate (Crawley & Nahlen. 2009). Most of these deaths are attributed to people living in endemic areas, although "travellers' malaria" has increased drastically over the recent years. After visiting endemic countries, approximately 25 000 travellers will contract malaria each year, with 100 of those fatal. Potentially, it is completely avoidable that any of these travellers should die (Ashley *et al*. 2006).

There are various factors which contribute to these dramatic numbers of infection and mortality. Global warming and the resulting climate change increase the epidemiological area of malaria (Stratton *et al*. 2008). Socio-economic factors like poverty, poor healthcare systems and infrastructure also contribute greatly to the number of infections. Population movement and even some water development projects all contribute to the spread of malaria. As malaria depends on humans as a carrier, limiting transmission also combats the spread of this disease. It cannot spread to any newcomers if a viable infected host is not in the population.

Malaria causes mortality through one of three ways: repetitive malaria infection which over time causes serious anaemia, an acute case of infection, and still-birth because of maternal malaria (Crawley & Nahlen. 2009). The biggest contributor to the high rate of mortality through malaria is drug resistance (Winstanley. 2000). *P. falciparum* is the most lethal form of malaria, compared to the other three strains*, P. ovale, P. malariae and P. vivax* (de Ridder *et al*. 2008). Chloroquine (CQ) drug resistant *P. falciparum* in Africa is of grave concern (Stratton *et al*. 2008). To date, studies have concluded that CQ resistance has resulted in a

4-8 fold increase in mortality. Increased mortality because of malaria infection can be directly linked to CQ resistance (Lewison & Srivastata. 2008). However, none of the current antimalarial prophylactic treatments are 100% effective (Gkrania-Klotsas & Lever. 2007).

Newer and more potent antimalarials that *P. falciparum* has not yet built up resistance to are of pinnacle importance to effectively combat this global scourge. Using a treatment with a different mechanism of action to the current drugs is one avenue to explore. Combination therapies like Artemisinin Combination Therapies, or ACTs, seem to be the way forward (de Ridder *et al*. 2008) in this regard. With the emergence of resistance, especially multi-drug resistance, artemisinin, and its more potent derivative, dihydroartemisinin are becoming more and more important in the fight against malaria (Gordi & Lepist. 2004). Although continued research to develop a viable vaccine is ongoing, chemotherapy continues to be the only treatment option (Gelb. 2007). The treatment policy adopted by some countries is no longer effective. Research and development into more effective drugs, like the artemisinins should be addressed (Geurin *et al*. 2002).

2.1.2 The burden of Malaria in Sub-Saharan Africa and South Africa

Malaria as an infectious disease, affects Africa in extreme measures (Plowe. 2008). The intensity in which it presents itself is usually double or four times that of other endemic areas globally. Africa also has the highest incidence of child mortality (0-5 years) than any other area (Alles *et al*. 1998). The problem is that for historical and operational reasons, most of Southern-Africa has done without any structured antimalarial plan (Alles *et al*. 1998).

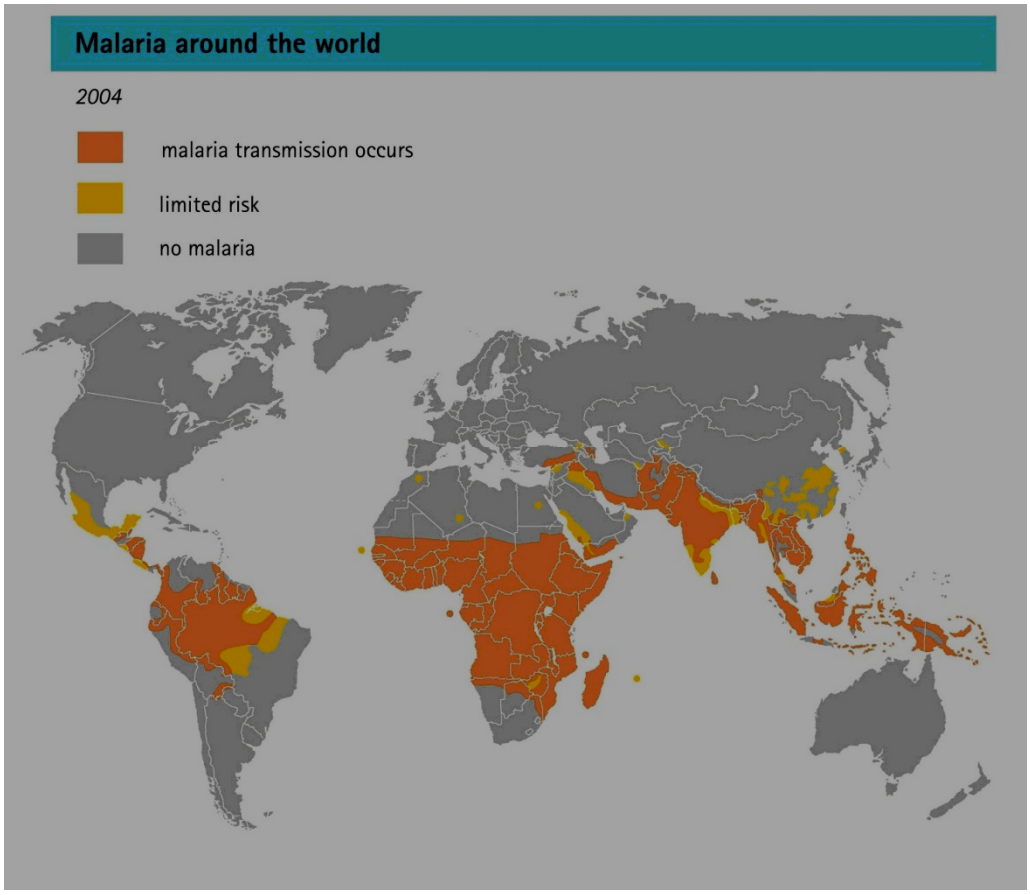


Figure 1.1: Malaria around the world (kevbrick.info/img/malaria).

South Africa as a country, has tried to implement control strategies over the years, as the spread of the disease is increasing.

Almost 10% of South Africa's total population of 49 million is at risk of contracting malaria, which translates to almost 5 million potential cases in endemic areas. In South Africa malaria is confined to low lying areas (below 1000 m) and mostly the north-east and tropical regions. The three provinces where malaria is an endemic because of geological factors are Kwazulu-Natal, Mpumalanga and the Limpopo (Figure 1.2). Cases of malaria have been reported as far as the Northern Cape and North-West provinces, although these seem to be limited to travellers and/or focal transmissions (Steyn *et al*. 2010). Malaria occurs mostly during the summer months because of seasonal rainfall, occurring from May –October (www.doh.gov.).

At this point in time malaria in South Africa is relatively under control, with little mortality compared with the rest of Southern Africa. This is ascribed to better control measures, treatment guidelines adhered to and strategies in line with global policy. South Africa as a whole has malaria under control because of effective management, funding and adequate research, and mostly through academic institutions (Tren & Bate. 2004).

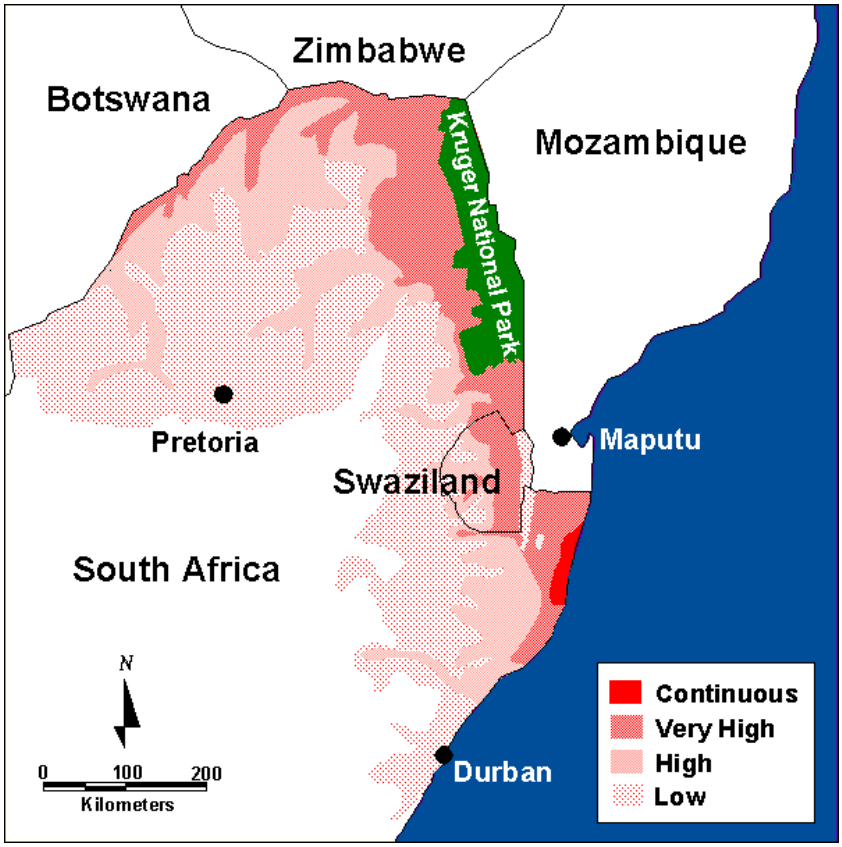


Figure 1.2 Malaria areas in South Africa (www.malaria.org.za.)

2.1.3 Epidemiology of Malaria

2.1.3.1 History and causes

The Greeks and Romans, as far back as the 6th century BC discovered the association between stagnant swamps and fever. This led to the drainage of these swamps, which in turn led to improvement of health and the increase in agricultural production. It led to various communities in Europe adopting the same strategy to the same affect. Although the strategy helped bring down the infection rate, the direct link was never established until the discovery of the mosquito as the vector of malaria (Konradsen *et al*. 2004).

The problem is that for historical and operational reasons, most of Southern-Africa has done without any structured antimalarial plan (Alles *et al*. 1998).

Malaria is caused by the protozoan *Plasmodium,* which is transmitted to humans when the female *Anopheles sp.* mosquito takes a blood meal from a human. She injects sporozoites directly into the circulation, which are then swiftly carried to the liver where they start to reproduce in a matter of minutes. This is the start of malaria infection (Ashley *et a*l. 2006).

2.1.3.2 Prevalence and distribution

Malaria distribution is heavily subjective in terms of numerous variables in data. Global warming and the resulting climate change, population movement and drug resistance all play major roles in the spread of malaria. These elements also influence the global disease distribution (CDC. 2004b). Malaria is a worldwide burden and is especially concentrated in the tropical areas of sub-Saharan Africa as shown by Figure 1.3.

A third of the worlds population live in areas where malaria is endemic, an estimated one billion people carry the protozoan at any given time. Africa is the hardest hit, with cases and numbers estimated at anything from 250 - 450 million infections annually. Mortality in a global context, is calculated at 500 000 to 3 million a year. Specific statistics are hard to come by as no proper and long term research has been done. Malaria is a difficult disease to be precise on, as most deaths occur in rural areas, where patients die at home (Geurin *et al*. 2002).

Countries where malaria is endemic are among the poorest in the world, and in most cases, income is only a third of non-endemic countries (Geurin *et al*. 2002).

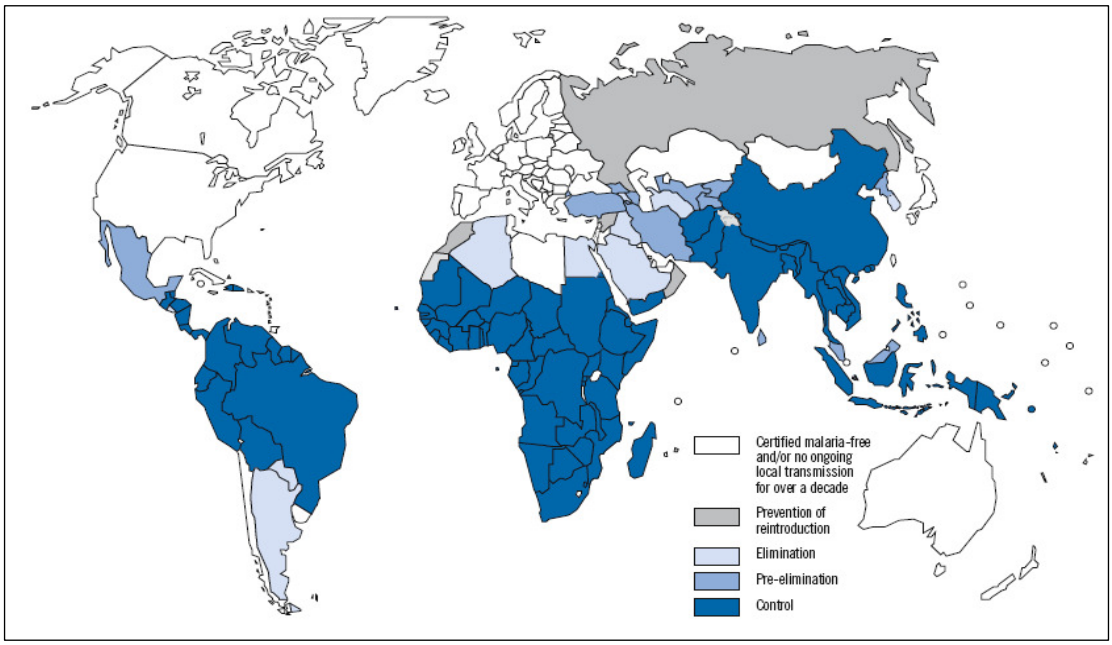


Figure 1.3 Phases of malaria distribution worldwide (www.mmv.org.)

2.1.4 Lifecycle of *P. falciparum*

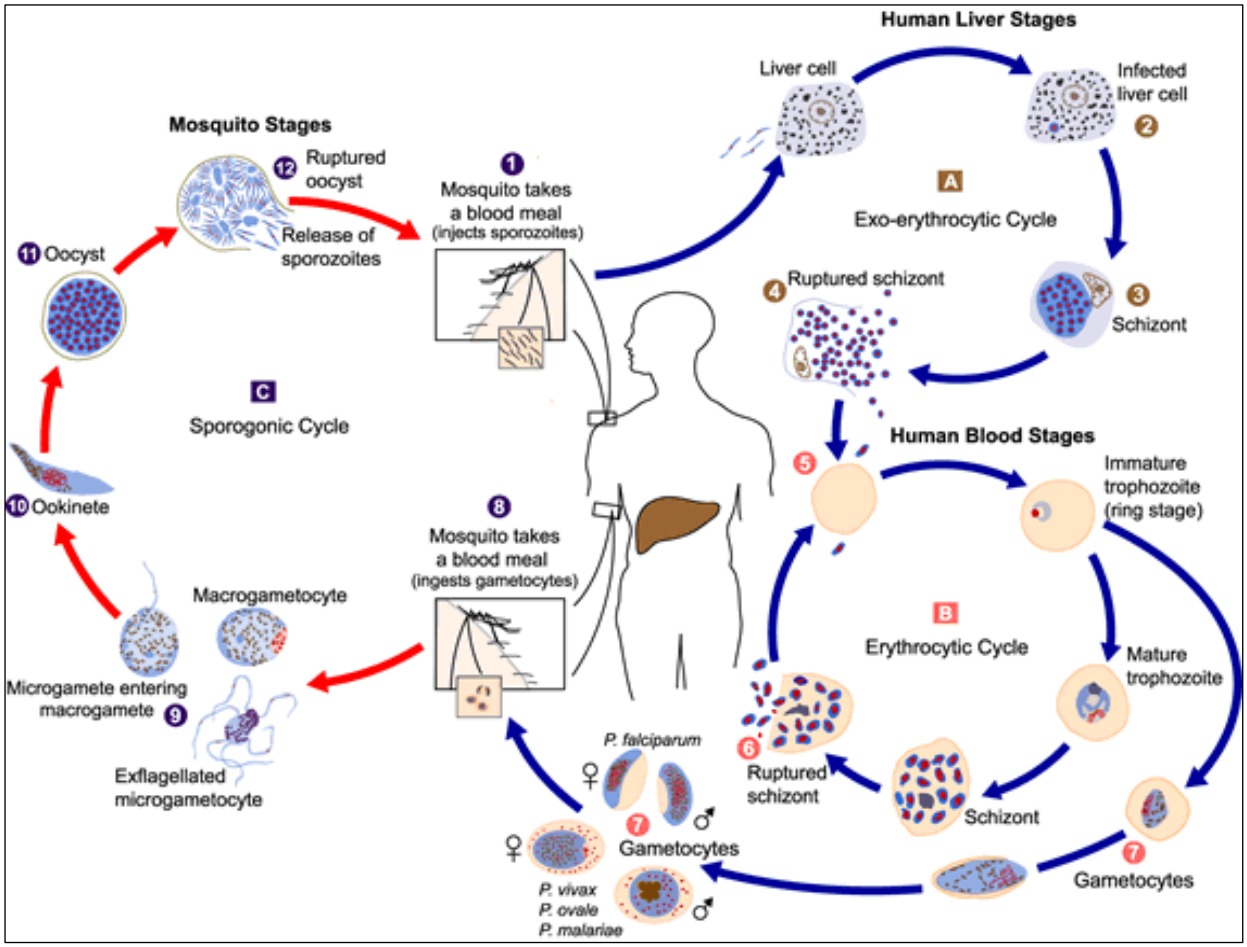


Figure 1.4 Lifecycle of *P. falciparum* (www.dpd.cdc.gov/dpdx)

2.1.4.1 Stages of infection

2.1.4.2 Pre-erythrocytic schizogony

After the female *Anopheles* mosquito takes a blood meal from a human, sporozoites enter the human body (1). Subsequently, these sporozoites are directly transported by hepatic circulation to the liver, where they penetrate liver cells, also called hepatocytes (2). After hepatocytic penetration has occurred, the asexual proliferation begins. It is in the hepatocytes that they develop into exo-erythrocytic schizonts (3). Herein lays thousands of merozoites. After a period of time the proliferation of thousands of merozoites causes an increase of pressure inside the hepatocytes, and the cell ruptures and the merozoites are

released into the systemic circulation where they invade erythrositic cells (4). The pathophysiology of malaria only starts to present at this stage, where the parasite leaves the liver and starts to infect the erythrocytes (Quast. 1999).

2.1.4.3 Erythrocytic schizogony

After the release from the liver, the merozoites infect the red blood cells (5) and either form asexual trophozoites, or sexual gametocytes through schizogony (6). This in turn causes the further proliferation of the asexual trophozoites, causing the erythrocytes to rupture, releasing more merozotes into the circulation to further infect other red blood cells (7) (Wiser. 2008).

When the red blood cells ruptures, along with the merozites, other antigens and toxins are released. This leads to the intermittent fever that is common in malaria infection (Steyn *et al*. 2010).

2.1.4.4 Sporogony

Both sexes of the gametocytes are ingested by the mosquito during a blood meal. This is the last stage in the cycle of malaria, and does not occur within the human host, but within the female *anopheles* mosquito herself (8). This is also referred to as the intermediate host phase. After ingestion by the mosquito, the gametocytes mature into micro- and macro-gametes (9). A zygote is formed from the fertilised female macrogamete. The stimulation of the zygote leads to the formation of an ookinete (10). The ookinete then penetrates the gut wall of the infected mosquito, forming an oocyst (11). Reproduction of sporozoites occur within the oocyst, and when maturity is reached, it bursts and releases the sporozoites back into the mosquito (12), where it travels up to the salivary glands, where it is transferred back into a human to complete the lifecycle of the parasite (Steyn *et al*. 2010).

2.1.5 Signs and symptoms of malaria infection

2.1.5.1 Uncomplicated malaria

In the beginning of infection, malaria symptoms are non-specific, almost akin to that of influenza. These include, fever, chill, headache, loss of appetite and even body aches. Fever patterns are common in malaria infection, with fever spikes every two days in *P. falciparum, P. vivax and P. ovale* malaria (tertian fever). In *P. malariae* infection, quartan fevers are more common, or fever every three days. These tie in with the specific lifecycle of each. Fever spikes are rarely seen, and is usually the result of untreated malaria infection. *P. falciparum* has the ability to progress extremely quickly into severe malaria (Ashley *et al*. 2006).

