Studies on the Molecular Mechanism of Action of Artemisinins

by

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August 2008
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This is to certify that I have examined the above MPhil thesis and have found that it is complete and satisfactory in all respects, and that any and all revisions required by the thesis examination committee have been made.

(Thesis Supervisor, Prof. Richard K. HAYNES)

(The Head of Department of Chemistry, Prof. Guochen JIA)

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Abstract

The causes and chemotherapies of malaria are briefly discussed. Among those treatments for malaria, artemisinins, the Chinese peroxidic sesquiterpene quinghaosu, is of special interest. The history, development, and particularly, mechanism of action of artemisinins are reviewed.

Despite the fact that artemisinin and its derivatives have been used to treat malaria for years, the mode of action of this compound class is still not understood, and highly controversial. In order to probe the antimalarial activity in relation one hypothesis for mechanism of action, namely binding to the calcium pump PfATP, it is appropriate to evaluate the binding of the enantiomer of artemisinin, and of artemisone, the new derivative which inhibits the pump at nanomolar level. Therefore, a preliminary study on the total synthesis of the unnatural enantiomer of artemisinin has been carried out. The methodology is based on a total synthesis of
artemisinin reported in the literature, but which uses enantiomers of the starting materials and intermediates. Cyclization of S-(−)-citronellal by the Prins reaction affords isopulegol which then is hydroborated and selectively protected at the primary hydroxyl group by triisopropylsilyl group. This was converted to the Robinson annelation precursor, (1S,3S,4R,8S)-9-[(triisopropylsilyl)oxy]-p-menthol after the Swern oxidation. However, unexpected difficulties arose in the course of conducting the conjugate addition. Different unsaturated acceptors, including methyl vinyl ketone and its α-trimethylsilyl derivative have been tried. The triisopropylsilyl protecting group was replaced by benzyl group, but still very low yields of conjugate adducts were obtained. Other means of alkylating the precursor, by using halide alkylating equivalent of MVK were briefly explored.

The idea that artemisinins may exert their activity by accelerating the consumption of crucial biomolecules involved in essential redox or cellular processes in parasites is examined. The reactions of artemisinin and its derivatives with cysteine, reduced glutathione, and other hydride donors such as NADH in the presence of electron-deficient compounds such as Methylene Blue for instance, have been monitored spectrophotometrically. Effect of artemisinins on the reduction of desferrioxamine-ferric iron complex and ferrioxamine E in the presence of ferrous iron chelating agent, BPS, are tested. Efforts to demonstrate the effect of artemisinins on redox systems including naphthoquinones and glutathione reductase have been made. In the presence of artemisinins, an intriguing finding has been shown on the spontaneous oxidation of leuco Methylene Blue. An advance in understanding the mechanism of action could be made by the observation that BNAH, a model of the crucial enzyme co-factor NADH, can be oxidized in the
presence of both ferrous sulfate and \textit{tert}-butyl hydroperoxide. However, artemisinin in the presence of ferrous sulfate was ineffective.
Chapter 1 Introduction

1.1 Introduction of Malaria

Malaria is one of the most life-threatening infectious diseases caused by protozoan parasites called *Plasmodium*. The parasite is transmitted to humans by the bite of female Anopheles mosquito. It is shocking to realize that 41% of the world's population lives in areas where malaria is transmitted and that every 10 to 30 seconds a child dies from malaria. There are approximately 300 million cases of malaria all over the world, claiming more than one million casualties. Spread of malaria depends largely on climatic factors such as temperature and humidity.\(^1\) There are four species of *Plasmodium* that can cause human disease;\(^2\) *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*. *P. falciparum*, in particular, is regarded by WHO as public health problem in Africa and South East Asia, where *Anopheles* mosquitoes, responsible for the spread of the disease,\(^3\) can survive and reproduce and malaria parasites can maintain their life cycle in the mosquitoes. *P. ovale* is the most common form of malarial while *P. falciparum* is the most severe form. The problem of malaria is gaining global concern due to the increasing resistance of *P. falciparum* to antimalarial drugs such as chloroquine. Organizations such as The US National Institutes of Health (NIH), the World Health Organization (WHO) and The Medicine for Malaria Venture (MMV) are joining for a number of programs aimed at finding new treatments for the disease and new means of controlling the mosquito vector. DDT, an effective pesticide used for killing anopheline mosquito, was abandoned because of its toxicity and environmental concerns. Since then, the population anopheline mosquito has replenished in areas where they were once eradicated. Besides, it is worth noticing that the number of imported cases of malaria has risen
up to over 2000 per year today since 1970 in Britain, largely because of the growth of global travel. The speculation that transmission of malaria may reestablish in previously endemic areas such as Europe and the United States is not totally impossible. Because of the problem of using pesticides, development of new chemotherapy is gaining weight on combating malaria.

1.2 Chemotherapy for Malaria

The plasmodium parasite has different stages of their life cycle in humans. Different antimalarial drugs that are used for treating malaria, deal with different stages of the parasites. Quinine and chloroquine kill the parasites within the red blood cells while proguanil and primaquine are proven to be lethal to the liver-staged parasites. Antimalarials, based on their structures, can be categorized into four: quinolines, antifolates, antibiotics and peroxides

1.2.1 Quinolines

Quinoline is a heterocyclic aromatic compound used as a building block for other compounds. The most famous drug of this type is quinine 1. It is a natural white crystalline alkaloid, natural occurring compounds that contains nitrogen atoms, found in the bark of Cinchona trees. Treatment of malaria with the bark has been used for several centuries. French and German chemists in about 1820 had succeeded in isolating the active principle from an alcoholic extract of the bark which the key ingredient, quinine 1, was structurally identified in 1907. Although it is still a clinical important drug for severe falciparum malaria, its use has been limited due to its side effects and short half life. Interestingly, an epimer of quinine 1,
quindine 2, is also effective for treating severe falciparum malaria but it is not widely used due its high cardiototoxicity.  

![Chemical structures of quinolines](image)

**Figure 1**: Structures of quinolines

With the success of quinine, research focus had been shifted to synthesizing variants of quinine. In fig. 1 are shown some quinolines synthesized throughout the years. Chloroquine 5, synthesized in 1934, was once the most successful drug for combating malaria. Chloroquine 5 is widely available, easy to be synthesized and relatively low production cost. Unfortunately, due to inadequate usage and widespread preventing treatment, chloroquine-drug resistance has been developed within *Plasmodium falciparum* parasites. Chloroquine usage for treating malaria is rare now. New potential uses of this cheap and widely available drug have been investigated.  

- 3 -
The antimalarial mechanism of quinoline-based drugs is still under debate. Possible modes of action include inhibiting dimerization of heme,$^{11,12}$ DNA binding and disruption of the parasite's food vacuole, a membrane-bound compartment that serve a variety of functions.

1.2.2 Antifolates

Folate, an anion form of vitamin B₉, is important for cells and tissues that rapidly divide. Cancer cells divide rapidly, and drugs that interfere with folate metabolism are used to treat cancer.$^{13}$ The success of antifolates in cancer treatment led to an exploration of this type of drugs in the treatment of other diseases, in particular parasitic diseases, that involve rapid cell division.

![Figure 2: Structures of antimalarial antifolates](image)

Antifolate agents used in the treatment of malarial infection are subdivided into two classes based on the enzymes that they inhibit. Inhibitors of dihydropteroate synthase (DHPS), and inhibitors of dihydrofolate reductase (DHFR), are the two
classes. Since DHPS and DHFR are effective by inhibiting different enzymes, combination of these two inhibitors is synergistic.\textsuperscript{14} Thus they are used in combination in the treatment of malaria. Despite the fact that the introduction of these drugs was associated with optimism, mutation of parasites under drugs stress, and the emergence of resistance to this class also have been decisive, and have made many of the drugs ineffective.\textsuperscript{15}

\subsection*{1.2.3 Antibiotics}

Antibiotics act against malaria parasites by prohibiting crucial protein synthesis within the parasites.\textsuperscript{16} Doxycycline and clindamycin (fig 3) are two most commonly used antibiotics for combating malaria.