2.1.5.2 Severe malaria

Manifestations of severe malaria are mostly associated with a broad spectrum of prostration and unrousable coma. The mortality rate of cerebral malaria patients is 20% in non-pregnant patients and 15% in children. Symptoms of cerebral malaria include;

- Unrousable coma
- Generalised convulsions
- Nonocytic anaemia
- Hypoglycemia
- Metabolic acidosis with respiratory distress
- Fluid and electrolyte disturbances
- Acute renal failure
- Acute pulmonary oedema
- Circulatory collapse
- Jaundice
- High fever
- Hyperparasitemia
- Prostration

In addition to the above mentioned symptoms, there is also a clinical manifestation after treatment for malaria called Post Malaria Neurological Syndrome (PMNS). Obvious symptoms include confusion, seizures and even tremors. The incidence of PMNS increases with mefloquine treatment and is therefore not recommended for use against severe malaria (Ashley *et al*. 2006).

2.1.6 Diagnosis

Access to good medical care and facilities is very limited in many areas where malaria is endemic. In places where medical facilities do exist, they mostly lack the necessary equipment needed for laboratory diagnosis. This creates a situation where malaria diagnosis is mostly done on a clinical evaluation or self-diagnosis. This means the patent tells the clinician they think it is malaria, or the clinician does a differential diagnosis as blood tests are

not always available. Clinical diagnosis however is very inaccurate at the best of times, as pyrexia is a common symptom even in areas where malaria is endemic. Many other diseases also present with fever and chills as an initial symptom. Symptoms of uncomplicated malaria are non-specific and overlap with other febrile infectious diseases.

The major limiting factor for malaria diagnosis is cost effectiveness. Should diagnosis be done on a clinical or parasitological basis? This is especially applicable in areas where malaria is endemic, as the population is infected with malaria at any given time, although clinical symptoms and pathology is absent. Many of these patients will test positive for malaria, but no symptoms are present. Microscopy is usually a luxury that most settings in Africa do not have, and when present the quality of the microscopy is poor. As a result, antimalarials are prescribed to treat any fever, irrespective of proper diagnosis (Geurin *et al*. 2002). This adds significantly to the problem of drug resistance. The need for rapid, cheap and accessible malaria diagnostic methods are thus just as important as vector control and treatment.

The chance of malaria infection should always be considered in any individual who travelled (even in transit) or lived in a malaria region. Diagnosis should always be a first consideration for idiopathic pyrexia, even if there is no known exposure. Cases in the US with no clear link to malaria have been documented, as well as nosocomial infection of malaria in the UK (Gkrania-Klotsas & Lever. 2007)

There are four specific diagnostic methods:

- Thick and thin light microscopy
- Fluorescent microscopy
- Polymerase chain reaction (PCR) based techniques
- Antigen detection models

2.1.6.1 Thick and thin light microscopy

This is the gold standard in malaria diagnosis as it is easy and cheap, as well as accurate. A thick and thin blood smear is taken and examined under a light microscope. Giemsa stains are usually used, although Field and Wright stains may also be used (Ashley *et al*. 2006). As the parasite lifecycle is based on a specific time period, smears should be taken 6 and 12 hours apart on consecutive days, for up to a week to assure accuracy. Thick smears are used for initial diagnosis and a thin smear for more detailed characteristics allowing for the

quantification of parasitised cells. This is a clinical indication as to the severity of infection. Counting the blood cells infected with asexual parasites in relation to a given number of white blood cells enables a clinician to evaluate parasite densities. This is then used to make a diagnosis and prognosis, as well as to choose the correct protocol for treatment (Gkrania-klotsas & Lever. 2007).

2.1.6.2 Fluorescent microscopy

The best known method is using an acridine base to stain the nucleus of the parasite, called Quantitive Buffy Coat technique, or QBC. Compared to normal light microscopy, it is 88 to 98% sensitive and 58 to 90% specific. The problem with this technique is that a specially trained technician is required, and with parasite counts lower than 100 parasites/µl the accuracy and sensitivity drops dramatically.

2.1.6.3 PCR based techniques

There are various PCR based techniques available and some recently developed techniques include real-time PCRs. PCR are used to detect DNA, mRNA or small sub-unit rRNA that is specific to *P. falciparum*. These tests are used as follow up techniques for diagnosis and treatment. PCR techniques are extremely sensitive compared to other tests. This is especially useful in cases of low parasitemia and early infection. The drawback of PCRs, however, is that they are very labour intensive, expensive, require expertise and specialised equipment. PCRs can be performed in one hour.

2.1.6.4 Antigen Detection Methods

These are primarily field techniques developed for rapid and easy detection. Recently they have become commercially available, using a dipstick or test strip with monoclonal antibodies designed to detect parasite antigens (Geurin *et al*. 2002).

A blood sample is taken by means of a finger prick, and tested for a parasite antigen, either the histamine rich protein 2 (HRP-2) that is only present in *P. falciparum* infection, or the parasite lactate-dehydrogenase (pLDH) antigen, present in all four types of malaria. These tests have a low sensitivity under 100 parasites/µl and are relatively expensive. These rapid diagnostic tests are hampered by their design limitations and quality assurance, but are easy and straight forward for evaluation by trained personnel. If these factors can be improved, new generation rapid tests may be useful as a diagnostic tool adjunct to light microscopy,

especially in regions without major expertise in tropical medicine (Gkrania-Klotsas & Lever. 2007).

Dipstick antigen capture assays are recommend for specific conditions where *P. falciparum* is suspected, in epidemics and emergencies, mobile clinics, where laboratories are ill equipped and where first line treatment is very expensive (Geurin *et al*. 2006).

2.1.6.5 Automated systems

An example of these systems is the Cell-Dyn 400 that has been designed for various purposes. The analyser is sensitive and accurate, with a 96% specificity and 62% sensitivity for malaria in general, and a 96% sensitivity *in P. falciparum* infection with patients with a parasitic count lower than 0.5%. Although very expensive and not available in most endemic areas, the advantage is that most advanced pathology laboratories and centres already have these systems in place and require no additional equipment (Gkrania-Klotsas & Lever. 2007).

2.2 Current antimalarial drugs

Presently, none of the current prophylactic antimalarials are 100% effective. The current research to address this problem, also seems to be left far behind by many other, and dare I say, “less important fields”. Any treatment in the medical field should be done with due consideration to the “Risk vs Benefit” principle. The risk relies on many factors, including duration in endemic area, degree of exposure and the occurrence of resistance in that specific area (Gkrania-Klotsas & Lever. 2007).

Treatment is usually given to anyone with symptomatic malaria. In the past, studies have shown that malaria treatment is usually based on efficacy and toxicity. The first antimalarial used, was derived from the Concha tree, namely quinine. These drugs were highly effective, until the first signs of clinical resistance were reported. After the resistance to quinine spread rapidly, chloroquine (CQ) was introduced just after World War II, as it was better tolerated. To combat the hypnozoitic phase, primaquine was also added to the treatment regime. A chloroquine analogue, amodaquine was also developed in the 1940s. Later it emerged that amodaquine led to neutropenia and hepatitis, and its use was discontinued as prophylactic drug. The advent of CQ resistance posed many problems, as well as the side affects of the other drugs in use. Fortunately, it led to the development of mefloquine and halofantrine. The use of doxycycline as a prophylactic was found by serendipity (Berman. 2004).

The antimalarials can be divided into 5 different classes:

- Quinolines and arylaminoalcohols
- Antifols
- Hydroxynapthaquinones
- Antibacterials
- Artemisinin and derivatives

2.2.1 Quinolines and arylaminoalcohols

2.2.1.1 Quinine

Quinine (1) is extracted from plant material and therefore relatively expensive compared to other drugs. Quinine is used in areas where CQ resistance is prevalent, and although it has a small therapeutic index, it is quite reliable against resistant *P. falciparum*. Quinine is mainly used globally as a parental drug in severe falciparum malaria. In certain countries it is also used for uncomplicated malaria. As an in-hospital treatment, quinine is quite successful, but because of its awkward dosage regimen, administration and symptomatic toxicity, it is unpractical for unsupervised outpatient therapy. Absorption Dissolution Metabolism Elimination (ADME) profiles show that a very small percentage of quinine is left unchanged and excreted, whereas most of the drug undergoes extensive hepatic biotransformation. During administration to the patient, blood glucose levels should be closely monitored, as quinine stimulates the secretion of insulin from the pancreatic islets.

When quinine is administered properly and within a strict dosage regimen, it is a relatively safe drug to use. If overdoses occur, adverse effects might include hypotension, seizures, heart arrhythmias and visual impairment. Case studies have shown that activated charcoal can decrease the amount of quinine in systemic circulation. Allergic reactions and hypersensitivity to quinine are rare, but rashes, leukopenia, DIC, bronchospasm and like pyrimethamine, pancytopenia have occurred in isolated cases. Some of the symptomatic toxicity like ototoxicity and GI disturbances does not need a dosage reduction, Quinine has a long list of drug interactions, and should always be given with due diligence. It is however safe to use in pregnancy, unlike most other antimalarials (Winstanley. 2000).

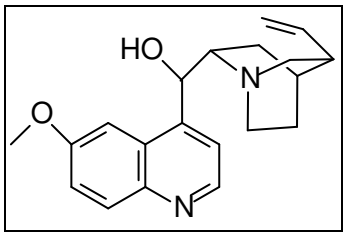


Figure 1.5 Quinine (1)

2.2.1.2 Halofantrine

Halofantrine (2) is similar to CQ, in that is has the same mechanism of action. It is usually given as treatment for multi-resistant malaria in uncomplicated cases. It does however, lead to the lengthening of the QT interval, which is a serious adverse effect. It is linked to ventricular arrhythmias, and has been associated to sudden deaths in patients. Its effect could be worsened by the combined administration of mefloquine. Therefore, halofantrine should in no instance be given to patients with pre-existing cardiac conditions, or patients with an already long QT interval. The absorption of halofantrine is increased by the intake of fatty food (Winstanley. 2000).

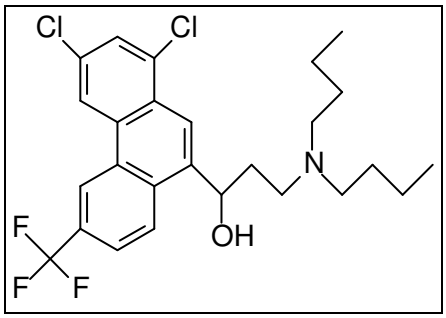


Figure 1.6 Halofantrine (2)

2.2.1.3 Chloroquine and congeners

Chloroquine (CQ) (3) is still the most widely used antimalarial in the world today, despite its wide-spread resistance. The proposed mechanism of action for CQ is that it binds to the parasitic ferriprotoporphyrin IX, where it antagonises the polymerisation of this toxic metabolite and creates heamozoin inert crystals. CQ is most commonly used for all four strains of uncomplicated malaria. Its use as parenteral first line treatment for severe malaria however has declined because of the prevalence of resistance (Winstanley. 2000).

When CQ is administered intravenously, is it very important to infuse it slowly, and to dilute it in a crystalloid. It is well tolerated by the general population, except for dark skinned races, where pruritis is a common adverse effect (Gkrania-Klotsas & Lever 2007). The problem is that for historical and operational reasons, most of Southern-Africa has done without any structured antimalarial plan (Alles *et al*. 1998). When the concentration of CQ in the plasma becomes too high, it leads to side effects like nausea, dizziness, malaise, headache and dysphasia to name but a few. In cases where CQ levels are very high, ECG abnormalities occur, as well as hypotension, two very serious adverse effects. In rare cases, bleaching of the hair, skin pigmentation and photosensitivity have been reported. It can also exacerbate epilepsy in some patients. It must be given with careful consideration, as its plasma half-life is 1-2 months. Either CQ or hydrochloroquine may worsen psoriases. Retinopathy is a risk, but is unusual in very small doses (Gkrania-Klotsas & Lever. 2008).

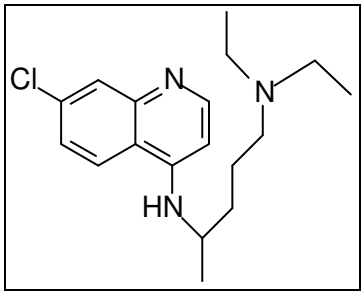


Figure 1.7 Chloroquine (3)

2.2.1.4 Mefloquine

As with the above mentioned halofantrine, mefloquine (4) also shares its mechanism of action with CQ. Absorption through the GI tract is sufficient, and it possesses a very long half-life (15-33 hrs), which makes it an ideal candidate for combination therapy with drugs that have a short half life. Aplastic aneamia and severe skin rashes have been reported, although serious idiosyncratic adverse reactions are rare. Dizziness and GI disturbances are common, like in most antimalarial agents, but these are mostly dose dependant symptoms. Acute encephalopathy, psychoses and extreme seizures have also been reported, but only in rare occasions. Mefloquine has however also been linked to an increase in stillborn babies if the mother was treated with mefloquine while pregnant (Winstanley. 2000)

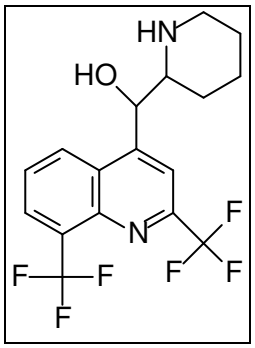


Figure 1.8 Mefloquine (4)

2.2.2 Antifols

2.2.2.1 Proguanil

Proguanil (5) was formulated to adhere to US and European standards. In recent studies, proguanil was tested in a placebo controlled trial in Zambia in patients for ten weeks and only few side effects were noted. The use of proguanil resulted in fewer side effects than mefloquine, which was used as a control in this study (Berman. 2004).

Proguanil is a biguanide and acts like a prodrug for an active metabolite, which also inhibits DHFR. In general it is a weak antimalarial, and as a monotherapeutic agent resistance is built up very quickly (Ashley *et al*. 2006). Both proquanil and its chlorinated counterpart, chlorproguanil, are metabolised by the cytochrome enzymes in the liver, to produce their active metabolite, cycloguanil and chlorocycloguanil. Both have the same mechanism of action in that they both inhibit parasitic dihydrofolate reductase (DHFR). Genetic polymorphism plays a role in the metabolism of these two drugs, although concrete clinical evidence is lacking because of this effect that proguanil, and not cycloguanil is used in combination with atovaquone. Specific adverse effects of proguanil use are rare, although mouth ulceration and GI intolerance have been recorded. Serious side effects however, are very unusual (Winstanley. 2000).

Chloroproguanil-dapsone is the latest addition to this class of drugs, and has been registered as Lapdap. As a combination therapy, it has been shown to be more effective against *P. falciparum* than sulphadoxine-pyrimethamine (SP). In this combination the most common side effects are haemolysis and methemglobinaemia, but are mostly attributed to dapsone. The exact cause of resistance to DHFR resistance is unknown, but it has to do with sequential accumulation of four point mutations, that effectively renders antifolates useless. Resistance has been found in South-East Asia, South America and Southern Africa (Ashley *et al*. 2006).

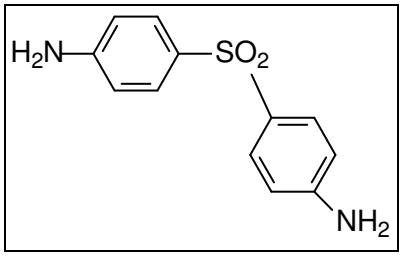


Figure 1.9 Proguanil (5)

2.2.2.2 Pyrimethamine combinations

The mechanism of action for pyrimethamine (6) is that it is a selective inhibitor of parasitic DHFR. It is not used as a monotherapy, and is only available as a synergistic combination with a sulfonamide like sulfadoxine in Fansidar. It is most commonly given as an oral treatment, although an IM formulation is available for patients with acute nausea. The half life for elimination is about 100 hours. Adverse effects are relatively rare, but suppression of the bone marrow has been documented, and may lead to transient pancytopenia or agranulocytosis.

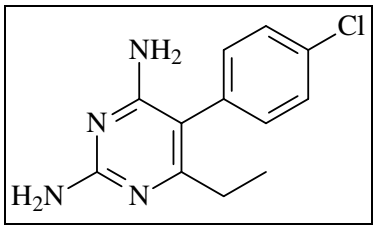


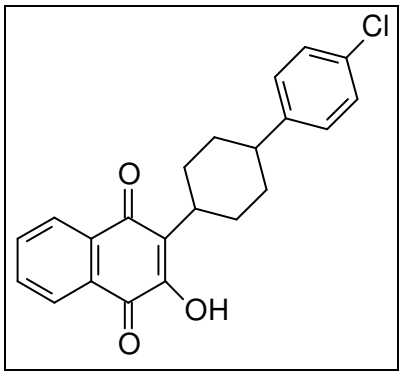
Figure 1.10 Pyrimethamine (6)

2.2.3 Hydroxynaphtalenes

2.2.3.1 Atovaquone

The mechanism of action for Atavaquone (7) is thought to be the inhibition of mitochondrial respiration in the parasite. This drug is usually given with proguanil for its synergistic effect, as mentioned above (Winstanley. 2000). Used alone, atovaquone resistance develops alarmingly quickly. The base for this quick build up of resistance is a single point mutation on the parasytic cytochrome gene (Ashley *et al*. 2006). Absorption of the drug in the gut is variable, but does increase with concurrent food intake. The metabolism of the drug is

simple, as it is eliminated unchanged through the bile. The one predominant character of atovaquone is that has an extensive half-life, 50 – 70 hours. Atovaquone is well tolerated, with just a few regular side effects being reported. Mild rashes are clinically observed, although occasionally severe. Drug induced fever has also been reported. Like most anti-malarials, it does also cause GI discomfort. CNS disturbances are also common, and it is because of this that treatment during pregnancy with atovaquone is not recommended (Winstanley. 2001). It should under no circumstance be given to infants that are >11kg, breastfeeding mothers or patients with severe renal impairment (Gkrania-klotsas. 2006).



(7)

Figure 1.11 Atovaquone

2.2.4 Antibacterials

Clindamycin and erythromycin are both weak and slow acting antimalarials, and are not recommended as monotherapy against malaria. Doxycycline is an option, but because photosensitivity is a common side affect, it might pose problems as malaria is most prevalent in the tropics and around the equator. As with any antibacterial, GI disturbances like nausea, diarrhoea and vomiting is common. Less frequent adverse reactions include dry mouth stomatitis, glossitis and oesophageal ulceration. Increased *Candida* susceptibility, especially in woman may also occur. In severe cases, pseudomembranous colitis, hepatotoxicity and pancreatitis have also been reported. The tetracyclines are useful in combination therapy with quinine or one of the artemisinins, except for the fact that it is contra-indicated in pregnant woman and children as it causes discolouration of teeth and bones, and can even cause fatal hepatotoxicity. In these cases it is safer to consider clindamycin as an option (Ashley *et al*. 2006).

2.2.5 Artemisinins

Artemisinin (10) was discovered in China during the 1970s, even though it use goes as far back as 2000 years as a cure for fever. It was found to be a highly effective antimalarial compound. Artemisinins combine potent and rapid onset of action with clinically absent resistance (Woodrow *et al*. 2004). With the emergence of drug resistance, artemisinins have become the first choice of treatment. Artemisinin, and its more potent derivative, dihydroartemisinin is converted primarily by hepatic metabolism by cytochrome P450, with a half life ranging from 0.5 – 3 hours after oral administration. The ester derivatives like artesunate (10a), and artemether (10b) and arteether (10c), the ester derivatives are all prodrugs of dihydroartemisinin (10d). Artemisinin and its derivatives have safely been used in the fight against malaria for over two decades now (Gordi & Lepist. 2004).

Artemisinins can be given to a patient through several routes of administration. Artesunate, the water soluble ester derivative can be given orally, intramuscular, intravenous or even rectally. The relatively short half life of artemisinin and its active metabolite, dihydroartemisinin confers the theoretical advantage it poses against other antimalarials in terms of resistance as the *P. falciparum* isn't exposed to artemisinin long enough to build up resistance. The disadvantage however of such a short half life, is the higher associated risk of recrudescent infections, especially when used as a monotherapy (Krishna *et al*. 2004).