![Structures of antimalarial antibiotics](image)

\textbf{Figure 3}: Structures of antimalarial antibiotics

Doxycycline is not permitted for use in children under the age of 8 years, the group that is most vulnerable to the disease. Although inhibition of protein synthesis is an effective means to kill parasites, a mutation changes the parasite enzymes affected by the drug in such a way that the antibiotic can no longer inhibit it. This is the main mechanism of resistance to the compounds that inhibit protein synthesis, such as the doxycycline antibiotics. Added to the problem, this form of resistance is transmitted genetically by the parasites to its offspring.
1.3 Artemisinins

1.3.1 Origin

Artemisinin, a key ingredient of an herbal treatment called Qīng hāo (青蒿), has been used to treat malaria, fever and chill in China for years. Its usage even can be traced back to Han dynasty (168 B.C.). An initiative was launched by Chinese government aimed at finding a treatment for malaria. About 200 traditional Chinese medicines were screened against malaria and Qīng hāo was the only one found to be effective. Since then, research focus had been turned to identifying the key principle of Qīng hāo. Eventually in 1971 at the Pharmaceutical Institute of the Academy of Traditional Chinese Medicine in Beijing scientists, screening candidates against rodent malaria parasites, successfully demonstrated that an extract of qīng hāo had shown a positive activity.\textsuperscript{17,18} In 1973 the active principle, named qīng hāo su compound 16 (Figure 4), was isolated. A research group, named the 'Qinghaosu Antimalaria Coordinating Research Group', was founded. All research aspects of Qinghaosu had been covered - from agricultural production of qīng hāo, pharmaceutical and clinical aspects, and chemistry and structure of qīng hāo su.\textsuperscript{17,19,20} The complete structure was elucidated in 1977.\textsuperscript{21,22} It became known as artemisinin.
1.3.2 Structure Determination

As it was found and developed, the structure of artemisinin 16 was deduced by Chinese scientists.\textsuperscript{20,23} Artemisinin 16 is a sesquiterpene lactone. Artemisinin 16 consists of a fused ring system incorporating a lactone ring and a 1,2,4-trioxane system (Figure 5), which is the pharmacophore for the antimalarial. Other naturally occurring peroxides which lack the third non-peroxidic oxygen atom are less active towards malaria.\textsuperscript{24,25}

Figure 4: Structure of artemisinin and its derivatives

Figure 5: Structure of artemisinin (qing hao su) with numbering according to CAS.
1.3.3 Physical Properties

The chemical and physical data is summarized in table 1 below.

<table>
<thead>
<tr>
<th>Table 1: Chemical and Physical data of artemisinin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Formula</strong></td>
</tr>
<tr>
<td><strong>Mol. Mass</strong></td>
</tr>
<tr>
<td><strong>Water solubility</strong></td>
</tr>
<tr>
<td><strong>Density</strong></td>
</tr>
<tr>
<td><strong>Melt. Point</strong></td>
</tr>
<tr>
<td><strong>Optical rotat.</strong></td>
</tr>
</tbody>
</table>

Artemisinin itself has a poor solubility in water or oil. So its derivatives have been designed in order to enhance its properties as a drug. Some, like artesunate ²⁰, are synthesized to be more water soluble while some are aimed to display a better oil solubility, such as artemether ¹₈ and arteether ¹⁹.

1.3.4 Antimalarial activities of artemisinin and its derivatives

Artemisinin has a very short half life and a fast rate of metabolism rate in the human body.²⁰,²⁷ It means that reappearance and growth of parasites in patients will occur after a short period of treatment if parasites have not been completely eradicated.¹⁷,¹₈,²⁸,²⁹,³⁰,³¹,³₂ These factors combined show that artemisinin itself is not a perfect treatment for malaria. Structural modification of artemisinin has been carried out in attempts to make the drugs better antimalarial, which also could last longer in the human bodies and be easier to administer.
Table 2: *In vitro* activities (IC$_{50}$ ng/mL) of artemisinins against *P. falciparum*

<table>
<thead>
<tr>
<th>Compound</th>
<th>D6$^{33}$</th>
<th>D6$^{34}$</th>
<th>W2$^{33}$</th>
<th>W2$^{34}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artemisinin 16</td>
<td>6.41</td>
<td>2.33</td>
<td>10.53</td>
<td>1.21</td>
</tr>
<tr>
<td>DHA 17</td>
<td>1.79</td>
<td>0.11</td>
<td>1.83</td>
<td>0.04</td>
</tr>
<tr>
<td>Artemether 18</td>
<td>3.34</td>
<td>-</td>
<td>4.19</td>
<td>-</td>
</tr>
<tr>
<td>Arteether 19</td>
<td>2.94</td>
<td>0.87</td>
<td>4.07</td>
<td>0.16</td>
</tr>
<tr>
<td>Artesunate 20</td>
<td>1.66</td>
<td>-</td>
<td>2.18</td>
<td>-</td>
</tr>
</tbody>
</table>

In Table 2 is summarized the *in vitro* activities of artemisinin and its derivatives. The activity is judged by the IC$_{50}$, a measure of the effectiveness of how much of a drug is needed to inhibit a given biological process by half. It can be seen from the table that most of the derivatives possess a high antimalarial activity. Although *in vitro* activity shows a good guide for screening drugs, important factors such as the route of administration and metabolism have not been taken into account.

1.3.5 Pharmacokinetics and Metabolism

Pharmacokinetic deals with the absorption, distribution, metabolism and excretion of artemisinins with human body. Usually the concentration of artemisinins in humans is at about a maximum within 1-3 hours after administration, if metabolism or hydrolysis of DHA is taken into account.$^{35,36}$ Among the derivatives, artesunate 20 is the most unstable, and is metabolized (hydrolysed) *in vitro* to DHA, a major metabolite of most artemisinins derivatives, at physiological pH.$^{37}$ Because of the instability, specific procedure for administration of artemesunate 20 is required to prevent the drug from deteriorating.$^{38}$ Table 3 shows some
pharmacokinetics parameters of artemisinin 16 and artesunate 20. Both drugs were taken orally as 250-mg tablets by healthy volunteers.

**Table 3: Pharmacokinetics parameters of artemisinin 16 and artesunate 20**

<table>
<thead>
<tr>
<th></th>
<th>Artemisinin</th>
<th>Artesunate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Max. Drug conc.</td>
<td>0.36 µg/ml</td>
<td>appearance rate constant 2.11 hr⁻¹</td>
</tr>
<tr>
<td>Peak time</td>
<td>100 min</td>
<td>elimination rate constant 1.18 hr⁻¹</td>
</tr>
<tr>
<td>Appearance half-life</td>
<td>0.62 hr</td>
<td>biotransformation half-life 0.33 hr</td>
</tr>
<tr>
<td>distribution half-life</td>
<td>2.61 hr</td>
<td>elimination half-life 0.65 hr</td>
</tr>
<tr>
<td>decline half-life</td>
<td>4.34 hr</td>
<td></td>
</tr>
</tbody>
</table>

As for metabolism of artemisinins, Artemisinin 16 is oxidized (phase I metabolism) by members of the P-450 enzyme family. Artemether 18 and arteether 19 are metabolized by CYP3A4, a member of the P450 enzymes mixed-function oxidase system which oxidatively removes the methyl or ethyl groups to give DHA. Rearranged products and products resulted from extracting an oxygen atom from peroxide bridge are found in trace amounts. Formation of these products does not necessarily involve enzymes. These products are formed in the intracellular fluid of liver cells.
**Figure 6:** Regiochemistry of metabolic hydroxylation of artemether and arteether to give products with hydroxyl groups at positions 5, 5a, 7 and 14 based on rat liver microsomal and microbial studies.

Dealkylation of the ethers and hydroxylations at positions 5, 5a, and 7 by P-450 lead to products which the newly added hydroxyl group is on the same side as the peroxide group while the peroxide is unchanged (Fig 6). It is worth noting that the hydroxylated metabolites are still effective for treating malaria, as the 1,2,4-trioxane system is left intact. The hydroxylated metabolites, however, may be subjected to conjugation of water-soluble groups via a phase II metabolic pathway.

### 1.3.6 Toxicity of Artemisinins

Artemisinins are toxic to living cells, both mammalian and parasitic cells. However, the cell types respond differently to artemisinins. Mammalian cells are more resistive to artemisinins than parasitic cells. Only a nanomolar concentration of artemisinins will be lethal to parasitic cells while usually micromolar concentrations are required to have the same effect to mammalian cells. In other words, parasitic cells are more vulnerable to artemisinins; this can be explained by the much greater efficiency of uptake of drug into the parasitized erythrocyte. The neurotoxicity of the major metabolite of artemisinins, DHA 17, is of concern, given
the proven neurotoxicity of artemisinin-type drugs in animal models.\textsuperscript{50,51} In an assay of the measuring the effect on viability and on neurofilaments of primary neuronal cells of rats and dogs,\textsuperscript{52} DHA shows a high neurotoxicity, even a concentration at which is effective against \textit{P. falciparum} in vitro. The pharmacophore of artemisinins, 1,2,4-trioxane system, is not exclusively responsible for the neurotoxicity. By comparing structures and activities of artemisinins derivatives, derivatives that are substituted at position C9 and C10, and stereoisomers at C10 have varying levels of toxicity. 10-Deoxoartemisinin shows a lower neurotoxicity than its parent compound. Some argue that radical production and inhibition of respiratory chain are possible causes of the neurotoxicity.\textsuperscript{52} High doses of artemisinins can produce neurotoxicity such as gait disturbances, loss of spinal and pain response and respiratory depression. These had raised people’s concern and definitely are an obstacle for artemisinins drugs development. Despite these, artemisinin derivatives rarely cause adverse effects in patients. No evidence for serious adverse effects has been found in a study of over 3500 patients in Thailand,\textsuperscript{53,54} but in no study was a long term follow up of patients carried out. Neurotoxicity is a delayed phenomenon, and is observed to take place in neuronal cells once drug is withdrawn.
1.4 Mechanism of Drug Action of Artemisinins

1.4.1 Introduction

Artemisinin 16 is an enantiomerically pure sesquiterpene lactone bearing an endoperoxide function that has been proven to be essential for antimalarial activity. The compounds obtained by removing an oxygen atom from the peroxide bridge or replacing one of the peroxidic oxygen atoms by carbon are totally inactive towards malaria parasites. Fully synthetic compounds that share the benefits of artemisinins without its disadvantages and which can be made at low cost are highly desirable. Clearly future development of new compounds requires a full understanding of mechanism of artemisinins inside infected patients. Although the mode of action of artemisinins is not well understood, it is apparent that artemisinins do not share the same mechanism with other antimalarials.55

1.4.2 Artemisinins in infected red blood cells

Antimalarial drugs mostly are stage-specific, except for artemisinins, which act on several stages. So the life cycle of Plasmodium parasites will be briefly discussed here. Once a person is bitten by an infected female Anopheles, Plasmodium parasites in the sporozoite stage will accumulate in liver cells (hepatocytes). The sporozoites undergo massive asexual reproduction, producing up to 30,000 daughter cells from each sporozoite called merozoites. The infected hepatocytes rupture, and the merozoites are released into the blood stream, to invade red blood cells for the next phase of maturation. After a few days, the merozoites mature through several stages into schizonts, which then cause rupture of the infected
erythrocyte, releasing thousands of merozoites which re-invade erythrocytes. It is this rupturing of red blood cells and reinvasion which causes the periodic fever in the infected patients. Parasites inside the infected red blood cells feed on the host’s hemoglobin and digest up to 30% protein in the cells. The protein is a source of amino acid for the parasites and the heme residues are converted by a biomineralization, enzyme-independent process, into hemozoin, a redox inactive ferric ion-heme compound, which is insoluble in physiological condition and ends up accumulating in the food vacuole of the parasites.\textsuperscript{56,57} A small amount of heme may be used by the parasites for its iron- metalloenzymes.

1.4.3 'Prodrug' Hypothesis

1.4.3.1 General

A prodrug is a pharmacological substance that is administered in an inactive or significantly less active form. Once administered, the prodrug is metabolised in vivo into an active metabolite. Carbon-centred radicals, carbocations, and/or stable end-products have been proposed as parasiticidal metabolites. In the past decades, there seems to be a general consensus established, that does not imply scientific reality, supporting the mechanism that artemisinins-derived radicals damage specific target of the parasites. A huge amount of publications have claimed that it has sufficient evidence to demonstrate the relationship between antimalarial activity and the active metabolites. Nonetheless, the hypothesis has left much to be desired. Further investigation should be carried out to confirm the mechanism that many subscribe to.
1.4.3.2 Artemisinins as Prodrugs

To many researchers, artemisinins themselves are not antimalarial active, but rather act as prodrugs which are required to be activated after being administrated. A putative theory of artemisinins activation requires the presence of ferrous iron. Many believe that ferrous iron is necessary for artemisinins to generate the antimalarially-active intermediates, which will be discussed in detail later on. Research has focussed on the nature of the 'activators' such as ferrous iron, where these activators are in the parasite, and the source of ferrous iron in infected cells. One of the obvious targets is the food vacuole (membrane-bound compartments) of the parasite. Digestion of haemoglobin and detoxification of haem by deposition in haemozoin occurs inside the food vacuole. After encountering ferrous irons, either 'free', that is, not incorporated in the haem, or haem-ferrous irons, unstable intermediates such as carbon centered radicals or stable end products are stated to be formed. The intermediates are thought to be lethal to parasites by reacting with vital biomolecules inside it while some claimed the end products are antimalarial active. This 'prodrug' theory of parasiticidal activity has been considered to be a 'random' process which is opposed to binding of intact artemisinin molecules to an active site without any activation.

1.4.3.3 Reductive Cleavage of Peroxides

It has been demonstrated that the endoperoxide bridge in artemisinins is necessary for its antimalarial activity. Peroxides are a known source of reactive oxygen species such as hydroxyl radicals and superoxides. This arouses the possibility of free radicals being involved in the mechanism of action. Free radicals
scavengers such as catalase, dithiothreitol, and α-tocopherol had been shown to antagonize the in vitro antimalarial activity of artemisinins. In contrast, free radicals generators such as riboflavin, miconazole and doxorubicin enhanced the activity.\textsuperscript{58}

How does the endoperoxide bridge break down to form radicals? The Fenton reaction,\textsuperscript{59, 60} generates oxygen centered, or alkoxyl radicals, and then carbon centered radicals and neutral product by reductive cleavage of peroxides. It is believed by groups of researchers that it is the reductive cleavage of the endoperoxide in artemisinins with ferrous ions generating free radicals is the key of antimalarial activity.

1.4.4 Non-specific, proposed mechanisms of action

As mentioned, artemisinins have different sensitivities towards infected red blood cells and normal (non-parasitized) cells. This difference can be explained by artemisinin drugs accumulation. It has been reported that artemisinins can accumulate in infected red blood cells. About 35% to 40% of partitioning of artemisinins in non-parasitized cell was observed. This accumulation is because of passive diffusion of the drug through the cell membrane. In contrast, partitioning of the same drug inside parasitized red blood cells is up to 55% to 60 %.\textsuperscript{61} This finding accords with an earlier study that DHA has a much higher accumulated concentration in infected red blood cells than in the normal one.
1.4.4.1 Activation by 'free' ferrous iron

As 'free' ferrous iron-decomposition of artemisinins is being considered, reductive cleavage of peroxides by ferrous iron (the Fenton reaction) is noticeable and has been discussed. The decomposition of artemisinins and its analogues has been intensively examined, but has also has prompted too much speculation over mechanism of action.\textsuperscript{62,63,64,65,66,67} The details for artemisinins decomposition are depicted in Fig 7.

Two different alkoxyl radical intermediates, labeled 'O-2' and 'O-1' radicals, are generated from cleavage of the peroxides by ferrous iron. Firstly, in the 'O-2' path, the radical very likely undergoes cleavage of the C3-C4 bond ('α-cleavage'). A 'sec0' C-4 radical \textbf{23} would be generated. The relatively stable end product \textbf{25}, formed from extrusion of ferrous iron, is not antimalarial active.
Figure 7: Pathways proposed for activation of artemisinin by ferrous iron leading to the seco C4 radical 23 and C4 radical 24 and the end-products 25 and 27. Neither 25 nor 27 possess antimalarial activity, and thus antimalarial activity is claimed to reside in the radicals 23 and 24 by their reacting with 'sensitive biomolecules' in the parasite.