Recently, research has been focused on the ester derivatives of DHA. Artesunate, a DHA ester has favourable physicochemical properties over DHA, with increased half-life and solubility. It is available for oral, rectal or intravenous administration. The rapid onset of action makes it an ideal candidate for patients with severe cerebral malaria (Barradell & Fitton. 1995). Parasitic clearance with artesunate is also much faster than the other non-ester artemisinin derivatives as showed in a recent meta-analysis. Recrudescence was also much lower when artesunate was added to monotherapy treatment (Adjuik *et al*. 2004). Singh et al proposed the synthesis of artemisinin ester derivatives that exclusively produces the α-isomer, which is the more potent and active isomer. The lipophilicity of ester derivatives (log P 5.85-6.95) are comparable to those of the ether derivatives (log P 5.51-6.91). Further research into ester derivatives is thus of great importance (Singh *et al*. 2008)

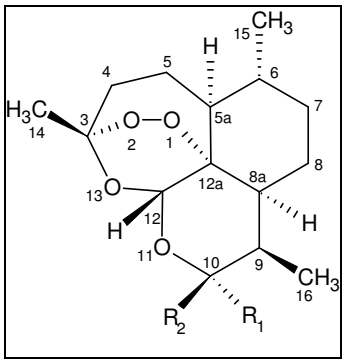


Figure 1.12 Artemisinin derivatives

Artemisinin	(10)
Artesunate	(10a): R_1 = H, R_2 = $OCO(CH_2)_2CO_2Na$
Artemether	(10b): R_1 = H, R_2 = OMe
Arteether	(10c): R_1 = H, R_2 = OEt
Dihydroartemisinin	(10d): R_1 = H, R_2 = OH

2.2.5.1 Mechanism of action

Various researchers consider that artemisinins themselves are not antimalarial per se, but act rather as prodrugs, requiring activation by either encounters with ferrous iron, or binding site activation (Krishna *et al*. 2004)

Artemisinin is a sesquiterpene trioxane lactone with an endoperoxide bridge, which acts as the pharmacophore (Haynes & Krishna. 2004). The yield from which artemisinin is extracted from the *Artemisia annua* plant varies greatly. Despite the growing reliance on artemisinin, and more specifically dihydroartemisinin prodrugs, their exact mechanism of action is still unknown. Various mechanisms have been proposed, and better insight and knowledge of these mechanisms are needed to target drug resistance, further drug research and to improve their judicious use.

2.2.5.1.1 Interference with plasmodial sarcoplasmic/endoplasmic Ca^{2+} - ATPase (SERCA)

Steric effects where shown to have an influence on the *in vitro* activity of artemisinin. The replacement of a methyl group at C3 of artemisinin and 10-deoxyartemisinin by much larger functional groups resulted in a decrease of activity. Drawing from these results, it was suggested that the molecule needs to be activated after binding to a specific site. As artemisinin is structurally similar to thapsigargin, an inhibitor of SERCA, it was hypothesized that artemisinins specifically inhibit *P. falciparum* SERCA. Inside the parasite, SERCA is responsible for the maintenance of calcium ion concentrations, which is important in the generation of calcium mediated signalling and the correct folding of post-transitional processing of proteins PfATP6, the only SERCA type calcium ATPase in *P. falciparum.*

Several studies show that artemisinin completely inhibited PfATP6 activity, and the inhibition was completely specific, not having an effect on other potential inhibition sites. Testing of DHA, as well as many other derivatives, showed a correlation between the concentration at which PfATP6 was inhibited, and the IC_{50} values. Based on the results from tests, conclusions were made that artemisinin binds to the same region in the protein as thapsigargin. This allows the peroxide bridge to be cleaved; forming carbon centred free radicals, leading to enzyme inactivation and death. This theory is also supported by the fact that certain point mutations on the SERCA decrease artemisinin activity *in vitro* (Golenser *et al*. 2006). There are many questions still posed to this mechanism of action, especially the role that ferrous iron plays in the activity of the drug and the specific interaction with the parasitic SERCA. Nevertheless, the mechanism is amenable, as long as further studies are done (Haynes & Krishna. 2004)

2.2.5.1.2 Interference with mitochondrial electron transport

Administration of artemisinin to particular monkeys that have *P. inui* resulted in mitochondrial size increase due to swelling. More recently a new hypothesis has come to light, and that is that artemisinin and its derivatives interfere, and are in fact also activated by components of the electron transport chain of the parasitic mitochondria*. S. cerevisiae*, a type of yeast, preferentially uses glycolysis to generate energy as long as a carbon based ferment source is available. This allows them to grow even when mitochondrial activity is minimal. Artemisinin added to *S. cerevisiae* grown on glycerol or ethanol strongly inhibited yeast growth. The addition of artemisinin also showed a depolarisation of the mitochondrial membrane.

In the mechanism proposed, artemisinin also causes local production of reactive oxygen species after activation. These factors combined may readily cause parasite death (Golenser *et al*. 2006).

2.2.5.1.3 Production of reactive species

It is also proposed that DHA kills malaria parasites by the formation of reactive species like free radicals that lead to the reduction of red blood cell anti-oxidants and glutathione. The basis of the heme activation is the supposition that artemisinin and the ferrous heme encounter each other in the food vacuole of the parasite (Haynes *et al*. 2006). Present in artemisinin and all its derivatives is the endoperoxide bridge, essential for anti-malarial activity. This was also proven by removing the additional oxygen, which led to inactivity. Based on the finding, it was proposed that the mechanism of action for the artemisinins involve free radicals due to the importance of the endoperoxide ring, mediated by iron. In the one hypothesis, it is suggested that the peroxide bridge is cleaved by divalent Fe^{2+} , the byproduct of hemoglobin digestion. Origin of the specific iron is debatable, although it was suggested that a cytosolic pool of iron was used by the parasite. This chelatable iron is involved in free radical production and therefore might activate artemisinin (Golenser *et al*. 2006).

2.2.6 Latest research – Artemisone and Malaria Vaccine

2.2.6.1 Artemisone: new artemisinin derivative

Current research on the artemisinins focus on artemisone, a new artemisinin derivative. It is an aminoartemisinin, and is currently in phase II clinical trials. It is stable at a neutral pH, and presents neurotoxicity in validated essays. Unlike other artemisinins, is does not need to be converted to DHA through the metabolism. It has enhanced pharmacokinetic properties compared to the other artemisinins in terms of half life, distribution and clearance. It has been proven to also be active against various other parasitic infections, and even displays promising anti-tumor properties. Further studies and research are underway (Haynes. 2009).

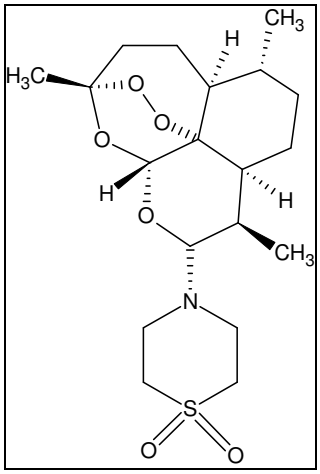


Figure 1.13 Artemisone

2.2.6.2 Malaria Vaccine

The possibility of a malaria vaccine in the future would greatly increase the global chance of eradicating malaria as a whole (Moorthy & Smith. 2007). Currently research into a malaria vaccine is ongoing. One candidate vaccine, RTS,S/AS02 shows promise. The mechanism of this particular vaccine is to induce immunity to a malaria antigen expressed in the liver stage of the infection and life-cycle. This aims to create sterilizing immunity to sporozoites before transformation to merozoites. The problem is that it does not provide complete protection, and studies are underway to evaluate its use in severe forms of malaria infection. No vaccine is thus presently available for the prophylaxis or treatment of malaria.

2.2.7 Treatment considerations when using antimalarials

It is usually impossible to measure drug sensitivity in a clinical setting, especially in an endemic area. The geographical origin and determination of species makes it easier to choose the correct treatment. Experimental treatment of malaria is a highly risky practice, but if the patient displays severe symptoms, even if the blood smear is consistently negative, treatment is justified. In all cases, even when there is no clinical evidence, it should be assumed that *P. falciparum* is present. As with any medical condition, a complete evaluation of risk vs benefit must always be undertaken before deciding on any course of treatment.

During the treatment of uncomplicated malaria, the primary goal is always to alleviate symptoms and to decrease potential morbidity. Radical cures are usually taken in non-endemic countries, but is unattainable in endemic areas due to cost. When treating severe malaria, the main objective is to save the patients life, and to lower the risk of sequelae. Plasma concentrations of the chosen drug must be achieved as rapidly as possible and with

a focus on safety as to ensure the rapid clearance of the parasites in the blood. When this has been achieved, supportive and symptomatic care can be implemented. Prevention of recrudescent infections and gametocyte eradication should only be focused on once the patient's life is no longer in danger (Winstanley. 2001).

2.3 Malaria drug resistance

2.3.1 Drug resistant malaria epidemiology

As there is currently no effective vaccine against malaria, treatment and prophylaxis rely completely on chemotherapeutic anti-malarial drugs. Resistance to these drugs is therefore an extreme concern for all endemic areas of malaria transmission (Hastings & Watkins. 2005).

P. falciparum sensitivity to anti-malarial treatment is most commonly determined by *in vivo* testing. The response of malaria to treatment is based on parasitic clearance, as well as the three degrees of resistance (RI, RII, RIII). These tests are applicable in areas where malaria is not endemic, but the problem becomes a little more complicated where malaria and malaria transmission is common.

There is a multitude of factors that influence the spread of resistance and the speed at which it develops. Malaria parasites as a species are not commonly mutagenic, and although natural gene mutations do occur in nature, it usually does so outside of drug effects. The various factors that contribute to drug resistance include the interaction of drug usage, physicochemical and pharmacokinetic properties of the drugs, human factors and vector/environmental factors. Most important is the maintenance of adequate drug concentrations in the systemic circulation of the patient treated. If complete parasitic clearance is not achieved it leaves the door open for the development of resistance. A sub-therapeutic level of optimal drug concentrations only kills off the weakest of the parasites, leaving the strongest to mutate and develop resistance (Wongsrichanalai *et al*. 2002).

Mutations that occur spontaneously to drugs are very rare, but once it occurs, there is a chance of specific selection. The latter occurs when a parasite that has built up some resistance comes into contact with a concentration of antimalarial drug sufficient to kill the susceptible but not the resistant parasites. If this happens the mutants will be selected and preferentially transmitted (Price & Nosten. 2001).

Drug resistant malaria, especially chloroquine resistant (a 4-aminoquinolone) *P. falciparum* was first observed about 50 years ago. Currently, CQ resistance is so prevalent that it has

almost become a redundant drug. *P. falciparum* is so adapt at developing a resistance, that most strains are widely resistant to most of the commonly used antimalarials, even SP and mefloquine. The resistance also develops at a very rapid pace, 10-15 years is enough. SP resistance is of the greatest concern, as it was the last of the cost effective, practical and widely used antimalarial used against CQ resistant *Plasmodium falciparum*. Artemisinin however, is a relatively new drug, with little clinical resistance observed as yet, making it very important in the fight against malaria (Wongsrichanalai *et al*. 2002).

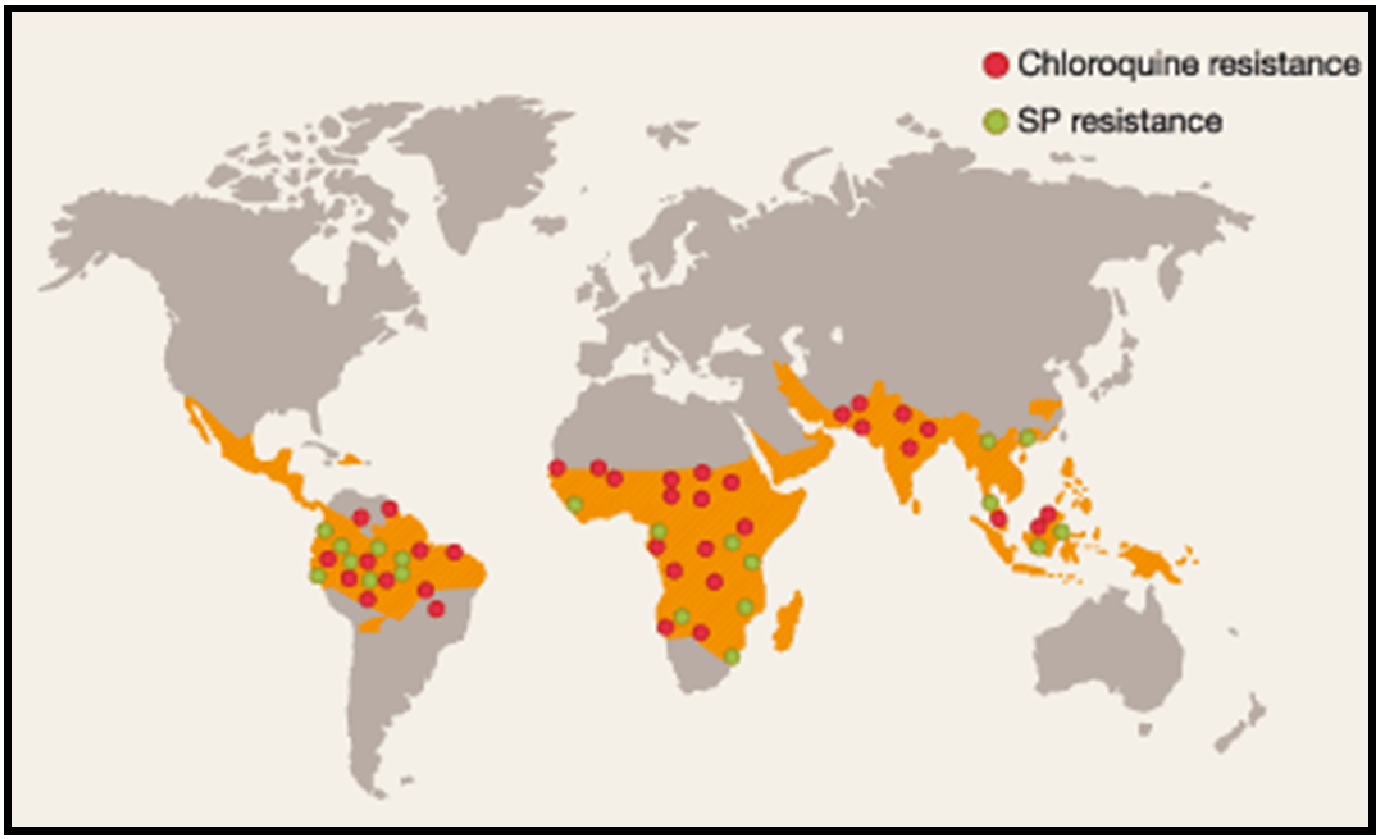


Figure 1.14: Global malaria drug resistance

Consequences of resistance

- Recrudescent infections
- Delay in initial therapeutic response
- Increased mortality
- More anaemia
- Increased gametocyte carriage
- Increased incidence of malaria
- Greater economic impact

Antimalarial drug	**Introduced**	**First reported resistance**
Quinine	1632	1910
Chloroquine	1945	1957
Proguanil	1948	1949
Sulphadoxine – pyrimethamine	1967	1967
Mefloquine	1977	1982
Atovaquone	1996	1996

Table 1. Drug resistance evolution globally

2.3.2 Established multidrug resistance

Multi-drug resistance is mainly established in South-East Asia, particularly at the Thailand border. The resistance is widespread, including for mefloquine, SP, and CQ resistance. The current treatment protocol includes the use of high dose of mefloquine combined with artesunate, (a dihydroartemisinin ester) .

Two important issues here are extremely relevant. Firstly, the efficacy of this specific combination must remain as there is currently no other alternative to the artemisinins. Secondly, the spread of multidrug resistance must be confined and even reduced. Certain areas in the Amazon basin may also be hotspots for multidrug resistance. At this point in time, the mefloquine-artesunate combination is the first line treatment for malaria in the Brazil-Peru border area.

2.3.3 Emerging multidrug resistance

In areas where CQ resistance is noted and there is a loss of clinical efficacy of SP and there is a possibility of resistance to a third class of drugs like the artemisinins. These areas are classified as emerging multi-drug resistant areas (Wongsrichanalai *et al*. 2002). The identification of emerging multi-drug resistant areas are of importance, as they usually appear in areas where malaria transmission is high, and control is poor. If these areas are identified early, it is possible to prevent further resistance through careful drug treatment strategies. The main concentrations of these potential areas are East Africa and the Amazon basin. As the quality of specific data for drug resistance is poor, especially in these areas, it is assumed as a precaution that regions which manifest widespread SP resistance are at risk of developing multi-drug resistance (Wrongsrichnalai *et al*. 2002).

Strategies to combat drug resistance:

- Improve prescribing patterns
- Improve therapeutic protocols
- Prevention of infection
- Combination therapy
- Development of new drugs
- Improving current drugs (Price & Nosten. 2001)

The biggest challenge in the global fight against malaria is by far the emergence of drug resistance globally, Africa being the cause for the most concern. As CQ became less and less effective, countries started using SP as a first line treatment. With the emergence of widespread SP resistance, the problem is epically more concerning. The loss of SP as an effective measure against malaria in these regions is creating multi-drug resistant strains of

P. falciparum. As most of these countries have limited resources, rational drug use is of pinnacle importance. Combination treatment with ACTs is the answer for the future, as well as early detection and rapid treatment protocols. With all of these factors combined, preventing further emergence of resistance might be possible (Wongsrichanalai *et al*. 2002).

What is alarming is the fact that the first signs of artemisinin resistance is eminent, and all indications are that artemisinins will become similarly redundant and useless as many other antimalarials. Unless a global policy is put into place, full blown resistance is envisaged within a few years. Most importantly is the drastic need to improve the drugs undesirable pharmacokinetics of these treatments by changing the formulations and designs (Golenser *et al*. 2006).

Article for submission

Chapter 3 contains the manuscript of an article to be submitted to Journal of Pharmacy and Pharmacology. The article contains the background, aims, all the experimental details and results of this study, including the physicochemical properties and *in vitro* biological results of the artemisinin derivatives. The article is prepared according to the Guide for Authors that can be found on the website of this journal (http://www.wiley.com/bw/submit.asp.), except that for easy reading figures, schemes and tables are inserted at their logical places as they would appear in the printed version.

Synthesis and antimalarial activity of ester derivatives of artemisinin

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Abstract

Objective The aim of this study was to synthesise a series of esters of the antimalarial drug artemisinin, to determine their physicochemical properties and to evaluate their antiplasmodial activity against strains of *Plasmodium falciparum*.

Method The esters were synthesized in a one-step reaction by derivatisation on carbon C-10 of artemisinin. The aqueous solubility of the esters was determined in PBS solution at two different pH values (7.4 and 5.5). The log P values were determined by using a PBS/octanol partition coefficient. The compounds were screened for antimalarial activity alongside DHA and CQ against the CQ sensitive D10 strain of *Plasmodium falciparum*.

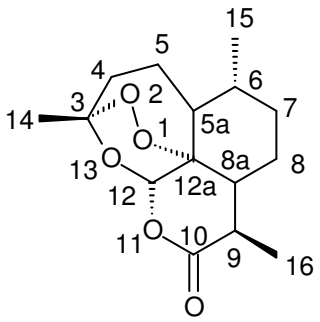
Key findings The esters were found less water soluble than DHA irrespective of the medium whether weakly acidic (pH 5.5) or neutral (pH 7.4). They also displayed higher water solubility at pH 5.5 than at pH 7.4. The majority of compounds were more lipid soluble than DHA in neutral conditions than in the acidic environment.