In contrast, in the O-1 path, a proton from C-4 would be abstracted by the oxygen radical which leads to the formation of 4-hydroxydesoxoartemisinin 27 through an unstable epoxide 26. Compound 27 shows no antimalarial activity. Despite the fact that compound 27 has been claimed to be a powerful alkylating agent and toxic to cells, the claim has been criticized.68,69,70 It is worth noticing that oxidation states of iron or of artemisinins have not changed. This means the formation of compound 25 and 27 is a catalytic reaction triggered in the presence of ferrous iron. The same isomerization could partially be done without iron by heating.
artemisinins at a rather high temperature for a short period of time (190 °C for 10 min). \(^71\) ESR studies of the carbon centered radicals have been carried out by using spin-trapping agents providing relatively stable radicals which are long lived enough for detection. \(^62,65,66,72\)

1.4.4.2 Activation by haem ferrous iron

Haem is thought to be another 'activator' of artemisinins.

Malaria parasites from the early trophozoite stage within the infected erythrocyte would use up haemoglobin of the host as a source of amino acids, which takes place at low pH of about 4 in the digestive food vacuole. \(^73\) In this feeding process, ferriprotoporphyrin IX (haem), will be released, which will dimerize to form haemozoin \(^28\). Haemozoin is insoluble in physiological environment and is highly crystalline. Most of the haemoglobin will be consumed and the released haem will be converted into haemozoin although a small fraction of haem is reported to be degraded by other pathways. \(^12\) The food vacuole is concentrated with haemozoin, which is the only detectable iron source in trophozoites. Moreover, the food vacuole contains almost 92% of the total amount of iron in *P. falciparum* trophozoites. Among the 92% iron source, 88% is in the form of haemozoin. In other words, the food vacuole of the trophozoites is flooded with haemozoin.
Both haemozoin and haem have been the focus of 'activation' studies. To make the 'activation' more plausible, the food vacuole, which contains haemozoin, is assumed to be a target of artemisinins. Reaction of artemisinins and haem has been studied, either within the parasites or in a chemical environment. Results show that artemisinins form an adduct with haem. To react with artemisinins, iron in haem has to be at the ferrous state. It has been claimed that the ferrous iron in haem could promote catalytic decomposition of artemisinins but this cannot be proven.\textsuperscript{74,75} Besides, the haem-artemisinin adduct found and isolated from parasites was verified to be non-toxic to the malaria parasite. One of the covalent adducts isolated from the laboratory reaction of artemisinin with Fe (II) protoporphyrin IX dimethyl ester followed by demetallation is compound 29.\textsuperscript{76,77} The others are formed by reaction of artemisinin at the \(\alpha\) and \(\gamma\) positions.
The adduct could be obtained by reacting artemisinin with ferrous haem dimethyl ester (Fe(II) protoporphyrin IX dimethyl ester) under highly reducing conditions in an aprotic solvent under an inert atmosphere – 10 equivalents of 2,3-dimethylhydroquinone in dichloromethane under argon - followed by removal of iron.\textsuperscript{76} It is also reported that the same adduct could be made using a large excess of glutathione in dimethyl sulfoxide at 37°C. As mentioned before, those conditions can not resemble biological system. There is no evidence that the adducts formed in vitro are structurally identical to those formed inside the parasites. Mass spectrum of the in vitro adducts has been compared to that formed in vivo. However, little can be known merely from comparing the mass spectra.\textsuperscript{77}

Protease inhibitors are enzymes that inhibit proteolysis (protein degradation). Hemoglobin degradation can be inhibited by using a protease inhibitor. Those antimalarials that exert their antimalarial action by binding to haem, like chloroquine, will be inhibited as less haem is available for binding. Intriguingly, protease inhibitors neither antagonize nor synergize artemisinin activity.\textsuperscript{104} In addition, screening of infected cells with fluorescent labeled artemisinins derivatives has demonstrated that the artemisinin derivatives distribute throughout the parasites
instead of localizing in the food vacuole, which is supposed to be the site of action in the haem activation idea. This distribution of artemisinins is in a good harmony with the results using electron microscopy.\textsuperscript{78} There is further proof that haem iron does not suffice to account for the antimalarial action of artemisinins. A group of researchers\textsuperscript{79} tested the role of haem iron by varying the culture conditions of the parasites. Two varied conditions have been tested. The first one is to increase oxygen concentration from 1% to 20% in which activity of artemisinin has been increased by 20-30%. However, if the parasites are cultured in an atmosphere containing 2% carbon monoxide, a 40-50% increase in antimalarial activity of artemisinin is noted. Under these conditions, any ferrous haem will be converted into the stable carbon monoxide adduct, which is presumably inert. The increased oxygen pressure will also result in less ferrous haem being present. The results are not compatible with the haem activation idea.
Figure 8: Proposed reductive scission of the endoperoxide by ferrous haem [Fe(II) protoporphyrin IX] leading to the seco C4 radical 23 and alkylated adduct 32 (pathway a), or by presumed loss of the seco C4 radical 31 from the haem (pathway b) to alkylate 'essential parasite protein' [(FeII)- and (FeIII)- = ferrous and ferric haem residue]. Adapted from refs. 80, 81 and 82.

The proposed reductive scission of artemisinins by haem is more or less the same as that with 'free' ferrous iron. The encounter of artemisinins and reduced haem (ferrous iron) in the food vacuole generates the seco C-4 radical which is now
attached to the ferric iron of the haem. In order for the seco C-4 radical to exert its supposed parasiticidal mode of action, it must be 'released' from the ferric iron in the haem and then the 'released' radicals are supposed to kill the parasites by encountering 'important biomolecules'. This is in itself remarkable because of the instability of carbon centered radicals.

Several critical requirements have to be fulfilled if haem-mediation is to hold true. First, the administrated artemisinins must encounter reduced haem (ferrous iron). It must be noted that the presence of ferrous haem during haemoglobin breakdown and haemozoin formation has not been established. The reduced haem obtained in a chemical environment was made under extremely reducing conditions in essentially anhydrous conditions, which do not exist in the parasite's food vacuole. The food vacuole of the parasite cannot be lipophilic, as apparently is assumed by those who support the haem activation idea. The pH of the food vacuole can be measured, it contains free Ca$^{2+}$, and fluorescence indicators,83 hardly lipophilic molecules, readily enter. That is, it is an aqueous environment. Second, the oxygen ligand of the seco C4 radical has to dissociate and escape from the ferric iron in the haem. Last, the 'liberated' seco C4 radical has to migrate and encounter important biomolecules of the parasites, an unlikely event, given that the highly unstable and reactive radicals will be destroyed, e.g. by reduction with thiols, or react with other molecules, e.g. oxygen, on the journey. It is not surprising that the seco C4 radical cannot be trapped by cysteine, or glutathione (GSH) which are used to reduce haem to provide ferrous haem. However, the seco- C4 radical does react, although in very poor yield, with cysteine or glutathione if free ferrous iron is used for reductive scission of the endoperoxide.

It is also proposed that formation of hemozoin is inhibited by the haem-artemisinin adduct.77 Then the 'liberated' seco C4 radical would alkylate histidine-
rich protein (HRP), an enzyme which is believed by some that is responsible for catalyzing formation of hemozoin in the parasite’s food vacuole.\textsuperscript{54,85} However, the role of HRP is this regard is unlikely; it is simply a statement that has no experimental evidence. It is easy to generate haemozoin by using aqueous conditions in laboratory experiments. The consequence of inhibition of haemozoin formation in the food vacuole would be the buildup of haem, which is stated, but not actually demonstrated, to be toxic to the parasites. It has been shown that the isolated haem-artemisinin adduct from parasitized red blood cells is antimalarial inactive \textit{in vitro}. Formation of hemozoin inside the parasites cannot be inhibited by the currently used artemisinin derivatives for malaria treatment.\textsuperscript{86}

\textit{1.4.4.3 Critical review of the idea of iron activation}

There are many unsolved puzzles that remain about the proposed and seemingly accepted mechanism of action of artemisinins. In principle, artemisinin and its derivatives could be acting as antimalarials via more than several mechanisms. Unfortunately, there is no direct evidence that points towards the validity of any of the proposed mechanisms. The idea of iron activation will be critically reviewed here.