The butyl (**12**) (IC_{50} = 3.2 μM), 4-nitrobenzyl (**16**) (IC_{50} =15.3 μM), 2-(acetyloxy) acetyl (**17**) (IC_{50} = 8.6 μM), and 2-phenylacetyl (**18**) (IC_{50} = 12.4 μM) esters showed on a 0.05 level statistically significantly better activity against the chloroquine sensitive D10 strain of *Plasmodium falciparum* than chloroquine itself while the decyl ester (**14**) (IC_{50} = 90.2 μM) was statistically significantly less potent. The activity of the octyl (**13**) (IC_{50} = 38.0 μM) and benzyl (**15**) (IC_{50} = 25.7 μM) esters did not differ from that of chloroquine. In comparison to dihydroartemisinin the propyl (**11**) (IC_{50} = 24.1 μM), octyl (**13**) (IC_{50} = 38.0 μM), decyl (**14**) (IC_{50} = 90.2 μM), and benzyl (**15**) (IC_{50} = 25.7 μM) esters proved to be statistically significantly less potent than DHA while the activity of the butyl (**12**) (IC_{50} = 3.2 μM), 4-nitrobenzyl (**16**) (IC_{50} =15.3 μM), 2-(acetyloxy) acetyl (**17**) (IC_{50} = 8.6 μM), and 2-phenylacetyl (**18**) (IC_{50} = 12.4 μM) esters did not differ from that of DHA.

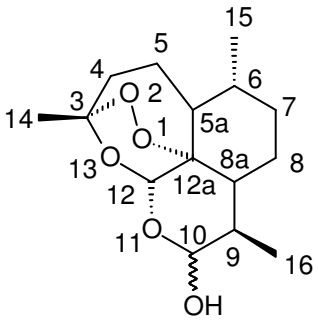
Keywords: Dihydroartemisinin, malaria, Plasmodium falciparum, ester prodrugs

1. **Introduction**

Each year 1-2 million people die of malaria, and 75% of them are African children [1]. Malaria chemotherapy based on standard drugs such as quinine is becoming especially more and more difficult because the malaria parasites, *Plasmodium falciparum*, causing the disease have developed widespread resistance against them, which warrants the search of new classes of natural and synthetic antimalarial drugs.



Artemisinin (ART) **1**



Dihydroartemisinin (DHA) **2**

Artemisinin (Qinghaosu **1**) is a sesquiterpene lactone endoperoxide isolated from an ancient Chinese herb *Artemisia annua* (Sweet wormwood). Artemisinin and its active metabolite dihydroartemisinin **2** exhibit a rapid onset of action and efficacy against blood stages of *P. falciparum*, and most importantly the preliminary phase of infection decreasing the prevalence of gametocytes in systemic circulation [2]. Thus, as a class, artemisinins have proven to be unusually valuable due to their brisk and potent antimalarial activity, lack of resistance and cross-resistance with other antimalarials, etc., which make them highly effective against multidrug resistant malaria caused by *Plasmodium falciparum*. Subsequently, the World Health Organization (WHO) recommends that Artemisinin Combination Therapy (ACT) should be given in all countries where the parasite has developed resistance to chloroquine (CQ). These ACTs associate fast-acting ART derived drugs with other antimalarials possessing longer half lives such as mefloquine. The practical values of these artemisinins, nevertheless, are impaired by their poor solubility in either oil or water, poor oral bioavailability, high rate of parasite recrudescence after treatment [3] and short plasma half lives [4].

Artemisinin has poor solubility in oil (0.012 mg/ml) and water (0.08 mg/ml) [5] and therefore can be formulated to be administered rectally, orally or as an intramuscular injection. DHA has a water solubility value of 0.13 mg/ml [6]. Oral administration poses a problem in patients suffering from severe malaria, as vomiting is a common symptom [7]. Derivatives of artemisinin such as the water

soluble artesunate (solubility value 1.2 mg/ml) [8] and the oil soluble artemether and arteether were synthesized to potentially overcome the physicochemical deficiencies of artemisinin. However, these derivatives still possess short half lives like artemisinin, which increases the risk of infection recrudescence when used as monotherapy [9].

In search of highly potent and long acting artemisinin derivatives, we synthesised and determined the solubility in both water and oil, and evaluated the activity of a series of esters of artemisinin.

2. Materials and methods

2.1 Materials

All the acyl chlorides used in the synthesis *viz.* propionyl, butyryl, octanoyl, decanoyl, benzoyl, nitrobenzoyl, acetyl salicyloyl and phenyl acetyl chlorides were purchased from Fluka (Johannesburg, South Africa). Anhydrous dichloromethane was purchased from Sigma-Aldrich (Johannesburg, South Africa). Dihydroartemisinin (10-α and 10-β diastereomers) was purchased from HuBei Enshi TianRanYuan Science and Technology Herbal Co., Ltd (Hangzhou, China). HPLC grade methanol was obtained from Labchem Ltd. (Johannesburg, South Africa). All the reagents and chemicals were of analytical grade.

2.2 General procedures

Thin-layer chromatography (TLC) was performed using silica gel plates ($60F_{254}$ Merck). Preparative flash column chromatography was carried out on silica gel (230-240 mesh, G60 Merck) and silica gel 60 (70-230 mesh ASTM, Fluka).

The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance III 600 spectrometer (at a frequency of 600.17 and 150.913 MHz, respectively) in deuterated chloroform ($CDCl_3$). Chemical shifts are reported in parts per million (δ ppm) using tetramethylsilane (TMS) as internal standard. The splitting pattern abbreviations are as follows: s (singlet), d (doublet), dd (doublet of doublets), ddd (doublet of doublet of doublets), dt (doublet of triplets), t (triplet), td (triplet of doublets), tt (triplet of triplet) and m (multiplet).

Low resolution spectra (LRESI) were ran on the Thermo Finnigan LXQ ion trap mass spectrometer in ESI mode. NaCl was added as a modifier to stabilize the compounds in ESI mode. The high resolution ESI (HRESI) mass spectra (MS) were recorded on a Thermo Electron DFS LXQ ion mass spectrometer in ESI mode and $[M+Na]^+$ were recorded.

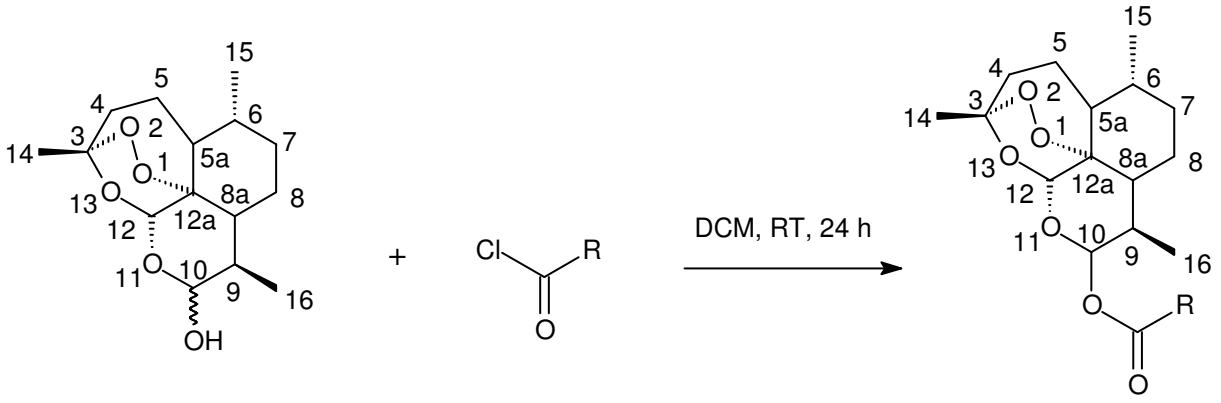
2.3 High performance liquid chromatography (HPLC)

The HPLC system consisted of an Agilent 1100 series auto sampler, Agilent 1100 series variable wave detector (VWD) and Agilent 1100 series isocratic pump. A Zorbax Eclipse XDB C18, 5 µm (150 x 4.60 mm) column was used and the Agilent Chemstation reverse A08.03 for LC systems software package was used for data analysis. The compounds were quantified using a gradient method (A = 0.2 % triethylamine in H_2O, pH 7.0, B = acetonitrile) at a flow rate of 1 ml/min with 20 µl standard sample injections. The gradient consisted of 25 % of solvent B (ACN) for 1 min, then increased linearly to 95 % of B after 8 min, and held for 15 min, where after the instrument was re-equilibrated to the starting conditions for 5 min. A calibration plot of peak area versus drug concentration for each compound showed excellent linearity ($0.993 < r^2 \leq 1$over the concentration range (0-2000 µg/ml) employed for the assays. The absorption maximum for dihydroartemisinin and all its derivatives was at 205 nm; and therefore this wavelength was consequently used for the HPLC detection.

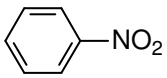
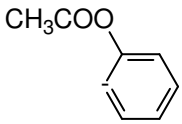
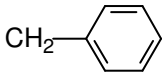
3. Experimental procedures

3.1. Synthesis of esters prodrugs of dihydroartemisinin 11-18

The synthesis of artemisinin esters was achieved using the general method reported by Singh *et al*. [10] (Scheme), and is described as follows: dihydroartemisinin **2** (7 mmol) and acyl chloride **3 - 10** (14 mmol, 2.0 equiv relative to dihydroartemisinin) were dissolved in 65 ml of anhydrous dichloromethane. The mixture was stirred at 25 ºC or room temperature for 24 h. The progress of the reaction was monitored by thin layer chromatography (TLC). After completion, the reaction mixture was evaporated to dryness *in vacuo*. The resulting residue was purified by flash chromatography eluting with DCM as mobile phase. All the synthesized compounds were solids, except **13** and **14** which were oils. ^{1}H and ^{13}C NMR chemical shifts as well as HRESI-MS data of compounds **11** -**18** are reported.



Dihydroartemisinin (DHA) **2** + Acyl chlorides **3** - **10** → Esters **11** - **18**

Esters	R
11	$-CH_2-CH_3$
12	$-(CH_2)_2-CH_3$
13	$-(CH_2)_6-CH_3$
14	$-(CH_2)_8-CH_3$
15	
16	
17	
18	

*3.1.1. 10α-dihydroartemisinyl propanoate **11***

Yield: 47.2%, off-white powder, $C_{18}H_{28}O_6$. ^{1}H NMR (600 MHz, $CDCl_3$): δ 6.16 (s, 1H, H-10), 5.51 (s, 1H, H-12), 2.09 – 1.95 (m, 2H, O-CO-*CH*$_2$), 2.59 – 1.74 (m, 5H), 2.16 – 1.96 (m, 2H), 1.88 (ddd, *J* = 25.2, 14.5, 10.9 Hz, 1H), 1.74 – 1.10 (m, 15H), 1.10 – 0.98 (m, 1H), 0.98 – 0.62 (m, 4H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 165.5 (-*C*OO-), 134.94 (C-3), 108.07 (*C*-10), 104.48 (*C*-12), 68.10 (*C*-12a), 51.39 (*C*-5a), 44.39 (*C*-8a). LRESI m/z: $[M+Na]^+$ 363, HRESI m/z $[M+Na]^+$ 363.13995 ($C_{18}H_{28}O_6Na$ requires 363.411).

3.1.2. 10α-dihydroartemisinyl butanoate ***12***

Yield: 18.7%. off-white powder, $C_{19}H_{30}O_6$. ^{1}H NMR (600 MHz, $CDCl_3$): δ 5.78 (d, *J* = 9.8 Hz, 1H, H-10), 5.42 (s, 1H, H-12), 2.59 – 2.48 (m, 1H, H-5a), 2.39 – 2.30 (m, 2H, O-CO-*CH*$_2$). ^{13}C NMR (151 MHz, $CDCl_3$) δ 172.41 (-*C*OO-), 104.42 (*C*-3), 91.63 (*C*-10), 91.47 (*C*-12), 80.14 (*C*-12a), 77.22 (*C*-5a), 77.00 (*C*-8a), 12.10 (R, -*C*H$_3$). LRESI m/z: $[M+Na]^+$ 377 and $[2M+Na]^+$ 731, HRESI m/z $[M+Na]^+$ 377.19350 ($C_{19}H_{30}O_6Na$ requires 377.438).

3.1.3. 10α-dihydroartemisinyl octanoate ***13***

Yield: 65.7%), clear oil, $C_{23}H_{38}O_6$. ^{1}H NMR (600 MHz, $CDCl_3$): δ 5.77 (d, *J* = 9.9 Hz, 1H, H-10), 5.42 (s, 1H, H-12), 2.59 – 2.48 (m, 1H, H-5a), 2.40 – 2.29 (m, 2H, -O-CO-*CH*$_2$. ^{13}C NMR (151 MHz, $CDCl_3$) δ 172.60 (-*C*OO-), 104.43 (*C*-3), 91.63 (*C*-10), 91.48 (*C*-12), 80.15 (*C*-12a), 51.58 (*C*-5a), 45.26 (*C*-8a), 14.05 (R, -*C*H$_3$). LRESI m/z: $[M+Na]^+$ 433 and $[2M+Na]^+$ 843, HRESI m/z $[M+Na]^+$ 433.26624 ($C_{23}H_{38}O_6Na$ requires 4233.544).

3.1.4. 10α-dihydroartemisinyl decanoate ***14***

Yield: 78.45%, clear oil, $C_{25}H_{42}O_6$. ^{1}H NMR (600 MHz, $CDCl_3$): δ 5.77 (d, *J* = 9.8 Hz, 1H, H-10), 5.42 (s, 1H, H-12), 2.67 – 2.42 (m, 1H, H-5a), 2.42 – 2.26 (m, 2H, -O-CO-*CH*$_2$-). ^{13}C NMR (151 MHz, CDCl3) δ 172.61 (-*C*OO-), 104.43 (*C*-3), 91.65 (*C*-10), 91.49 (*C*-12), 80.15 (*C*-12a), 51.60 (*C*-5a), 45.28 (*C*-8a), 12.11 (R, *C*H$_3$). LRESI m/z: $[M+Na]^+$ 461 and $[2M+Na]^+$ 900, HRESI m/z $[M+Na]^+$ 461.28753 ($C_{25}H_{42}O_6Na$ requires 461.597)

3.1.5. 10α-dihydroartemisinyl benzoate ***15***

Yield: 70.5%, clear crystal, $C_{22}H_{28}O_6$. ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.09 (dd, *J* = 8.2, 1.0 Hz, 2H, Ar, *o*-H), 7.60 – 7.48 (m, 1H, Ar, *p*-H), 7.42 (t, *J* = 7.8 Hz, 2H, Ar, *m*-H), 5.98 (d, *J* = 9.8 Hz, 1H, H-10), 5.50 (s, 1H, H-12), 2.79 – 2.65 (m, 1H, H-9), 2.36 (ddd, *J* = 17.7, 13.9, 4.0 Hz, 1H, H-8a), 1.10 - 0.83 (m, 6H, H-15, -16). ^{13}C NMR (151 MHz, $CDCl_3$) δ 165.27 (-*C*OO-), 133.27 (Ar, *p*-*C*), 130.09 (Ar, *o*-*C*), 128.26 (Ar, *m*-*C*), 104.40 (*C*-3), 92.49 (*C*-10), 91.56 (*C*-12), 80.17 (*C*-12a). LRESI m/z: $[M+Na]^+$ 411 and $[2M+Na]^+$ 799, HRESI m/z $[M+Na]^+$ 411.17726 ($C_{22}H_{28}O_6Na$ requires 411.454).

3.1.6. 10α-dihydroartemisinyl 4-nitrobenzoate ***16***

Yield: 94%, yellow solid, $C_{22}H_{27}O_8$. ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.27 (s, 4H, Ar), 6.00 (d, *J* = 9.8 Hz, 1H, H-10), 5.51 (s, 1H, H-12), 3.14 – 3.03 (m, 1H, H-9), 2.82 – 2.63 (m, 1H, H-8a). ^{13}C NMR (151 MHz, $CDCl_3$) δ 163.49 (-*C*OO-), 150.76 (Ar, *o*-*C*), 131.21 (Ar, *m*-*C*), 104.55 (*C*-3), 93.37 (*C*-10), 91.69 (*C*-12), 80.10 (*C*-12a). LRESI m/z: $[M+Na]^+$ 456 and $[2M+Na]^+$ 889, HRESI m/z $[M+Na]^+$ 456.18084 ($C_{22}H_{27}O_8Na$ requires 456.452).

3.1.7. 10α-dihydroartemisinyl 2-(acetyloxy) acetate ***17***

Yield: 84.5%, off-white solid, $C_{24}H_{30}O_8$. ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.08 (ddd, *J* = 17.7, 7.9, 1.6 Hz, 1H, Ar, *o*-H), 7.55 (td, *J* = 7.9, 1.7 Hz, 1H, Ar, *p*-H), 7.30 (td, *J* = 7.7, 0.9 Hz, 2H, Ar, *m*-H), 5.98 (d, *J* = 9.8 Hz, 1H, H-10), 5.48 (s, 1H, H-12), 2.74 – 2.55 (m, 1H, H-9), 2.38 (s, 3H, Ar-O-CO-*CH$_3$*), 2.09 – 1.97 (m, 1H, H-8a). ^{13}C NMR (151 MHz, $CDCl_3$) δ 169.93 (Ar-O-*C*O-), 163.34 (-*C*OO-), 104.40 (*C*-3), 92.64 (*C*-10), 91.59 (*C*-12), 79.84 (*C*-12a). LRESI m/z: $[M+Na]^+$ 469 and $[2M+Na]^+$ 915, HRESI m/z $[M+Na]^+$ 469.18333 ($C_{24}H_{30}O_8Na$ requires 469.49).

3.1.8. 10α-dihydroartemisinyl 2-phenylacetate ***18***

Yield: 40.1%, off-white solid. ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.36 – 7.20 (m, 5H, Ar), 5.76 (d, *J* = 9.8 Hz, 1H, H-10), 5.42 (s, 1H, H-12), 3.75 – 3.60 (m, 1H, Ar-*CH$_2$*-), 2.60 – 2.47 (m, 1H, H-5a), 2.36 (td, *J* = 14.1, 3.8 Hz, 1H, H-4). ^{13}C NMR (151 MHz, $CDCl_3$) δ 170.35 (-*C*OO-), 129.39 (Ar, o-C), 128.50 (Ar, m-C), 127.13 (Ar, p-C), 104.46 (*C*-3), 92.20 (*C*-10), 91.53 (*C*-12), 80.12 (*C*-12a). LRESI m/z: $[M+Na]^+$ 425 and $[2M+Na]^+$ 827, HRESI m/z $[M+Na]^+$ 425.19375 ($C_{23}H_{30}O_6Na$ requires 425.487).

3.2. Physicochemical properties

3.2.1 Solubility

The aqueous solubility values (Sw) of crystalline compounds **11**, **12** and **15**-**18** as well as DHA **2** were obtained by preparing solutions in phosphate buffer at pH 7.4 and pH 5.5 (Table 1). The slurries were stirred in a water bath at 37 ºC for 24 h. An excess of solute was present at all times to provide saturated solutions. After 24 h, the solutions were filtered and analyzed directly by HPLC to determine the concentration of solute dissolved in the solvent. Both the oils **13** and **14**, after 24 h stirring, the mixtures were allowed to stand at room temperature before the aqueous layers were carefully separated and analyzed by HPLC. The experiment was performed in triplicate for each compound. The average results are reported in Table 1.

3.2.2 Experimental log P

Equal volumes of *n*-octanol and phosphate buffer solution of pH 7.4 and pH 5.5 (Table 1) were saturated with each other under vigorous stirring for at least 24 h. Accurately weighed 2 mg of each derivative was dissolved in 0.75 ml of pre-saturated n-octanol, the solution was then stoppered and agitated for 10 min in 2 ml graduated tubes (0.5 ml division). Subsequently 0.75 ml of pre-saturated buffer was transferred to the tubes containing the before mentioned solutions.

The tubes were stoppered and agitated for 45 min then centrifuged at 4 000 rpm (1503 g) for 30 min. The *n*-octanol and aqueous phases were allowed to separate at room temperature for 5 min,

where after their volume ratio (v/v; n-octanol:buffer) was determined. The volume ratio was found in all cases to be 1. The *n*-octanol and aqueous phases were analysed by HPLC. From this data the concentrations of the derivative in both phases were determined. The log P values were calculated as logarithmic ratios of the concentrations in the *n*-octanol phase to the concentrations in the buffer. The experiment was performed in triplicate and the results expressed as averages and are listed in Table 1.