The capability of carbon centered radicals being the actual parasitical agents is questionable. Carbon centered radicals can undergo oxidation with high valent metal ions, e.g. Cu\textsuperscript{2+}, can be reduced, \textit{e.g.} by thiols, and undergo diffusion controlled reactions with oxygen. It is noted that seco-artemisinin and carba analogues of artemisinin still possess antimalarial activities, even though they seem not so capable of generating C-radicals (Fig 9).
Figure 9: Totally synthetic 5-nor-4,5-secoartemisinin derivatives possess antimalarial activity, but are less likely to generate carbon-centred radicals once the peroxide bridge is cleaved. Relative to artemisinin 16 [IC$_{50}$ 100% W2, D6]; compound 33 6% W2, 20% D6; compound 34 75% W2, 108% D6; compound 35 14% W2, 7% D6. The carba-analogues 37 and 38 relative to artemisinin 16 [IC$_{50}$ 100% W2, D6]; compound 37 3.8% W2, 2.5% D6; compound 38 16% W2, 32% D6.

The only structural difference between compound 33 and 34 is the methyl group at position six, but there is a huge difference in antimalarial activity among these two. Compound 34 is equally potent with its parent artemisinin while compound 33 has only 10% of the activity of artemisinin. And compound 35 has approximately the same activity as compound 33. In these cases of compound 33, 34, 35, scission of the peroxide by ferrous iron will form alkoxy radicals. The alkoxy radicals are no longer restricted in the vicinity of carbon atoms having abstractable hydrogen atoms as the case in the parent artemisinins.$^{87,88}$ Besides, compound 36, a rearranged DHA derivative, is also an active antimalarial.$^{89}$ Some compounds (37 and 38) are clearly capable of generating carbon centered radicals with ferrous iron, but they are poor antimalarials. The correlation of reactivity with ferrous iron and antimalarial activity also is not very strong. Various derivatives of DHA that have amino groups at C-10 are relatively less reactive towards ferrous iron in 1:1
acetonitrile to water, and still are highly effective antimalarials. In contrast, 10-deoxo-10-DHA 39 is readily decomposed by ferrous iron under the stated conditions, although it too is a better antimalarial drug than artemisinin. The idea of ferrous iron activation fails to accommodate the observation that the third non-peroxidic oxygen atom enhances activities, as shown above.

The idea of haem activation is also reviewed here. Haem is released by parasites as a result of catabolism. The haem in the ferric state will dimerise to form haemozoin, which is relatively inert. Artemisinins do react with haem both in vitro and in vivo to form adducts; however, the iron in haem must be in the ferrous state. In in vitro studies, the haem-artemisinin adduct can be isolated in high yields (80-85%) from reactions conducted in dimethyl sulfoxide using ferrous haem generated by reduction with a large excess of sodium dithionite. It has been proven that the haem-artemisinin adduct does not have any antimalarial activity. Several other adducts have also been isolated from an incubation of Plasmodium infected red blood cells with artemisinin. Mass spectral examination shows that these adducts have a molecular weight corresponding approximately to the sum of artemisinin and haem, but it does not provide any evidence about the structure of the adducts.

Antimalarial activity also does not correlate with the reactivity of artemisinins with haem as well. There are artemisinins derivatives that are inert to haem under biomimetic aqueous conditions, but are really potent antimalarials. An example is 10-deoxoartemisinin, which is chemically more stable than DHA or artemisinin, but is totally inert to ferrous haem in aqueous acetonitrile. 10-Deoxoartemisinin also does not inhibit haemozoin formation. In addition to that, the concentration of artemisinins used in in vitro studies of haem-artemisinins interaction is far outside the range that artemisinins exert its antimalarial activity in humans (nanomolar ranges). It has been argued that the target site of artemisinins is
food vacuole of the parasites. However, there are studies that show otherwise\textsuperscript{104,91} - artemisinins do not localize to the food vacuole. Also, only about one tenth of radio-labelled artemisinin is found in the haemozoin fraction after it was exposed to parasites. As mentioned, malaria parasites have different stages in their life cycle. Artemisinins are potent against all intraerythocytic stages of the parasites. It has been shown both \textit{in vitro} and \textit{in vivo} studies\textsuperscript{92,93} that artemisinins target the tiny ring stages of parasites; however, this stage does not generate haemozoin.

\textit{1.4.5 Specific proposed mechanism of action}

\textit{1.4.5.1 Binding of the intact molecule to an active site}

In vitro antimalarial activity of artemisinin was shown to be sensitive to steric effects. Based on this observation, it had been suggested that antimalarial activity of artemisinins would be unleashed once artemisinin, an intact molecule, binds to an ‘active site.’\textsuperscript{65,94,95,96} Thus the idea of site specific binding is associated with antimalarial activity.
Figure 10: Artemisinin and epi-artemisinin and derivatives

If larger groups are used in place of the methyl group at C3 of artemisinin 16 and compound 39, the antimalarial activity is diminished. Similarly, if large groups at C4, C5 or C12 in artemisinin are introduced on the same side as the endoperoxide, antimalarial activity is markedly diminished. In artemisinin itself, inversion of configuration of C-9 such that the methyl group is now on the same face of the molecule as the peroxide as in compound 40 also attenuates its activity. Structure-activity relationships, including comparison of antimalarial activities of artemisinin derivatives and analogues relative to artemisinin, have been summarized.
Figure 11: The energy minimized (AM1) structure of 10-deoxoartemisinin 42 (left) and carba analogue 38 (right) showing potential density surfaces, no solvation. Possible H-bond acceptor areas are associated approximately with red surfaces (H-atoms omitted for clarity).

The carba analogue 38 has a 3-D structure that can be superimposed upon that of 10-deoxoartemisinin 42, but analogue 38 has only 3% of the activity of 10-deoxoartemisinin 42. To explain the difference, it was initially suggested that the non-peroxidic oxygen atom was required to stabilize a cationic intermediate produced by ring opening of the intact peroxide, once it is activated by acting as a hydrogen-bond acceptor, or by electrostatic interaction with an electrophilic site at the receptor site.

What kinds of binding may be involved? Four classes of interaction are involved between a receptor and its substrate on a protein surface or in a protein cleft. They are ionic, dipole-dipole or induced dipolar interactions, and hydrogen bonding. The 1,2,4-trioxane system, with three electronegative oxygen atoms, is capable of associating a relatively large negative potential, which enable artemisinins to react with unsaturated hydrophobic surfaces such as benzene rings via induced
dipolar interactions. Besides, oxygen atoms in the trioxane acting as hydrogen bond acceptors may also be possible.

![Chemical structures](image.png)

**Figure 12**: Examples of products resulting from initial ring opening of each of DHA and artemisinin.

To support the idea that artemisinins undergo ring opening to provide free hydroperoxide, it is noted that the ether derivative artemether is metabolized to DHA, and artesunate is hydrolyzed to DHA. It is clear that the latter must undergo ring opening. It rearranges in solution or on silica gel to the hemiperacetal 36, which displays antimalarial activity against *P. falciparum* in vitro. On silica gel, the reaction proceeds to give the 2-deoxyartemisinin 21, which arises via Kornblum-de la Mare fragmentation-closure on 36. Dihydroartemisinin 17 also undergoes ring-opening and aldol reclosure under basic conditions to give the hydroperoxy hemiacetal 43. Artemisinin 16 in methanol containing potassium carbonate gives the rearranged peroxy hemiacetal 44. The open hydroperoxide from artemisinin is easily intercepted by a secondary amine through oxygen transfer to a tertiary amine, as pointed out above. Thus, ‘unzipping’ of the artemisinin 16 and dihydroartemisinin 17 nuclei are facile.

For artemisinins which do not bear a group at C-10 which drives the ring opening, it is possible that the role of the third non-peroxidic oxygen atom is to
stabilize the cation which would form on ring opening of the peroxide bridge (Fig. 13).

**Figure 13:** ring opening of artemisinin derivatives driven by protonation of the peroxide bridge. The ring opened hydroperoxide could provide alkoxy radicals or hydroxyl radicals which could contribute to oxidative stress.

In short, the unique electronic ability of the trioxane to provide hydroperoxide through generation of an oxo-stabilized cation upon heterolysis of the C3-O2 bond might be one reason why artemisinin and its derivatives are more active as antimalarial agents than the corresponding isosteric peroxides.

1.4.5.2 Proposal for the active site

An alternative hypothesis has been suggested based on the supposed structural similarities between the sesquiterpene moieties of artemisinins and in thapsigargin 45. A brief review of thapsigargin, its target site – SERCAs, and its relationship with artemisinins is now presented.
Figure 14: Structure of the sesquiterpene lactone thapsigargin (left) showing its approximate disposition bound in mammalian ATP6.

Thapsigargin 45 is a sesquiterpene lactone bearing polyketide side chains isolated from a plant called Thapsia garganica. It is an extremely potent and specific inhibitor of sarco/endoplasmic reticulum Ca\textsuperscript{2+}-transporting (SERCA) adenosine triphosphatases (ATPases) from a wide variety of organisms.\textsuperscript{102} Ca\textsuperscript{2+} is an important messenger in cells but a large amount of calcium ions is toxic to the cells. So the concentration of Ca\textsuperscript{2+} will be regulated carefully. The regulation system is depicted in figure 15.
Figure 15: The model of transporting Ca\textsuperscript{2+} through calcium channel by sarcoplasmic reticulum Ca\textsuperscript{2+}-transporting ATPases (SERCAs). Bcl-2 and Bax/Bak are ancillary transporters which are turned on in response to low or high Ca\textsuperscript{2+} levels in the endoplasmic reticulum.\textsuperscript{103}

Under normal conditions, Ca\textsuperscript{2+} continuously cycles between the ER and mitochondria. Ca\textsuperscript{2+} is pumped into the ER by Ca\textsuperscript{2+} ATPases (SERCA), and released by IP\textsubscript{3}-gated channels (IP\textsubscript{3}R). Ca\textsuperscript{2+} enters mitochondria by a Ca\textsuperscript{2+} uniporter (mCU) and is released by a Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (mNCE). The ER Ca\textsuperscript{2+} load reflects the balance between Bcl-2 and Bax/Bak proteins.\textsuperscript{103} Much of the Ca\textsuperscript{2+} is captured by mitochondria located near Ca\textsuperscript{2+}-release channels in the ER membrane. This close connection allows mitochondria to modulate, propagate, and synchronize Ca\textsuperscript{2+} signals and to prevent depletion of Ca\textsuperscript{2+} in the ER by recycling Ca\textsuperscript{2+} back to the ER.

In summary, SERCAs act to reduce cytosolic free calcium concentrations by actively concentrating Ca\textsuperscript{2+} into membrane bound stores. This activity is critical to cellular survival because it allows intracellular signalling by large increases in Ca\textsuperscript{2+} concentration to recur, by rapidly restoring resting concentrations. It follows that
inhibition of the SERCA transporter will have a substantial effect on Ca\(^{2+}\) levels, and result in cell death.

It was suggested that artemisinins may act in a similar way to thapsigargin, but with particular specificity for the SERCA of malarial (PfATP6) in contrast to mammalian pumps.\(^{104}\) Several independent lines of evidence in favour of this hypothesis were accumulated. PfATP6 was functionally expressed in Xenopus laevis oocytes, and the inhibition of PfATP6 activity produced by a variety of artemisinin derivatives was assessed. Artemisinin itself has an inhibitory constant that is very similar to that of thapsigargin, when assayed with PfATP6 in this system. As thapsigargin inhibits SERCAs with a 1:1 stoichiometry,\(^{105}\) this confirms the specificity of interactions between artemisinins and PfATP6. In figure 16 is shown the calculated binding mode of an artemisinin derivative superimposed over that of thapsigargin.

![Figure 16: Binding mode of artemisinin derivative (12-10-ethylacetic)-O-benzyldeoxo-artemisinin, 40; gray ball and stick model. TG; yellow cylinder model) in PfATP6. Figure is generated using DS ViewerPro 5.0 for Windows.\(^{106}\)](image-url)
The concentrations required to inhibit PfATP6 are submicromolar, whereas we have seen before that against other putative targets, values for interaction with artemisinins are in the micromolar range. Furthermore, there is a correlation between assays of artemisinin inhibition of PfATP6 in oocytes and those of the killing of parasites in vitro.

If thapsigargin and artemisinin interact with the same target (Figure 17), then competition between thapsigargin and artemisinins should occur when both are used simultaneously to kill parasites. This finding seems to be confirmed by isobologram analysis. The localisation of PfATP6 was assayed by visualising fluorescent thapsigargin in living parasites and it is similar to that visualised for a fluorescent artemisinin derivative using confocal microscopy. Moreover, these distributions conform to observations made by others using electron microscopy, where artemisinin localises to cytosolic membrane-bound structures (and not to the food vacuole). Furthermore, the earliest changes after artemisinin exposure of parasites do affect these membrane-bound organelles (and mitochondria, which can act as sinks for excess intracellular calcium).
Figure 17: Energy-minimized models of the rabbit SERCA1 thapsigargin-binding site and the corresponding region of a model of PfATP6 containing artemisone (right). Electrostatic interactions are predicted to exist between Lys252 and Glu255 on helix M3 in the mammalian SERCA1. In PfATP6 Lys260 is predicted to bond to the L6/7 loop above helix M7.\textsuperscript{109}

A highly specific binding site model should be very sensitive to structural changes of the substrates. If artemisinins do have non-covalent interaction with a protein target, proposed to be PfATP6, to unleash their potency, enantiomeric forms of the artemisinin drugs should have different antimalarial activities. Research aimed at finding out the antimalarial activity of enantiomeric forms of synthetic peroxides has been carried out.\textsuperscript{110} Chloroquine-sensitive, chloroquine-resistant, and multidrug-resistant strains of \textit{P. falciparum} were selected to analyze the antimalarial activity of enantiomers of trioxanes, structurally related to artemisinin. Surprisingly, the enantiomers showed equally potent antimalarial activities. In addition, the docking to SERCA is not equivalent to the structure-activity relationships of the artemisinins conducted by CoMFA studies.\textsuperscript{111} Bulky groups added to artemisinins do not necessarily deteriorate the potency. In the study, it showed that whether the
activity of artemisinins increase or decrease depends on the nature of the added groups.

The points seem to weaken the hypothesis that binding to a specific protein site is essential for the activation. Nevertheless, there are many unanswered questions that remain about this proposed mechanism of action of artemisinins, such as the role of ferrous iron in drug activity, the molecular sites of interaction with parasite SERCAs and the cellular consequences of SERCA inhibition. In addition to that, it is unclear how structurally diverse peroxides such as 10-deoxoartemisinin 39, the pyrimidinylpiperazine derivative, trioxolane and its close relatives 112 and a steroidal tetroxane 113 that possess antimalarial activities in vitro ranging from 1.5-11.4 nM may bind into the TG cleft to inhibit parasite PfATP6. For example, the trioxolane, OZ OZ277 (RBx6660) is two order of magnitude less effective in inhibiting PfATP6 (K_i = 7700 nM) than is artemisinin (K_i 79 nM). 112
The peroxide group of artemisinin has aroused the notion that the parasiticidal activity of artemisinins is due to oxidative stress mediated by enhanced levels of reactive oxygen species within the infected red blood cells.114,115

Reactive oxygen species (ROS) include oxygen ions, free radicals, and peroxides, both inorganic and organic. They are generally very small molecules and are highly reactive. In other words, free radicals associated with an oxygen atom or molecules containing oxygen atoms can either produce free radicals or are chemically activated by them are called ROS. Examples are shown in figure 18.

![Figure 18: Some examples of reactive oxygen species (ROS).](image)

There are many sources of ROS within the cells, such as aerobic respiration, β- oxidation of fatty acids which proceed via hydroperoxides, microsomal cytochrome P450 metabolism of xenobiotic compounds, stimulation of phagocytosis by pathogens or lipopolysaccharides, and arginine metabolism. The ROS are closely regulated within the body. Before reaching a damaging level, ROS are removed from the cells by the action of superoxide dismutase or SOD, catalase, or glutathione peroxidise (GSH-P). If the regulation system malfunctions, ROS can induce alteration of essential molecules such as polyunsaturated fatty acids in membrane lipids, essential proteins, and DNA itself.
Efforts aimed at seeking the connection between oxidative stress and artemisinins have been made. Consequential effects associated with enhanced oxidative stress like enhanced lipid peroxidation,\textsuperscript{116,117} synergism with pro-oxidants such as riboflavin, miconazole and doxorubicin, and antagonism with free radical scavengers such as catalase, dithiothreitol, \(\alpha\)-tocopherol (vitamin E),\textsuperscript{58} and ascorbic acid had been demonstrated after administration of artemisinins.
**Figure 19**: Formation and biotransformation of ROS in biological systems. Adapted from ref.115 and $O_2^\cdot-$, superoxide radical; SOD, superoxide dismutase; GSH, reduced glutathione; GSSG, oxidised glutathione; GSH-P, glutathione peroxidase; GSH-R, glutathione reductase; HO\(^\cdot\), hydroxyl radical; R\(^\cdot\), secondary radical; Vit E, vitamin E; Vit E\(^\cdot\), vitamin E radical; NADPH, reduced nicotinamide-adenine-dinucleotide phosphate; Ag, antigen; Ab, antibody; C5a, complement protein cleavage product.