3.3 *In vitro* antimalarial activity

The esters were tested 6-fold against a strain of *P. falciparum*, namely D10. *In vitro* cultures of the asexual erythrocyte stages of *P. falciparum* was maintained using a method postulated by Trager and Jensen (1976) [11]. Quantitative assessment of antiplasmodial activity *in vitro* was determined by parasitic lactate dehydrogenase assay using a modified method used by Makler *et al* (1976) [12]. The method was as follows: the test samples were prepared as a 2 mg/ml stock solution in 10% DMSO and sonicated to enhance solubility. Stock solutions were stored in a freezer at -20℃ Celsius. Further dilutions were prepared on the day of testing. Chloroquine and dihydroartemisinin were used at a comparative reference drugs for all samples. A full dose-response analysis was performed on all the ester prodrugs to determine the IC_{50} value, or the concentration inhibiting 50% of parasitic growth. Samples were tested at concentrations ranging from 1000 ng/ml to 2 ng/ml. 10 dilutions were used in total. Samples were then re-tested at a starting concentration of 100 ng/ml. The parasites showed no measurable effect being exposed to the solvents used. The IC_{50} values were calculated using a non-linear dose-response curve fitting analysis using Graph Pad Prism version 4.0.

3.4 Statistical analysis

The following statistical procedures were used to test if there were statistical significant differences between the mean IC_{50} values of the test compounds **11** to **18** and each of the standards, dihydroartemisinin (DHA) and chloroquine (CQ). This was done for the D10 strain. One-way analyses of variance (ANOVA) were done to determine if statistical significant differences exist between the means of the test compounds and each of dihydroartemisinin and chloroquine in general. Normal probability plots on the residuals were done in each analysis to assure that the data were normally distributed [13]. Levene's tests were done to assure homogeneity of variances. In both analyses homogeneity of variances could not be assured and thus Welch's *p*-values were reported. Dunnett's tests were done to determine which of the test compounds' means differ statistically significantly from the means of each of the standard compounds. Tukey multiple comparison tests were done to determine if test compounds' means differ statistically significantly from one another for the D10 strain omitting the standard compound from the analyses in order to

determine which of the test compounds were more effective than the other test compounds. These procedures were done using Statistica [14]. All tests were done on a 0.05 significant level. The results are given in Table 2.

Table 2: Physicochemical properties of dihydroartemisinin and its esters **11**-**18**

Compound	R	*J* (Hz)	Isomer	M_w (g/mol)	S_w (µM) [a] pH 7.4	SD	Log D [a] pH 7.4	S_{oc} (µM)	S_w (µM) [a] pH 5.5	SD	Log D [a] pH 5.5	S_{oc} (µM)
DHA		NA		284.35	1.10	0.09	0.09	1.30	3.11	0.08	0.29	6.06
11	Propyl	ND		340.41	0.31	0.01	0.76	0.76	0.42	0.04	0.28	0.80
12	Butyl	9.8	α	354.43	0.30	0.01	0.69	1.47	0.90	0.01	0.70	4.51
13	Octyl	9.9	α	410.54	0.18	0.04	0.90	1.43	0.47	0.05	0.21	0.76
14	Decyl	9.8	α	438.59	0.47	0.01	0.94	4.09	0.49	0.03	0.94	4.27
15	Benzyl	9.8	α	388.45	0.25	0.07	0.49	0.77	0.55	0.07	0.16	0.80
16	4-Nitrobenzyl	9.8	α	433.35	1.03	0.08	0.35	2.30	1.37	0.05	0.60	5.45
17	2-Acetyloxybenzyl	9.8	α	446.49	0.70	0.02	0.30	1.40	0.87	0.03	0.20	1.38
18	Phenyl	9.8	α	402.48	0.71	0.01	0.68	3.4	0.85	0.01	0.33	1.82

Substituent (R), molecular weight (M_W), coupling constant between H-10 and H-9 (J), aqueous solubility (S_w), octanol solubility (S_{oc}), standard deviation (SD), partition coefficient, log D (*n*-octanol: PBS, pH 7.4, 5.5). [a] Determined experimentally, each value represents the mean and SD of 3 measurements.

4. Results and discussion

4.1 Chemistry

The new esters of dihydroartemisinin were prepared by treating DHA **2** with an appropriate acyl chloride **3** – **10** in anhydrous dichloromethane (DCM) medium at room temperature for 24 hours. The yield of the purified products ranged from 18.7 to 94% . They were exclusively 10α epimers according to the literature [10] and their structures were confirmed by NMR spectroscopy and MS spectrometry.

(See Table 1)

4.2 Aqueous solubility and experimental log D

The synthesized compounds **11**-**18** as well as the parent **2** showed decreased aqueous solubility in comparison to DHA irrespective of the substituent (*n*-alkyl or aryl) and the medium (weakly acidic-pH 5.5 or neutral-pH 7.4). The aqueous solubility was more pronounced in the weakly acidic medium than in the neutral one.

4.3 *In vitro* antimalarial activity

All the compounds showed better activity against the D10 strain than CQ, apart from the two oils, **13** and **14**. Esters **12**, **17** and **18** showed a statistically better activity than chloroquine against the D10 strain of *P. falciparum*. Compared to DHA, only ester **12** showed comparable activity, and the rest were all less active. The IC_{50} results are reported in Table 2.

Table 3: Descriptive statistics of IC_{50} values, results of ANOVAs and Dunnett's tests, and antimalarial activity of esters of artemisinin **11 – 18,** dihydroartemisinin (DHA) and chloroquine (CQ)

Compound	Chloroquine sensitive D10 strain of *Plasmodium falciparum*					
	n[a]	Mean IC_{50} (μM)	S.D. x 10^{-3}	ANOVA*: p*-value (Welch)	Dunnett: *p*-value	
					DHA	CQ[b]
11	6	24.1	7.4		0.000321*	0.917582
12	6	3.2	1.7		1.000000	0.000020*
13	6	38.0	3.8		0.000007*	0.202029
14	6	90.2	17.6		0.000007*	0.000007*
15	6	25.7	10.0		0.000107*	0.994026
16	6	15.3	7.2		0.077231	0.040907*
17	6	8.6	1.1		0.838876	0.000665*
18	6	12.4	2.2		0.283691	0.007774*
DHA	6	3.8	1.9	0.00*		
CQ	6	28.3	5.4	0.00*		

* Statistically significant at 0.05 levels, [a] replicates (n), [b] chloroquine (CQ) was tested as diphosphate salt.

5. Discussion

5.1 Chemistry

The series of artemisinin esters (*n*-alkyl **11**-**14** and aryl **15**-**18**) were synthesized by coupling of an acyl chloride **3** - **10** to dihydroartemisinin **2** in DCM as a reaction medium at room temperature for 24 hours. The α-isomers were exclusively isolated. The $J_{H-9:\ H-10}$ coupling constant was used to determine the configuration at the C-10 position of the esters. In the case of the α-isomer, the H-9: H-10 coupling constant is quite large = 9-10 Hz [15]. This is indicative of a *trans* configuration. All the compounds possessed a $J_{H-9\ :\ H-10}$ coupling constant of 9.8 Hz, except for compound **11**, which had no detectable coupling constant.

In the ^{1}H NMR spectrum of dihydroartemisinin the signals for H-12 and H-10 for the 10β-isomer, having a higher R_f value, appeared at δ_H 5.41 (s) and 4.8 (s) respectively whereas for the 10α-isomer they appeared at δ_H 5.33 (s) and 4.43 (s) respectively. In the α-series the signals due to OCH_2 and H_{10} appeared up field compared with those in the β-series [16]. The ^{13}C NMR spectrum of dihydroartemisinin showed signals at 104.99, 99.40, 88.62, 81,42, 52.87, 45.51, 37.65, 36.84, 34.61, 32.28, 25.60, 22.99, 22.72, 20.00 and 14.87 ppm corresponding to C-10, C-3, C-12, C-12a, C-5a, C-8a, C-9, C-4, C-7, C-6, C-14, C-5, C-8, C-15 and C-16 respectively. Thus, the presence of the dihydroartemisinin moiety was confirmed in the spectra of all the esters.

The chemical structures of the title compounds **11** - **18** were further confirmed by NMR, LR- and HRMS. LRESI data showed the molecular ions $[M+Na]^+$ m/z of compounds **11**, **12, 13, 14, 15, 16, 17** and **18** at 363, 377, 433, 461, 411, 455, 425 and 469, respectively. The HRESI-MS confirmed the exact mass of **11** [363.13995], which corresponds to the formula $C_{18}H_{28}O_6Na$, of **12** [377.19350] for $C_{19}H_{30}O_6Na$, of **13** [433.26624] which corresponds to $C_{23}H_{38}O_6Na$, of **14** [461.28753] which corresponds to $C_{25}H_{42}O_6Na$, of **15** [411.17726] for $C_{22}H_{28}O_6Na$, of **16** [456.18084] for $C_{22}H_{27}O_8Na$, of **17** [469.18333] for $C_{24}H_{30}O_8Na$, and of **18** [425.19375] for the formula $C_{23}H_{30}O_6Na$.

5.2 Aqueous Solubility and experimental log D

Aqueous solubility and lipophilicity influence the way a drug molecule passes through biological membranes and barriers to ultimately enter the systemic circulation. The drug molecules must posses some lipophilic properties to permeate biological membranes and hydrophilic properties to be taken up in the systemic circulation, but a more hydrophilic nature is

needed in order for efficient drug delivery. The logarithm of ratio of octanol solubility to water solubility (log P), is thus found in almost every physicochemical analysis for reportedly being related to drug absorption [17, 18]. The distribution coefficient (log D), a pH dependent version of the partition coefficient (log P) was determined experimentally in phosphate buffer at two distinctive pH values (7.4 and 5.5), chosen to mimic the cytosolic and parasite digestive vacuole environments, respective. The lipid solubility of all compounds were evaluated by calculating the solubility in octanol (S_{oc}) from the experimental S_w and the log D data using the equation S_{oc} = log D + log S_w.

Overall, the esters as expected were found less water-soluble than dihydroartemisinin regardless of the medium being weakly acidic or neutral. They were also more soluble in the acidic medium with S_w values ranging from 0.4 to 1.4 μM than in the neutral one where S_w values varied from 0.3 to 1.03 μM. With the exception of esters of **11** and **15,** all others displayed higher lipid solubility than DHA at physiological pH. This was expected as for the formation of each ester, the hydrogen bonding therefore polar site *viz*. 10-OH of dihydroartemisinin is masked by a notoriously lipophilic group; butyl for ester **12**, octyl for **13**, decyl for **14**, 4-nitrobenzyl for **16**, 2-acetyloxybenzyl for **17**, and phenyl for ester **18**. The S_{OC} values of **11** and **15** respectively 0.76 and 0.77 μM were inexplicably lower than that of DHA (1.5 μM). All the esters were also found less lipophilic than DHA in the weakly acidic medium (pH 5.5) which somewhat unexpected and inexplicable.

Overall, no structure-aqueous solubility and structure-lipophilicity relationships could be found during this study. However, the results clearly indicate that these artemisinin esters both *n*-alkyl or aryl were more soluble in the weakly acidic medium than they were in the neutral one.

5.3 *In-vitro* Antimalarial activity

The series of newly synthesized dihydroartemisinin ester compounds were tested *in vitro* against the D10 chloroquine (CQ) sensitive strain of *P. falciparum*. All the esters showed better activity than chloroquine, except **13** and **14** which were less active. The octyl ester **14** is an oil with a lipid solubility of 4 μM and a water solubility of 0.5 μM in either weakly acidic or neutral medium. This poor aqueous solubility could be the main explanation of its poor activity because a balance between aqueous and lipid solubility is required for efficient biomembrane penetration through passive diffusion. Similarly, the poor performance of ester **13** can be attributed to poor water solubility regardless of the activity testing medium. In this heterogeneous series, the compounds **12 (**IC_{50} = 3.2 μM), **17** (IC_{50} = 8.6 μM) and **18** (IC_{50} = 12.4 μM), all with *p*-value (Dunnett) < 0.05

showed potency significantly better than CQ (IC_{50} = 28.3 μM). The esters **11**, **15**, **16** and **18**, all had relevantly equal IC_{50} values making their activity similar. The butyl ester **12** with an IC_{50} value 3.2 x 10^{-3} μM was the most active ester of all presumably due to a better lipophilicity/hydrophilicity balance. All the esters proved to be less potent than DHA against the D10 strain.

6. Conclusions

A series of artemisinin esters were synthesized through derivatisation at the 10-OH position. All compounds were subjected to NMR and MS techniques and their structures were confirmed. The physicochemical properties such as distribution coefficient (log D) and aqueous solubility was determined in both weakly acid and neutral media. The esters were less water soluble than DHA irrespective of the medium either weakly acidic (pH 5.5) or neutral (pH 7.4). They also displayed higher water solubility at pH 5.5 than at pH 7.4. The majority of compounds were more lipophilic soluble than DHA in neutral conditions in comparison to the acidic environment.

The butyl (**12**) (IC_{50} = 3.2 μM), 4-nitrobenzyl (**16**) (IC_{50} =15.3 μM), 2-(acetyloxy) acetyl (**17**) (IC_{50} = 8.6 μM), and 2-phenylacetyl (**18**) (IC_{50} = 12.4 μM) esters showed on a 0.05 level statistically significantly better activity against the chloroquine sensitive D10 strain of *Plasmodium falciparum* than chloroquine itself while the decyl ester (**14**) (IC_{50} = 90.2 μM) was statistically significantly less potent. The activity of the octyl (**13**) (IC_{50} = 38.0 μM) and benzyl (**15**) (IC_{50} = 25.7 μM) esters did not differ from that of chloroquine. In comparison to dihydroartemisinin, the propyl (**11**) (IC_{50} = 24.1 μM), octyl (**13**) (IC_{50} = 38.0 μM), decyl (**14**) (IC_{50} = 90.2 μM) and benzyl (**15**) (IC_{50} = 25.7 μM) esters proved to be statistically significantly less potent than DHA while the activity of the butyl (**12**) (IC_{50} = 3.2 μM), 4-nitrobenzyl (**16**) (IC_{50} =15.3 μM), 2-(acetyloxy) acetyl (**17**) (IC_{50} = 8.6 μM), and 2-phenylacetyl (**18**) (IC_{50} = 12.4 μM) esters did not differ from that of DHA.

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5. Summary and final conclusion

Malaria worldwide is increasing, with the rapid spread of drug resistant strains being the driving factor. The global consequence is a major increase in the economic and health problems it poses, especially in endemic areas, and more recently, the increase of potential infection for travellers. Malaria treatment should be implemented as soon as possible, preferably with an Artemisinin Combination Therapy or ACT. In non complicated malaria cases, out patient treatment with oral drugs is sufficient. In cases of severe or cerebral malaria, hospitalisation is required for IV treatment (Ashley *et al*. 2006). The rising prevalence of multi-drug resistant strains is the driving force behind increased research into alternative therapies (Price & Nosten. 2001).

Discovery of artemisinin or qinghaosu in the 1970's by Chinese researchers paved the way for a whole new field of discovery in malaria treatment. As far back as the 16th century, extracts of the wormwood plant or *Artemisia annua* was used as a cure for idiopathic fever (Zhang & Wu. 2007).This new compound possessed an array of promising qualities including potent, rapid anti-malarial action with a wide therapeutic index.

Furthermore, there was an absence of any clinically relevant resistance, the main problem with all current antimalarials (de Ridder *et al*. 2008).

Artemisinins and its active metabolite dihydroartemisinin (2) exhibit a rapid onset of action and efficacy against blood stages of P. falciparum, and most importantly the preliminary phase of infection. It also decreases the prevalence of gametocytes in systemic circulation (Golenser et al. 2006). Pure artemisinin has poor solubility in oil and water, and therefore can be formulated to be administered rectally, orally or as an intramuscular injection. However, oral administration poses a problem in patients suffering from severe malaria, as vomiting is a common symptom (de Ridder et al. 2008). Several semi-synthetic derivates of artemisinin were created to potentially overcome its physicochemical deficiencies, like the water soluble artesunate and the oil soluble artemether and arteether.

Recently, research has been focused on the ester derivatives of DHA. Artesunate, a DHA ester has favourable physicochemical properties over DHA, with increased half-life and solubility. It is available for oral, rectal or intravenous administration. The rapid onset of action makes it an

ideal candidate for patients with severe cerebral malaria (Barradell & Fitton. 1995). Parasitic clearance with artesunate is also much faster than the other non-ester artemisinin derivatives as shown in a recent meta-analysis. Recrudescence was also much lower when artesunate was added to monotherapy treatment (Adjuik *et al*. 2004). *Singh et al, 2008* proposed a synthesis of artemisinin ester derivatives that exclusively produces the α-isomer, which is the more potent and active isomer. The lipophilicity of ester derivatives (log P 5.85-6.95) are comparable to those of the ether derivatives (log P 5.51-6.91). Further research into ester derivatives is thus of great importance (Singh *et al*. 2008).

Once the complete structure of artemisinin was solved, various modifications have been researched into improving the water and oil solubility of dihydroartemisinin, the most used and clinically useful metabolite of artemisinin. The short half-life of dihydroartemisinin is an important factor in its clinical superiority over other antimalarials with regards to resistance. The disadvantage of dihydroartemisinin however, is because of its short half-life, it has an increased risk associated with recrudescent infections when used as monotherapy (Krishna *et al*. 2004).

In order to completely eliminate *P. falciparum* and to combat the possible emergence of artemisinin resistant strains, combinations with other long acting drugs are necessary. Currently ACTs are the recommended 1st line treatment for uncomplicated malaria by the WHO (Golenser *et al*. 2006).

The aim of this study was to evaluate the effects of estrification of DHA on the physicochemical properties and its activity against CQ-resistant strain of *P. falciparum*.

In order to achieve this goal, the following objectives were set:

- Synthesis of ester derivatives of artemisinin and to confirm their structures using various analytical techniques.
- Experimentally determine and evaluate the relevant physicochemical properties such as the aqueous solubility and the partition coefficient for the synthesised dihydroartemisinin ester prodrugs and to compare the experimental aqueous solubilities and the partition coefficients of the synthesised prodrugs to that of the known anti-malarial dihydroartemisinin.

- Evaluate the *in vitro* antimalarial efficacy of the dihydroartemisinin ester prodrugs against the chloroquine sensitive D10 strain of *Plasmodium falciparum* in comparison to that of the reference drugs, chloroquine and dihydroartemisinin.

The artemisinin derivatives were successfully synthesised via synthetic organic methods and their structures were verified by NMR spectroscopy and MS spectrometry.

Aqueous solubility and lipophilicity influence the way a drug molecule passes through biological membranes and barriers to ultimately enter the systemic circulation. The drug molecules must posses some lipophilic properties to permeate biological membranes and hydrophilic properties to be taken up in the systemic circulation, but a more hydrophilic nature is needed in order for efficient drug delivery. The logarithm of ratio of octanol solubility to water solubility (log P), is thus found in almost every physicochemical analysis for reportedly being related to drug absorption [17, 18]. The distribution coefficient (log D), a pH dependent version of the partition coefficient (log P) was determined experimentally in phosphate buffer at two distinctive pH values (7.4 and 5.5), chosen to mimic the cytosolic and parasite digestive vacuole environments, respectively. The lipid solubility of all compounds were evaluated by calculating the solubility in octanol (Soc) from the experimental Sw and the log D data using the equation Soc = log D + log Sw.

Overall, the esters as expected were less water-soluble than dihydroartemisinin regardless whether the medium was weakly acidic or neutral. They were also more soluble in the acidic medium with S_w values ranging from 0.4 to 1.4 μM than in the neutral one where S_w values varied from 0.3 to 1.03 μM. With the exception of esters of **11** and **15,** all others displayed higher lipid solubility than DHA at physiological pH. This was expected as for the formation of each ester, the hydrogen bonding therefore polar site *viz*. 10-OH of dihydroartemisinin is masked by a notoriously lipophilic group; butyl for ester **12**, octyl for **13**, decyl for **14**, 4-nitrobenzyl for **16**, 2-acetyloxybenzyl for **17**, and phenyl for ester **18**. The S_{OC} values of **11** and **15** respectively 0.76 and 0.77 μM were inexplicably lower than that of DHA (1.5 μM). All the esters were also found less lipophilic than DHA in the weakly acidic medium (pH 5.5) which somewhat unexpected and inexplicable.

Overall, no structure-aqueous solubility and structure-lipophilicity relationships could be found during this study. However, the results clearly indicate that these artemisinin esters either *n*-alkyl or aryl were more soluble in the weakly acidic medium than they were in the neutral one.

The series of newly synthesised dihydroartemisinin ester compounds were tested *in vitro* against the D10 chloroquine (CQ) sensitive strain of *P. falciparum*. All the esters showed better activity than chloroquine, except **13** and **14** which were less active. The octyl ester **14** is an oil with a lipid solubility of 4 μM and a water solubility of 0.5 μM in either weakly acidic or neutral medium. This poor aqueous solubility could be the main explanation of its poor activity because a balance between aqueous and lipid solubility is required for efficient biomembrane penetration through passive diffusion. Similarly, the poor performance of ester **13** can be attributed to poor water solubility regardless of the activity testing medium. In this heterogeneous series, the compounds **12 (**IC_{50} = 3.2 μM), **17** (IC_{50} = 8.6 μM) and **18** (IC_{50} = 12.4 μM), all with *p*-value (Dunnett) < 0.05 showed potency significantly better than CQ (IC_{50} = 28.3 μM). The esters **11**, **15**, **16** and **18**, all had relevantly equal IC_{50} values making their activity similar. The butyl ester **12** with an IC_{50} value 3.2 μM was the most active ester of all presumably due to a better lipophilicity/hydrophilicity balance. All the esters proved to be less potent than DHA against the D10 strain.

Due to the limited number of compounds tested (8 in total), it was statistically impossible to draw structure-activity relationship (SAR) deductions from the data received, and the statistical analysis is of such a nature that definitive statements could not be made.

A series of artemisinin esters were synthesised through derivatisation at the 10-OH position. All compounds were subjected to NMR and MS techniques and their structures were confirmed. The physicochemical properties such as distribution coefficient (log D) and aqueous solubility determined in both weakly acid and neutral media. The esters were found less water soluble than DHA irrespective of the medium either weakly acidic (pH 5.5) or neutral (pH 7.4). They also displayed higher water solubility at pH 5.5 than at pH 7.4. The majority of compounds were more lipophilic soluble than DHA in neutral conditions in comparison to the acidic environment.

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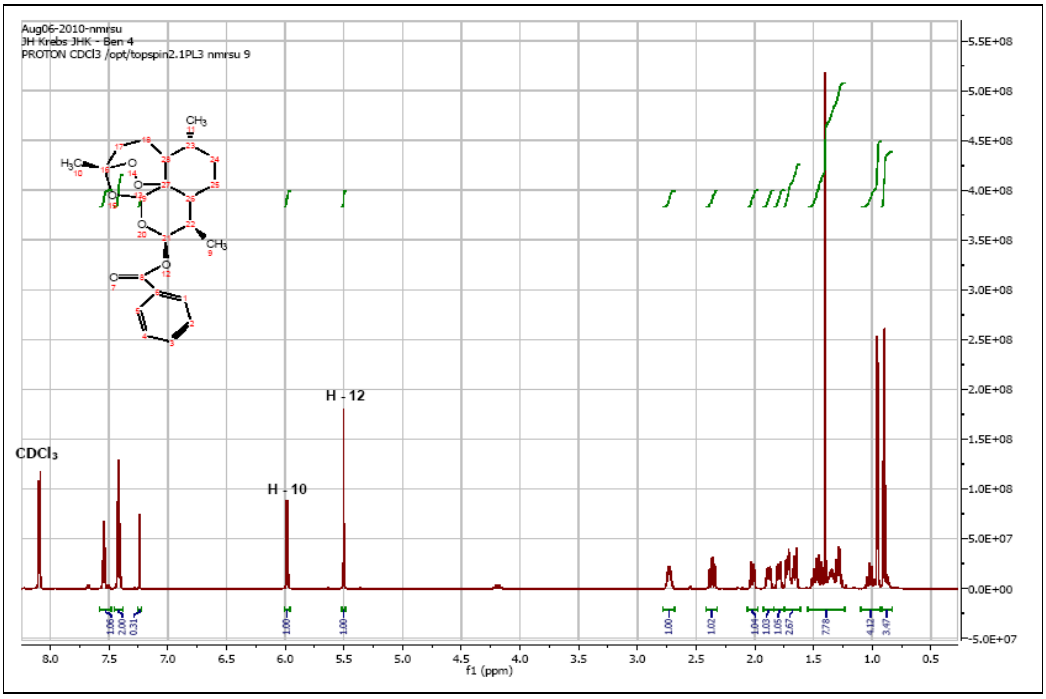
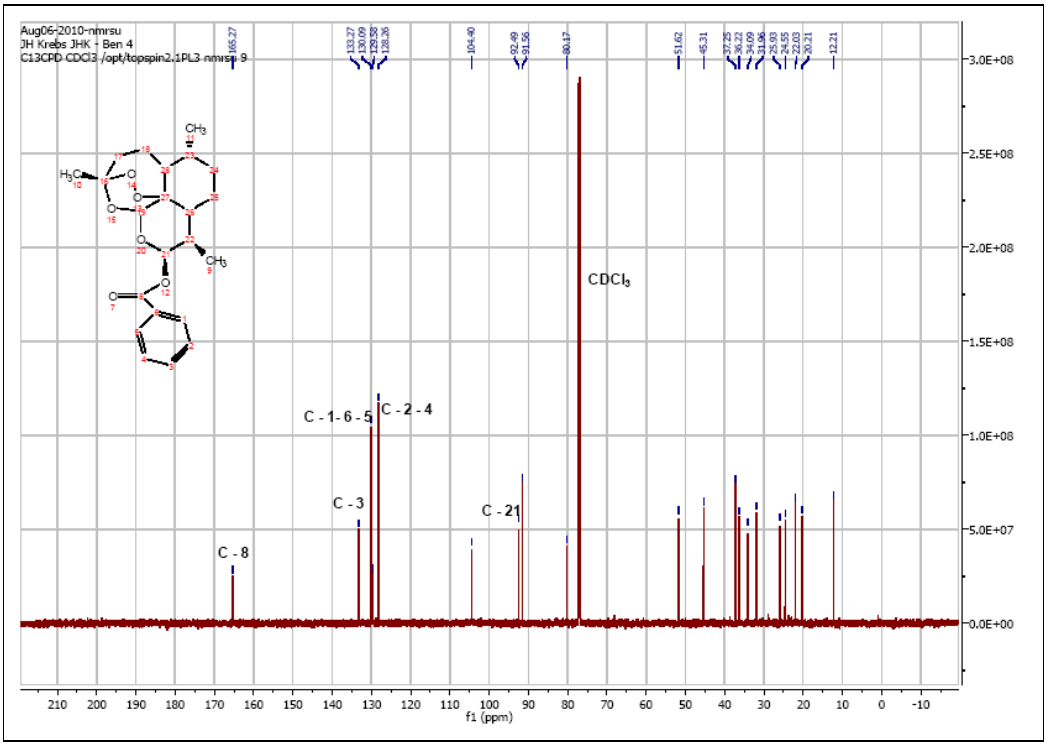
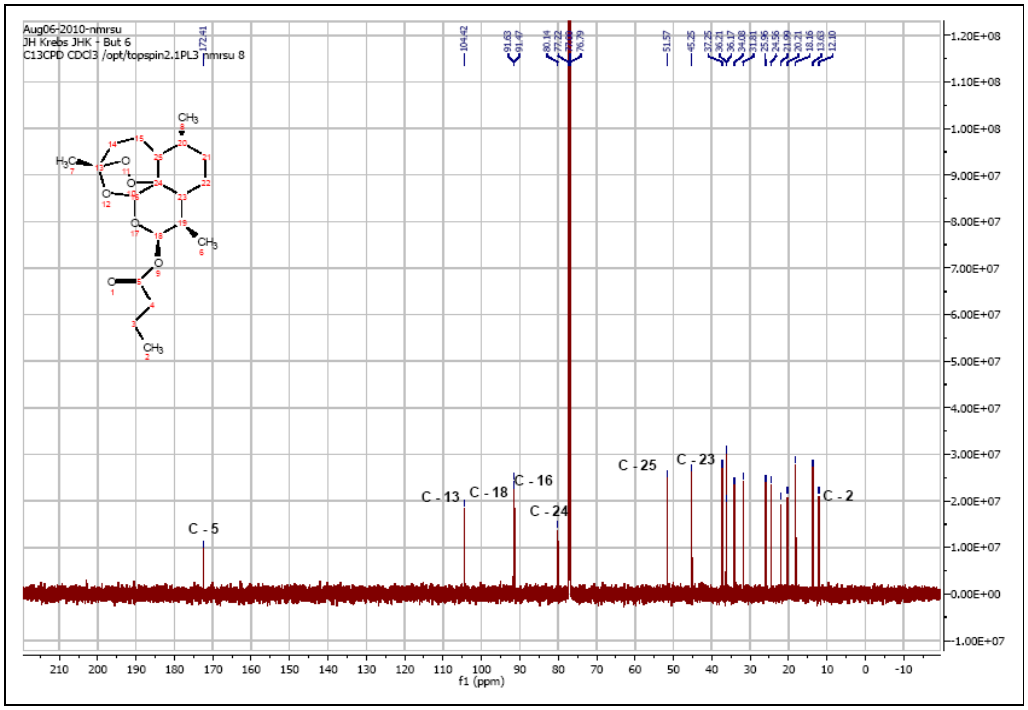
^{1}H NMR spectrum of 10α-dihydroartemisinyl benzoate



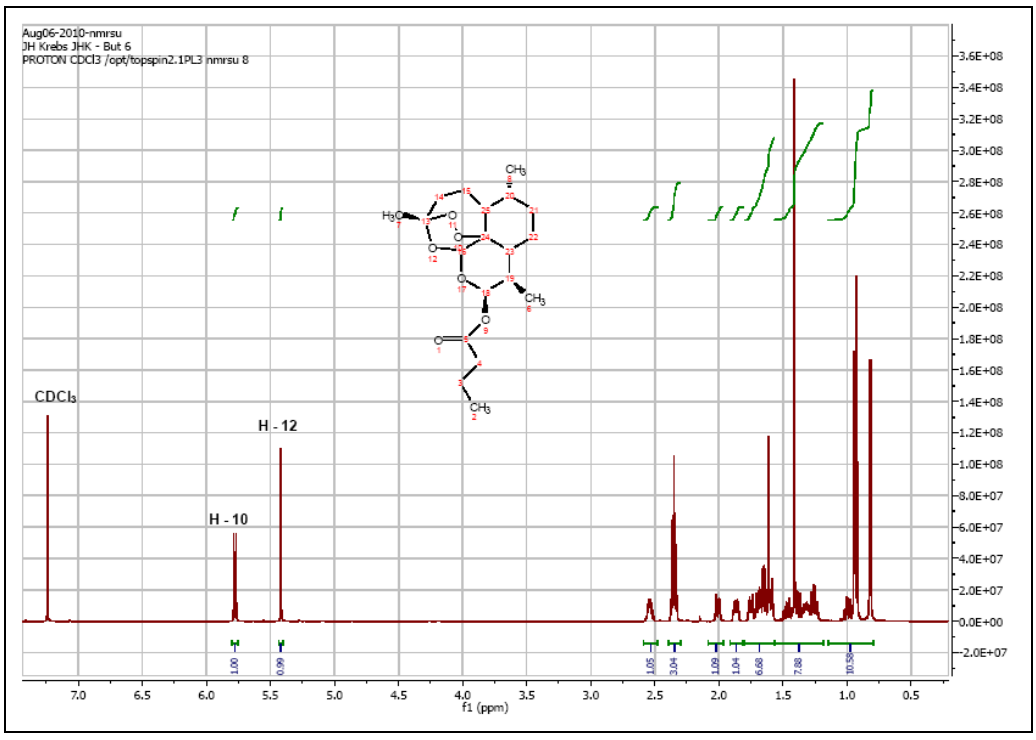
^{13}C NMR spectrum of 10α-dihydroartemisinyl benzoate



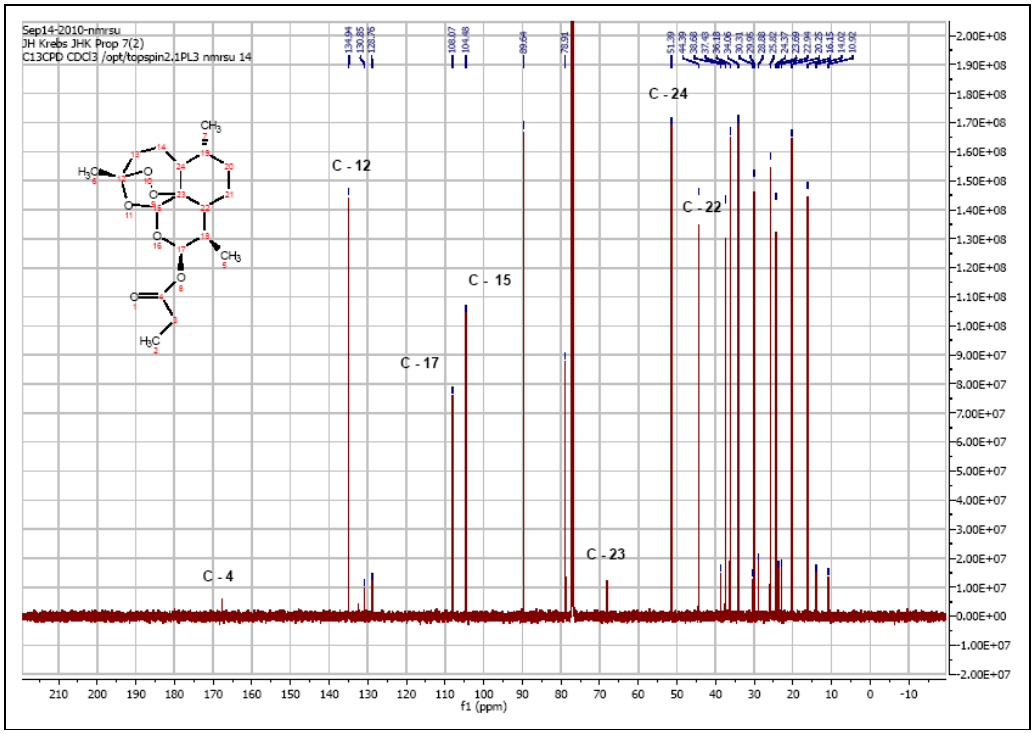
^{1}H NMR spectrum of 10α-dihydroartemisinyl butanoate



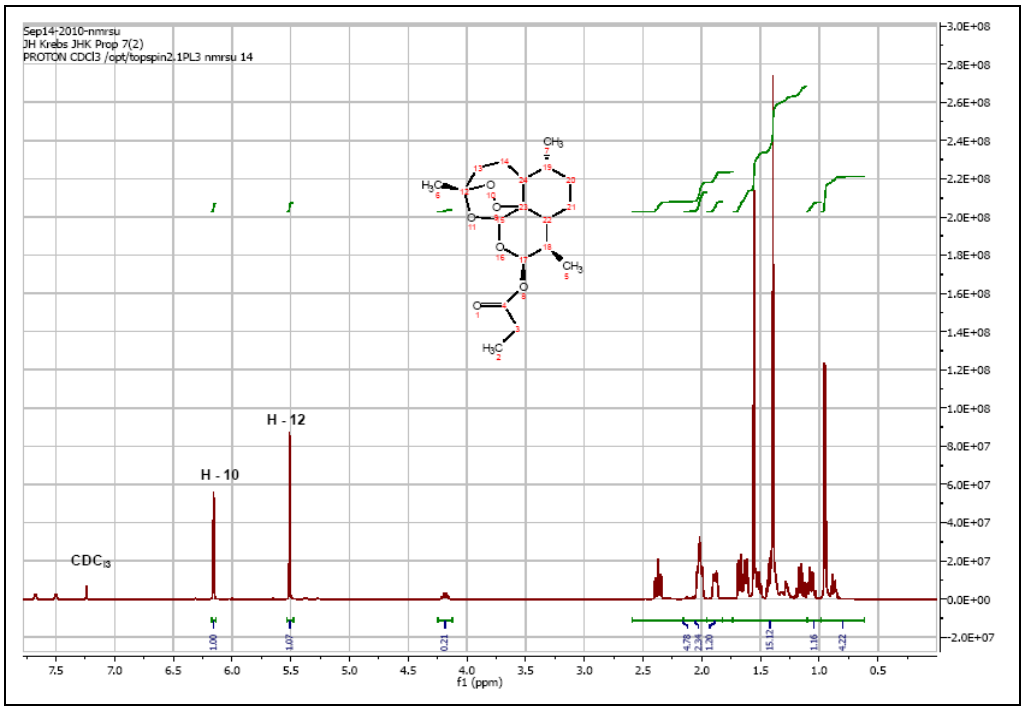
^{13}C NMR spectrum of 10α-dihydroartemisinyl butanoate



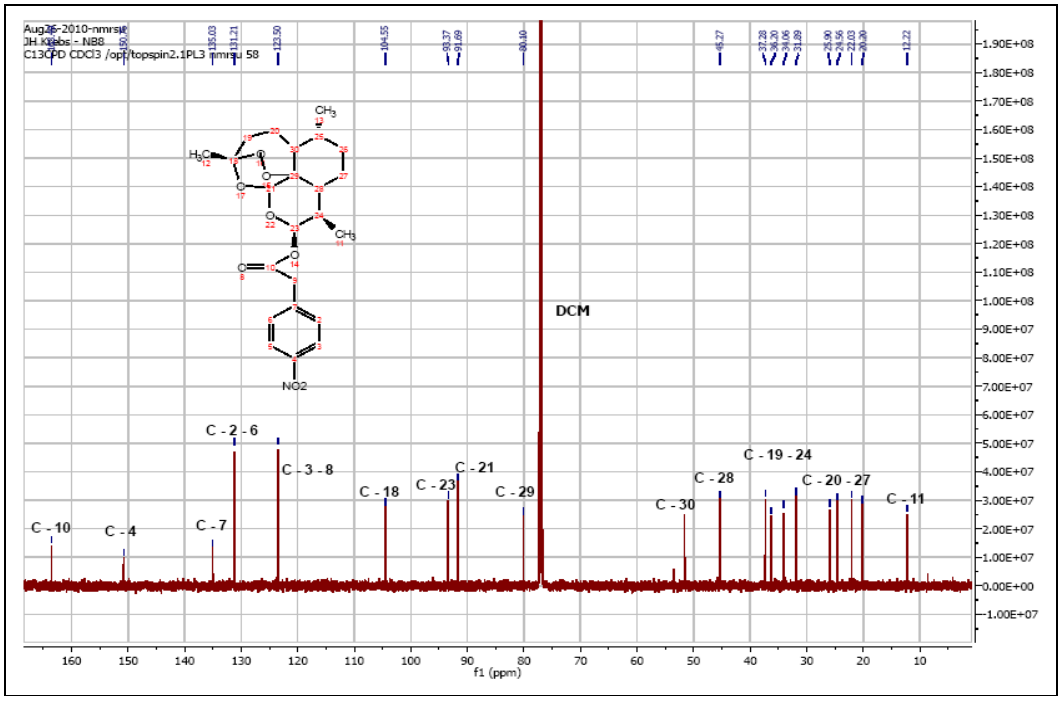
^{1}H NMR spectrum of 10α-dihydroartemisinyl propanoate



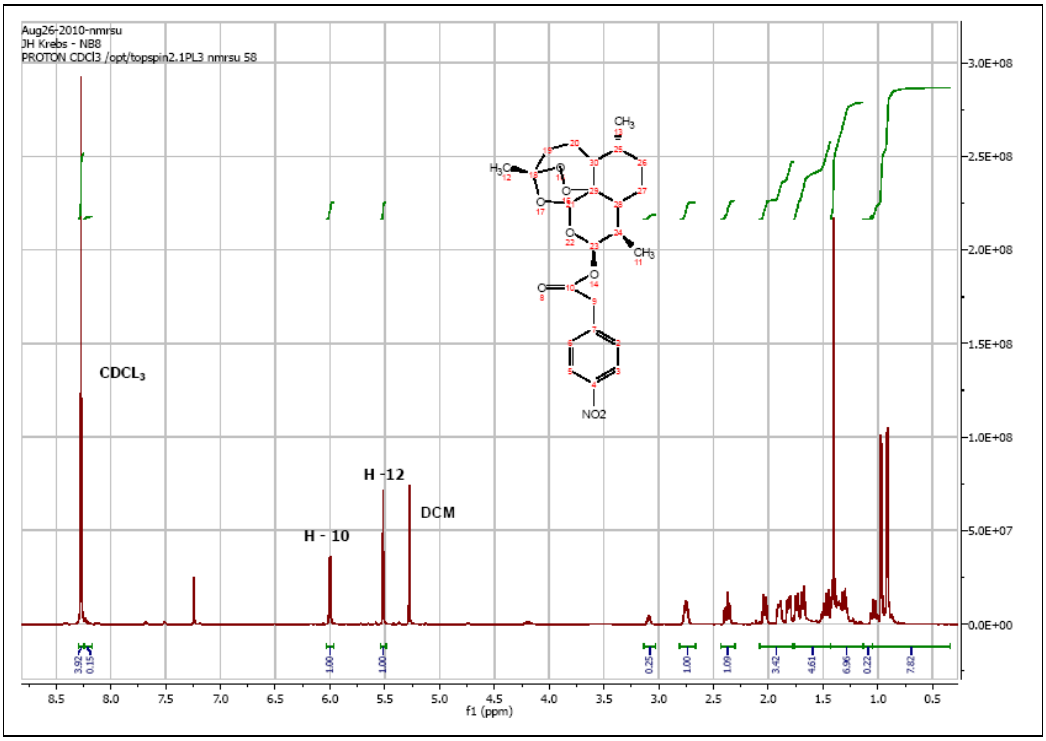
^{13}C NMR spectrum of 10α-dihydroartemisinyl propanoate



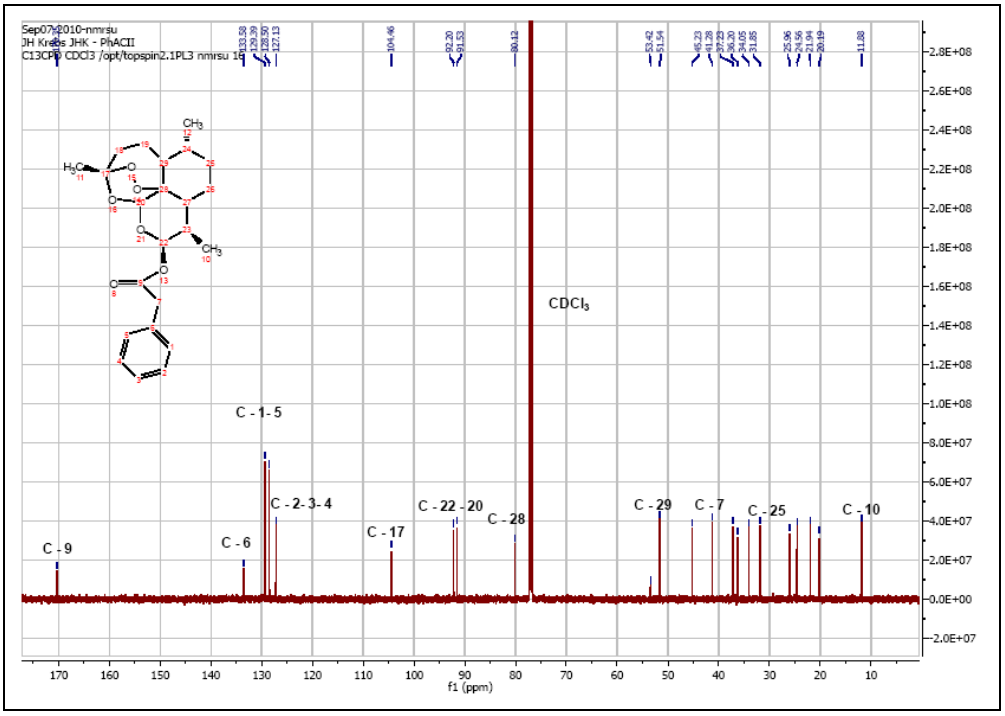
^{1}H NMR spectrum of 10α-dihydroartemisinyl 4-nitrobenzoate



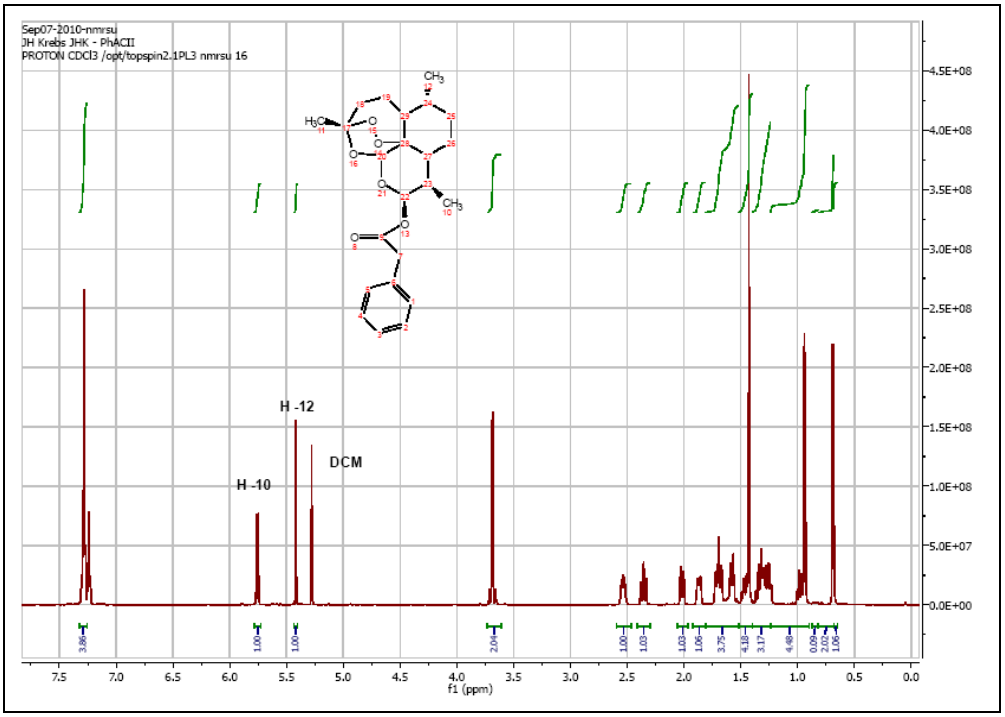
^{13}C NMR spectrum of 10α-dihydroartemisinyl 4-nitrobenzoate



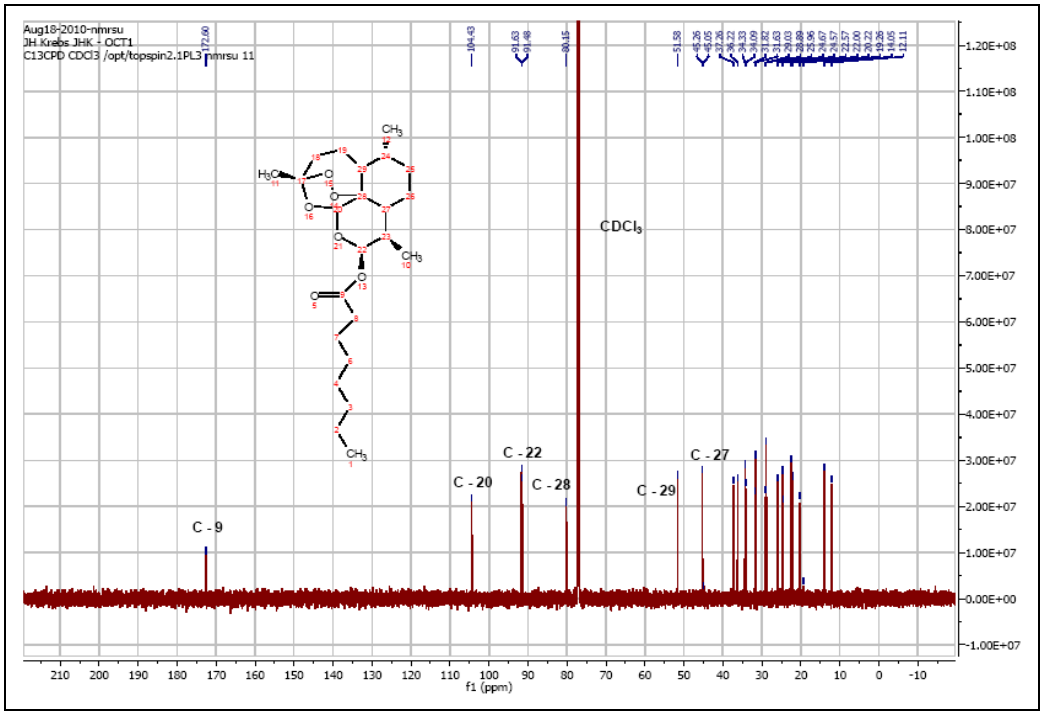
^{1}H NMR spectrum of *10α-dihydroartemisinyl 2-phenylacetate*



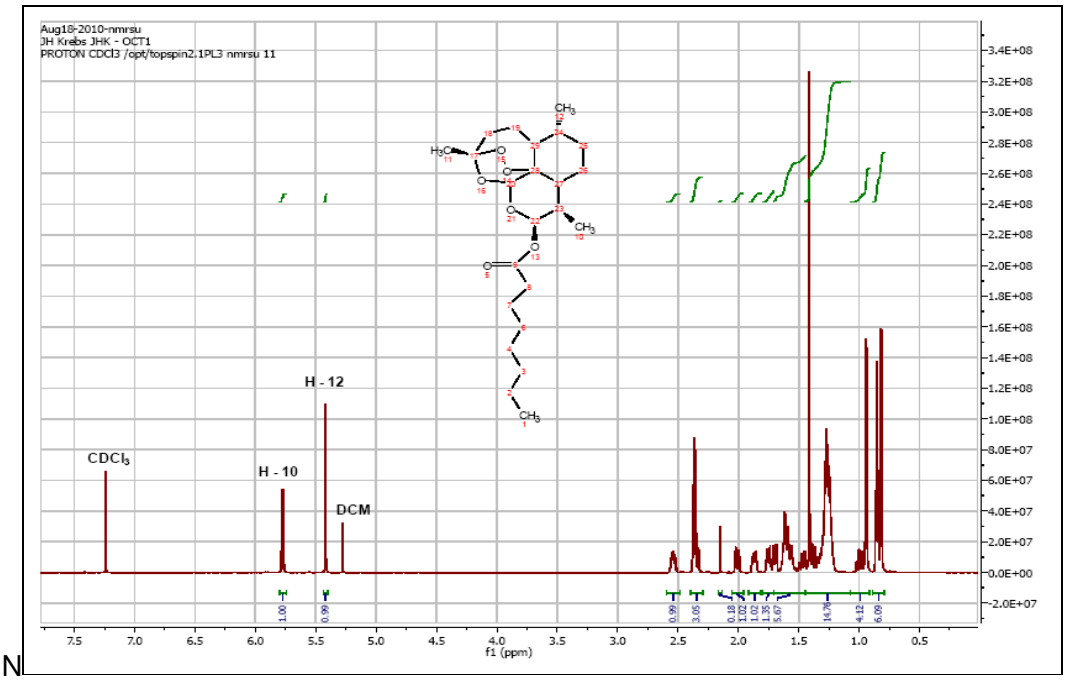
^{13}C NMR spectrum of *10α-dihydroartemisinyl 2-phenylacetate*



^{1}H NMR spectrum of 10α-dihydroartemisinyl octanoate

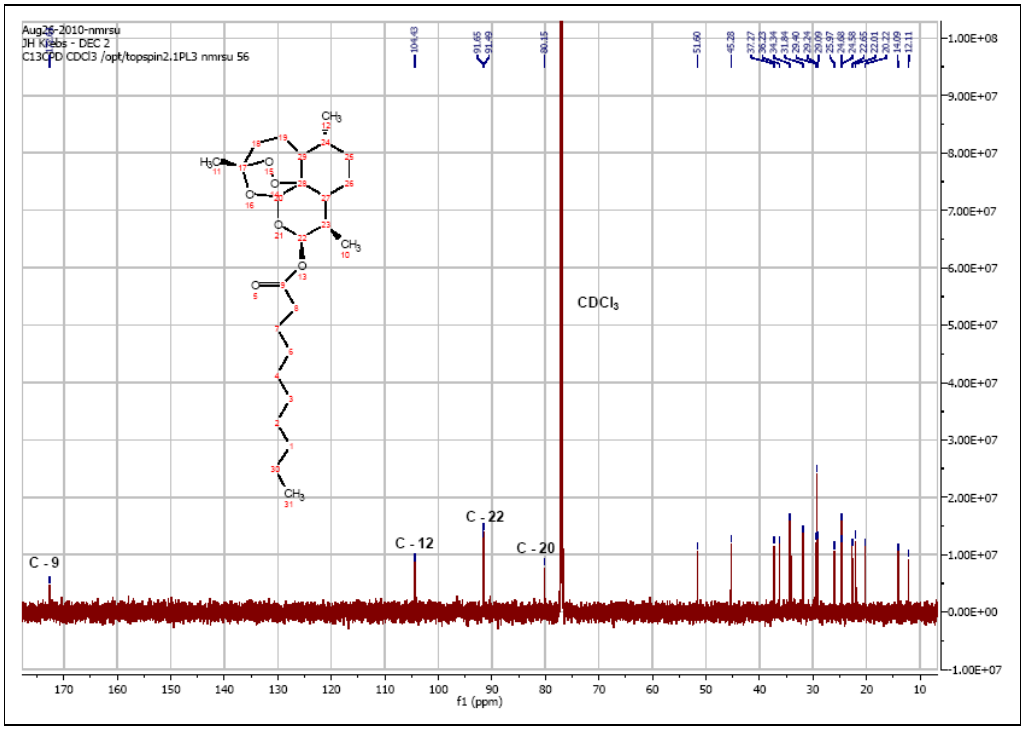


^{13}C NMR spectrum of 10α-dihydroartemisinyl octanoate

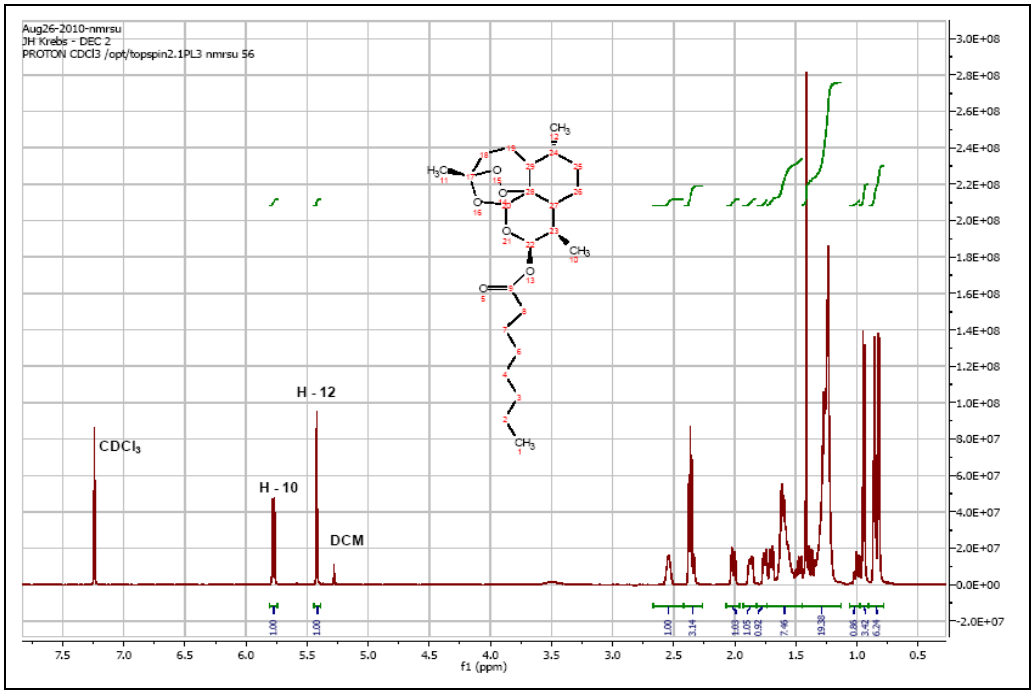


N

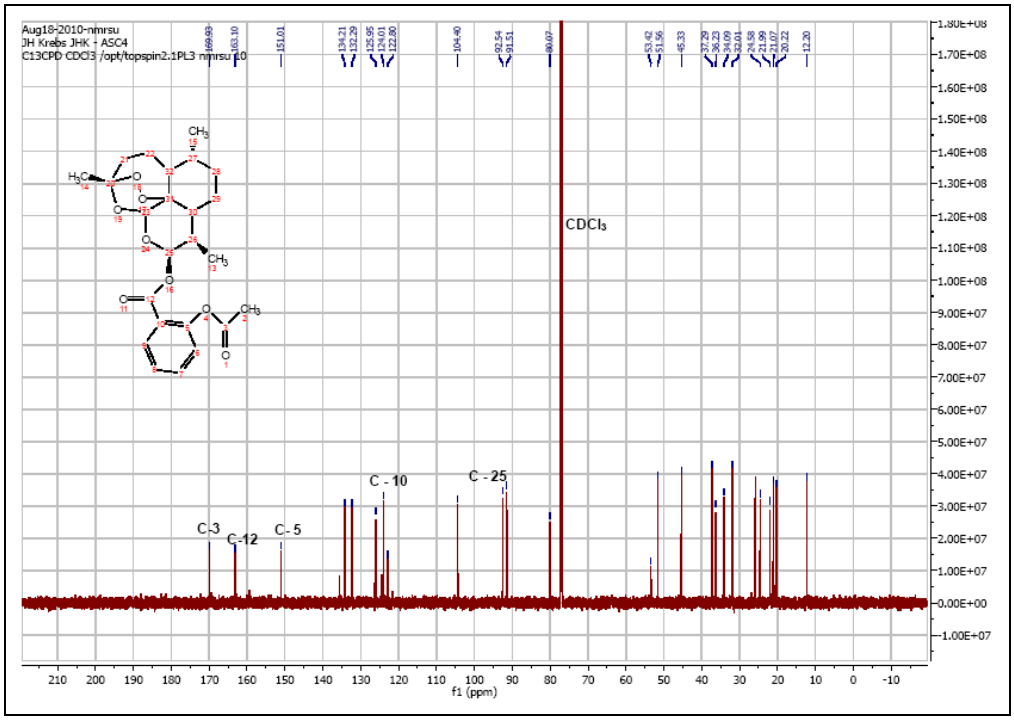
^{1}H NMR spectrum of 10α-dihydroartemisinyl decanoate



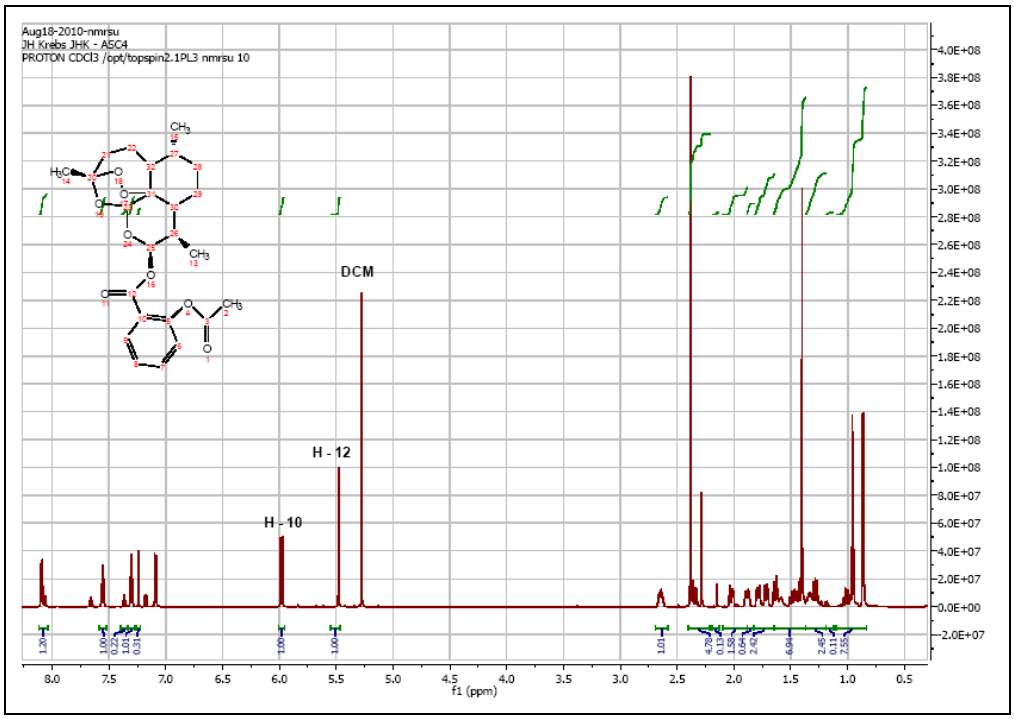
^{13}C NMR spectrum of 10α-dihydroartemisinyl decanoate



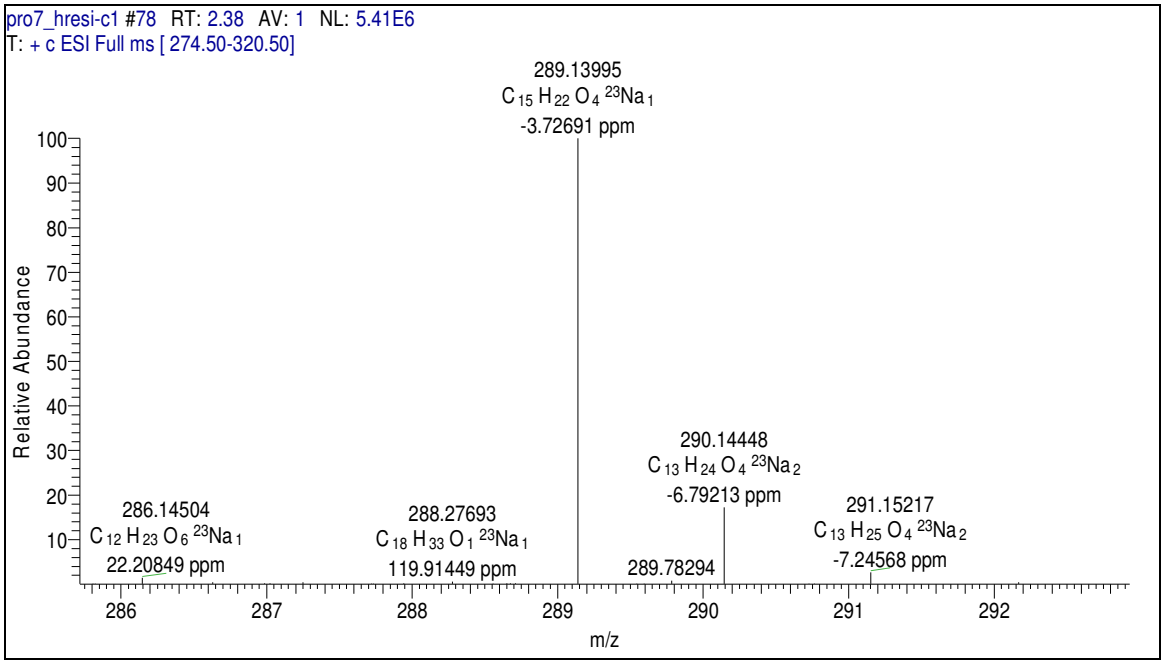
^{1}H NMR spectrum of 10α-dihydroartemisinyl 2-(acetyloxy) acetate



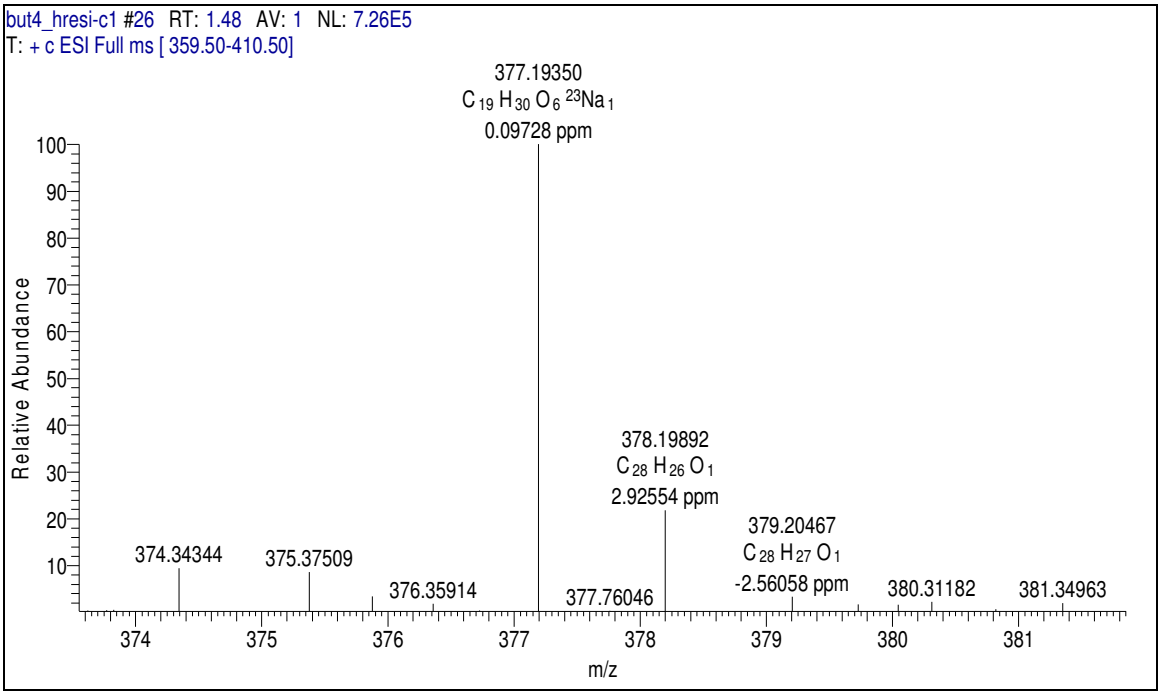
^{1}H NMR spectrum of 10α-dihydroartemisinyl 2-(acetyloxy) acetate



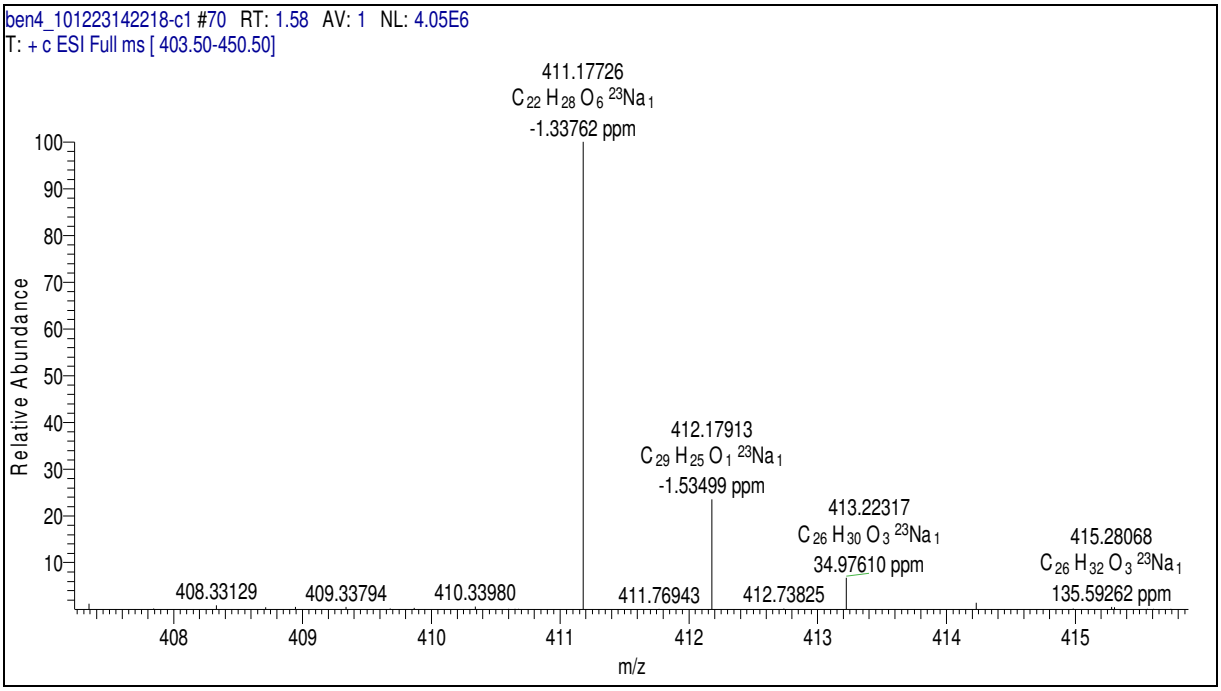
HRESI MS spectrum of spectrum of 10α-dihydroartemisinyl propanoate



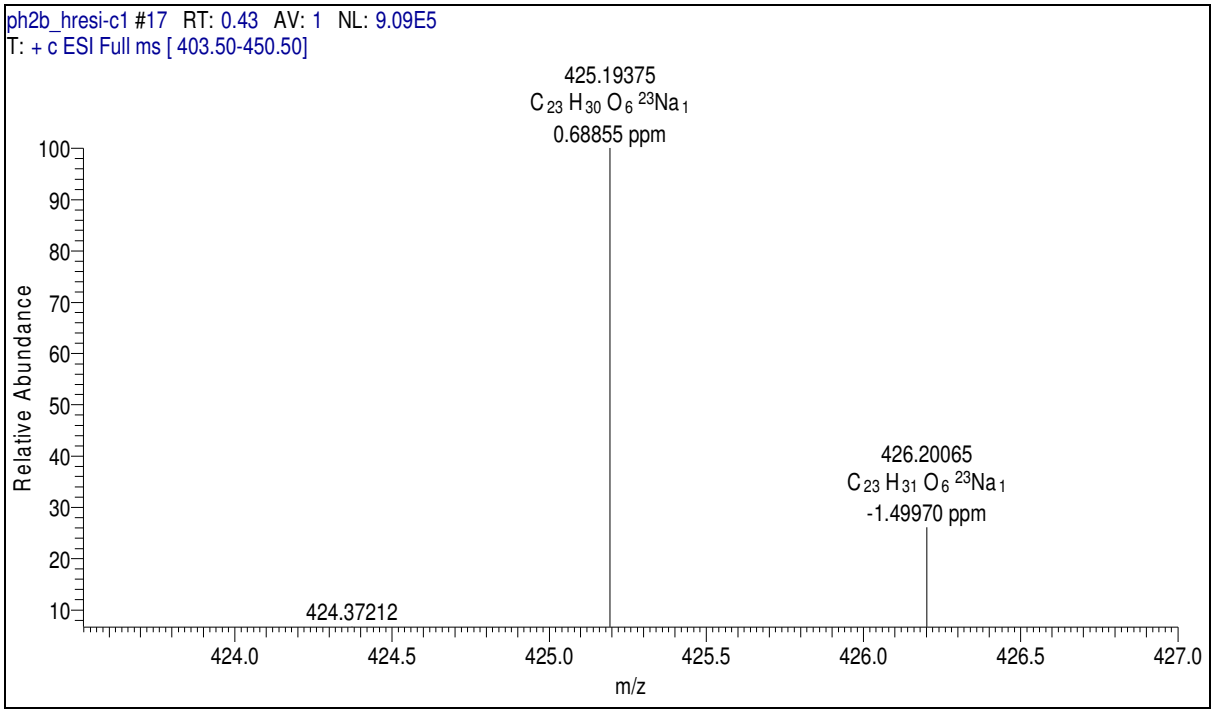
HSRESI MS spectrum of 10α-dihydroartemisinyl butanoate



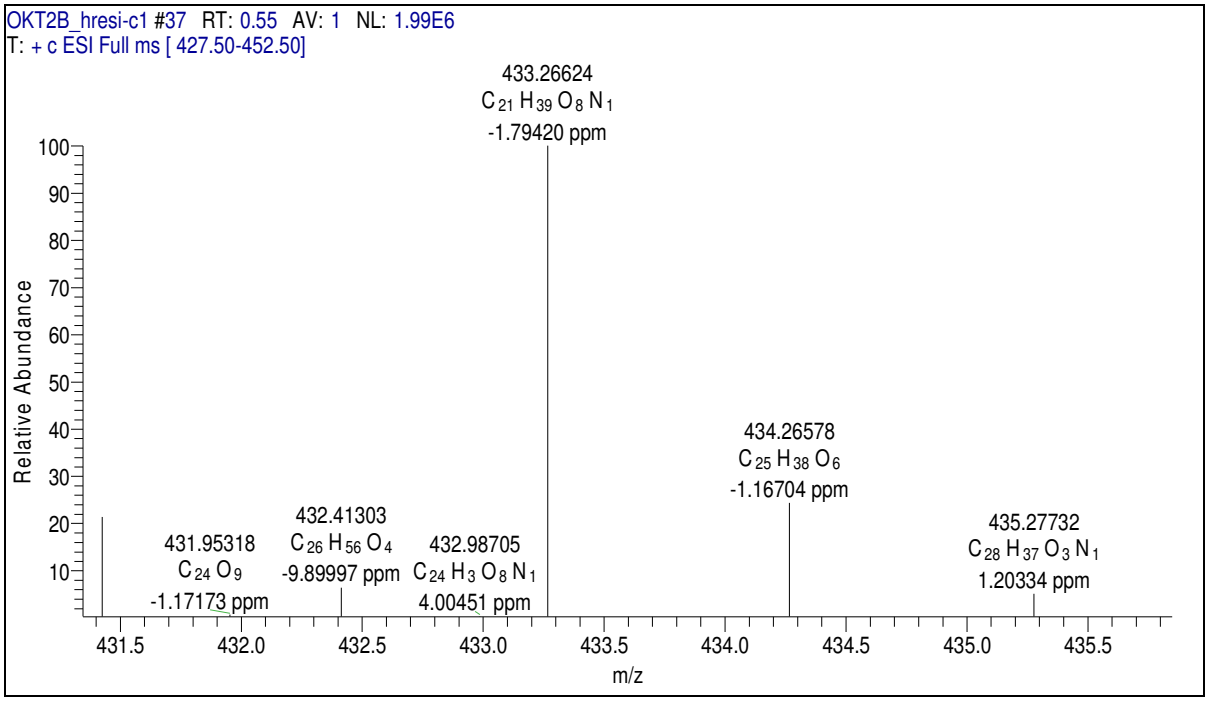
HRESI MS spectrum of 10α-dihydroartemisinyl benzoate



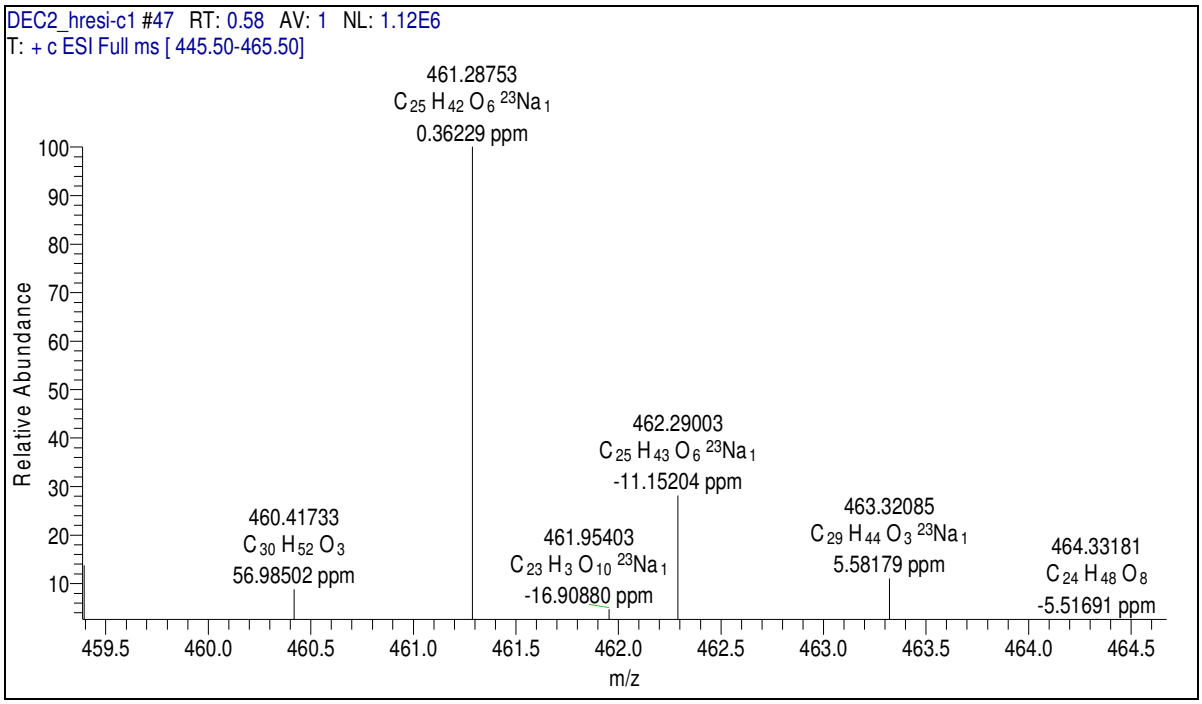
HRESI MS spectrum of *10α-dihydroartemisinyl 2-phenylacetate*



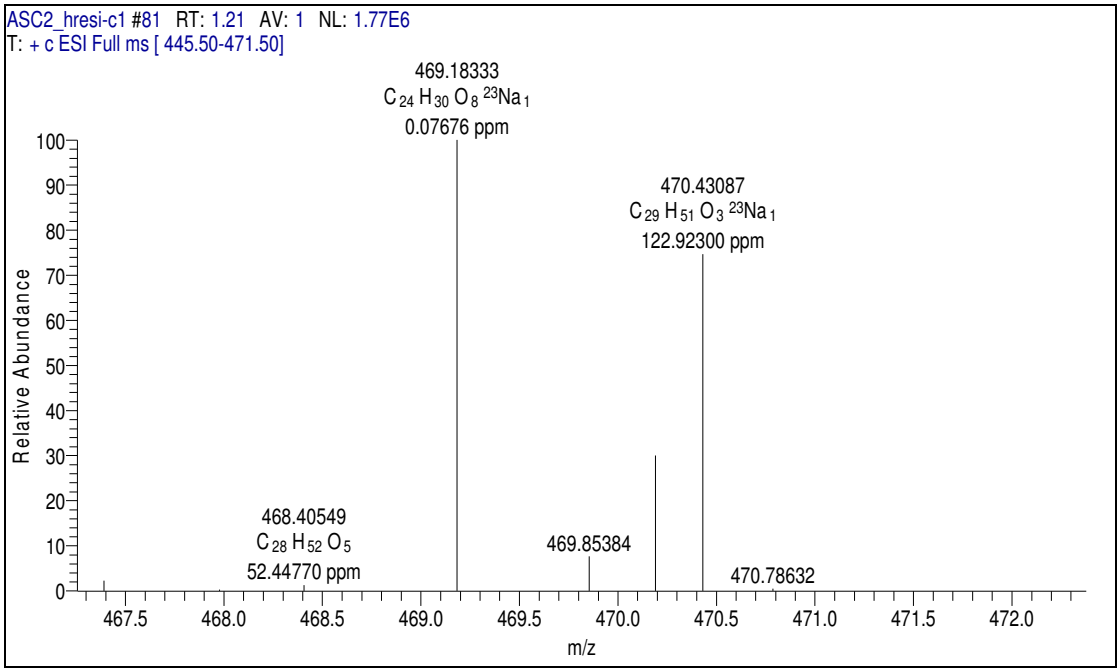
HRESI MS spectrum of 10α-dihydroartemisinyl octanoate



HRESI MS spectrum of 10α-dihydroartemisinyl decanoate



HRESI MS spectrum of 10α-dihydroartemisinyl 2-(acetyloxy) acetate



HRESI MS spectrum of 10α-dihydroartemisinyl 4-nitrobenzoate