The general interpretation for parasite death and haemolysis is that these are mediated by reactive oxygen species whose presence, greatly enhanced by the
exogenous peroxide, eventually overwhelms the parasite anti-oxidant defence systems. How does this relate to artemisinins? The way of artemisinins acting to enhance oxidative stress has not been addressed. Upsetting the redox balance in intracellular reduced glutathione (GSH), which oxidizes it to oxidized glutathione (GSSG) according to a heterolytic process, might be one of the possibilities. Although malaria infection is known to induce oxidative stress in erythrocytes, GSH levels in parasitized cells are not markedly depressed as compared to non-parasitized cells. However, as artemisinin is antagonized by glutathione, it is possible that the drug is decomposed by reduction in the presence of glutathione transferases (GSTs). Cytosolic GSTs detoxify electrophilic xenobiotics by catalyzing the formation of GSH conjugates and exhibit glutathione peroxidase activity towards hydroperoxides. When artemisinin was incubated with glutathione, NADPH and glutathione reductase (GSSG-R), NADPH oxidation was stimulated. Using human recombinant GSTs, artemisinin was similarly shown to stimulate NADPH oxidation. Thus, it was considered that GSTs may contribute to the metabolism of artemisinin in the presence of NADPH and GSSG-R. A model was proposed in which artemisinin reacts with GSH to provide GSSG, the latter is then converted to GSH via glutathione reductase. The last reaction may then result in the depletion of NADPH via GSSG-reductase. The ability of artemisinin to react with GSH in the presence of GST may be responsible for the NADPH utilisation observed in vitro and suggests that cytosolic GSTs may contribute to metabolism of artemisinin and related drugs in vivo, as discussed above.

Another piece of evidence which suggests that artemisinin is involved in modulation of oxidative stress is the behaviour of ceramide 46, a sphingolipid consisting of sphingosine and a fatty acid amide. Ceramide 46 depresses GSH levels when administered to erythrocytes parasitized with P. falciparum, and it also
significantly decreases the number of parasites, an effect which was abolished by sphingosine-1-phosphate, a ceramide antagonist. Artemisinin was shown to increase levels of intracellular ceramide, again an effect which was abrogated by sphingosine-1-phosphate. It was argued on the basis of these observations that artemisinin induces parasite death by increasing endogenous ceramide and decreasing GSH levels.120

![Figure 20: Structures of ceramide 46 and artemisinin-GSH adduct 47.](image)

It has been found that dihydroartemisinin has no effect on GSH/GSSG ratios and activities of superoxide dismutase (SOD), catalase and glutathione peroxidase (GSH-P) in unparasitized erythrocytes. However, in parasitized cells, lower GSH levels and lower catalase and GSH-P activities were seen at drug concentrations as low as 0.5-1.0 ng/mL, that is, within the therapeutic range. However, it is important to note that parasites in the presence of DHA were affected to a greater extent than the antioxidant levels.121 Thus, it appears likely than mere depletion of parasite antioxidants such as GSH may not be a prerequisite for the parasite death.
In relation to the oxidative stress idea, no model chemical study has been carried out on the reactivity of thiols in general with artemisinin and its derivatives. In particular, the formation of GSSG via direct oxidation of GSH by artemisinin has apparently never been carried out. However, artemisinin and derivatives have been shown to react with GSH in the presence of ferrous iron, that is under reducing conditions, in aqueous medium to give the adduct 47 in very low yield. Finally, it is known that the Fenton reaction, to be discussed below, converts peroxides into alkoxyl radicals. Whilst generation of such radicals from artemisinin may be held to enhance ROS, and thereby accelerate lipid and/or protein damage, it is of interest to note that alkoxyl radicals per se have never been considered to be the likely bioactive agents from artemisinin.
1.5. Thesis Project

It is clear that current theories on mechanism of action of artemisinin antimalarials are inadequate. Based on the obvious precept that artemisinins are peroxides, and that the malaria parasite is operating in an environment which is oxidatively stressed, it is considered that artemisinins are more likely to be acting as agents which exert their effect in an oxidative mode. Because they are so potent against the malaria parasite, they are likely to be knocking out crucial enzymic processes involving catalytic cycles, perhaps by destruction of a co-factor. One such co-factor may be NADPH,\textsuperscript{119} as discussed above.

In order to probe the biological chemistry of the artemisinins in relation to their mechanism of action, the reactions with cysteine and other natural thiols, other hydride donors such as NAD(P)H and FADH, and other oxidizable substrates in the presence of electron-deficient substrates such as methylene blue, other dyes, and naphthoquinones such as menadione require examination. The proposed work is based on the hypothesis that artemisinins may exert their activity by accelerating decomposition of crucial biomolecules such as cofactors involved in various redox or other cellular processes, and which thereby may affect PfATP6, and hence calcium homeostasis in the parasite.

Methylene blue, a member of the thiazine dye group, has been extensively used in in diverse areas. It acts as a photosensitiser for the treatment of cancer and has been used as a standard, for example in a test to detect any growth of bacteria in fresh milk.\textsuperscript{122} Methylene blue has been proven to be an active antimalarial drug that inhibits \textit{Plasmodium falciparum} glutathione reductase.\textsuperscript{123} It is also reported that methylene blue is a non-competitive inhibitor and subversive substrate of GR and thioredoxin reductase (TrxR).\textsuperscript{123,124,125}
The 'subversive substrate' idea means that methylene blue essentially subverts the role of NADPH as an essential co-factor for GR and TrxR (Scheme 1); the methylene blue oxidizes the NADPH, to NADP+, and the methylene blue is reduced to leuco-methylene blue. The leuco methylene blue is then oxidized by oxygen back to methylene blue, which continues the catalytic cycle (Fig. 21 below).

In particular, a synergistic effect of artemisinin with MB is found with chloroquine-resistant *P. falciparum*. The origin of this synergism is intriguing, and an understanding of this combination effect of MB, even leuco-MB, with artemisinin may be intriguing and may reveal the mode of action of artemisinin, a potent antimalarial drug used worldwide. An investigation based on the redox properties of the couple, methylene blue and leuco-methylene blue, will therefore be carried out in the presence of artemisinin.

**Scheme 1**: Dithiol-based redox system of *Plasmodia*\textsuperscript{125}
Project Objectives

The project is divided into two parts

i. To probe the antimalarial activity and binding of structurally modified artemisone:

Artemisone is an especially effective inhibitor of PfATP6: it displays 100-fold greater inhibition of the *P. falciparum* transporter PfATP6 than does artemisinin, and it inhibits the *P. vivax* transporter PvATP6 at picomolar levels. Because artemisinin antagonizes thapsigargin (TG), an inhibitor of mammalian SERCAs, the assumption is made that artemisinins bind at the TG site, a cleft situated in the transmembrane surface between the M3 and M5 helices. An energy-minimized model constructed from amino acid sequences in the cleft indicates that artemisone binds by hydrophobic interactions, with the peroxide exposed (Fig. 17). However, it is clear that another event must take place to render artemisone an effective inhibitor. Artemisinins lacking the peroxide bridge are not antimalarials, although they would appear to fit into the cleft. In addition, structurally diverse antimalarial active peroxides such as artesunate 20, trioxolane 50, a steroidal tetroxane and structurally simpler peroxides do not obviously fit into the cleft, although these possess antimalarial IC\textsubscript{50} values ranging from 4.4-11.4 nM. Structurally simple enantiomeric trioxanes exert essentially identical antimalarial activities in *vitro*.
although the interpretation is ambiguous.\textsuperscript{128} Therefore, other possibilities must be addressed. Artemisinins may bind elsewhere, such as the ATP region, in allosteric fashion to the transporter locking it in a conformation\textsuperscript{129} unable to bind TG. In this respect, it is interesting to note that isolates of \textit{P. falciparum} which are 30 fold more resistant to artemether carry a single amino acid mutation (S769N) which is not associated with the TG binding site in PfATP6.\textsuperscript{130} Alternatively, artemisinins may affect binding of an endogenous, as yet unknown, substrate to PfATP6. Other regions of the endoplasmic reticulum may be affected by the artemisinins which influence the transporter.\textsuperscript{131} Intriguingly, \textit{tert}-butyl hydroperoxide (TBH), which expresses \textit{in vivo} \textmu M antimalarial activity in mice,\textsuperscript{132} increases intracellular Ca\textsuperscript{2+} levels in pheochromocytoma cells. Pretreatment with TG abolishes the effect of TBH, whereas pretreatment with TBH abrogates the TG-induced increase in Ca\textsuperscript{2+}. Thus, TBH causes Ca\textsuperscript{2+} release from the TG-sensitive SERCA pool.\textsuperscript{133} Similarly, hydrogen peroxide (HP) increases cytosolic Ca\textsuperscript{2+} concentrations in pancreatic cells. Pretreatment with TG inhibits HP-induced changes in cytosolic Ca\textsuperscript{2+}.\textsuperscript{134} Thus, the effects exerted by TBH and HP mimic, albeit likely much less effectively, those of the artemisinins, even though TBH and HP are unlikely to bind to the TG site. A consideration of these findings together with those of antitumour activity of the artemisinins as discussed above suggests that peroxides in general have a profound effect on calcium transporters and the effect may not depend on 'classical binding' to the transporters.

Because artemisone is an exquisite inhibitor of PfATP6, and is so active as an antimalarial drug, we will prepare the enantiomer and in an independent project with an overseas group, evaluate both binding to PfATP6, and antimalarial activity as one means of testing the foregoing precept. The retrosynthetic analysis is given in
Scheme 1: the transformation to the previously unreported ent-artemisinin 16* and then ent-artemisone 56* will follow closely the published procedures for the total synthesis of artemisinin reported by Avery, and conversion of artemisinin via DHA into artemisone. Inactivity of 56* both against the PfATP6 target and the malaria parasite would indicate that 'classical' binding associated with a chemical event involving the peroxide in 56 is likely to occur. We would then need to examine more closely the nature of the binding process. On the other hand, if 56* is equipotent with 56, then a fundamental examination of those chemical processes of biological importance which are known to be, or likely to be, affected, by peroxides, and which are relevant to intracellular Ca\(^{2+}\) homeostasis would have to be carried out.

ii. To probe potential biological chemistry of the artemisinins in relation to mechanism of action:

Methylene blue (MB) is active against all blood stages of the malaria parasite with which the artemisinins interact. It also synergizes their antimalarial activity,
that is each drug is more active in the presence of the other than it is alone. MB inhibits *P. falciparum* glutathione reductase (GR), a parasite-specific enzyme which requires NADPH, and provides reduced glutathione (GSH) for the parasite antioxidant defence system. Unlike the mammalian enzyme, GR does not function via a heterolytic process involving selenocysteine, and thiol radicals are implicated in the reduction of GSSG. MB acts as a subversive substrate; it inhibits the reduction of GSSG and is a redox-cycling agent - that is, it is reduced by the enzyme at the expense of NADPH and is reoxidized by oxygen or other peroxides. The other key parasite enzyme which provides thiols for the antioxidant system is thioredoxin reductase (TR). TR reduces thioredoxin disulfide to the thiol in a reaction which also requires NADPH. In *P. falciparum*, hydroperoxides are reduced by thioredoxin, a reaction catalyzed by thioredoxin peroxidases. MB also acts as a subversive substrate of TR. Other redox active antimalarial drugs such as menadione (2-methyl-1,4-naphthoquinone) also act as inhibitors of these enzymes. The redox-active doxycycline, although not yet shown to be such an inhibitor, acts synergistically as an antimalarial drug with artemisinin.

Artemisinins, therefore, may be implicated in the action of these drugs with the enzymes or their substrates, possibly by acting as electron-transfer agents in the presence of the redox active drugs. In order to initiate work in this area, we shall examine the rate of anerobic oxidation of thiols (cysteine, GSH) and biological reductants [NAD(P)H, FADH] in the presence of MB and quinones such as menadione, anthraquinone 2-sulfonic acid and others, and establish the effect of artemisinins (artemisone) on the overall rate and stoichiometry of these reactions. It is emphasized that there is no precedent for this proposal in the literature, which in the absence of preliminary data, is admittedly speculative. However, artemisinins are exquisitely active antimalarial drugs, and the putative binding to the TG cleft in
PfATP6 aside, a catalytic involvement may account for this. It may even become apparent that once artemisinins become bound in the TG cleft, or elsewhere, in PfATP6, with the exposed and desolvated peroxide, then they may better function in their role of destroying the parasite enzymes or cofactors crucial for antioxidant defence. Thus, in the absence of reductants, the peroxide, endogenous or otherwise, then exert their changes on Ca^{2+}-flux, as described above and initiate apoptosis.
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Chapter 2 Result and Discussion

2.1 To probe the antimalarial activity and binding of the enantiomer of artemisone

The objective of this project is to synthesize the enantiomer of artemisinin and thereby the enantiomer of artemisone, a potent antimalarial drug. It was proposed that artemisinins may be 'activated' for antimalarial activity on complexation, or binding, of the intact molecule, into an 'active site'. Because artemisinin is a sesquiterpene lactone, a connection was perceived to exist with thapsigargin, a sesquiterpene which has polyketide side chains.

Thapsigargin, a sesquiterpene lactone isolated from *Thapsia garganica* is an extremely potent and specific inhibitor of sarco/endoplasmic reticulum Ca$^{2+}$-transporting (SERCA) adenosine triphosphatases (ATPases) from a wide variety of organisms. It is believed that thapsigargin and artemisinin compete for the ATP6 site in the *Plasmodium falciparum* malaria parasite, that is, that artemisinin is a competitive antagonist with thapsigargin. Thus, it was proposed that both thapsigargin and artemisinin function in a similar way in that the parasite crucial enzyme, PfATP6, is inhibited. With the inhibition of this enzyme, the intracellular level of Ca$^{2+}$ ions cannot be regulated, and this leads to the death of the parasite.
To verify this hypothesis, the most effective way is to test the potency of the enantiomer of artemisone as an antimalarial drug. A mode of action of inhibitory binding to an active site is extremely sensitive to the three dimensional structure and its chirality. If the enantiomer of artemisone is shown to be less effective as an antimalarial drug as artemisone, the binding hypothesis would be strengthened in explaining the mechanism of artemisinins drug. The outstanding biological activity and the need to seek the mechanism of artemisinins therefore incited us to develop a synthetic route toward enantiomer of artemisone.

The most intriguing molecular feature of artemisinins is the 1,2,4-trixoane ring, which may also be regarded as a bridging peroxide group. The peroxide moiety is essential for activity. In a retro synthetic perspective, artemisinins could be regarded as a ketal-acetal- lactone sytem formed by loss of water from hydroperoxy-aldehyde 61. There are various ways of getting to this intermediate. One such compound for that would be the enol methyl ether 62, which was used in an efficient synthesis.¹

![Scheme 3](image.png)

**Scheme 3:** Retro-synthesis of artemisinin 16 according to the literature (ref. 1).

Total synthesis of artemisinin can be traced back as early as 1983.² According to a later synthesis, (-)-isopulegol was considered to be a good starting compound for the synthesis.
Scheme 4: A proposed synthesis path to the enantiomer of artemisone

The plan was to follow the synthesis as depicted in Scheme 4. Whilst parts of these are adapted from the literature, the step to introduce the oxygen by proceeding from compound 70 to 71 to 72 would be completely new. Here the idea is to use hydrogen peroxide or an alkyl hydroperoxide in an acid catalyzed hydroperoxidation of the exocyclic double bond. This would by-pass the use of singlet oxygen traditionally used on another synthetic intermediate, and which always gives low yields of oxygenation products.
**Scheme 5**: Cyclization of S-(-)-citronellal 63 to (+)-isopulegol 64 by the Prins reaction; i. SnCl$_4$ (0.1 equiv), CH$_2$Cl$_2$, N$_2$, 0 °C, 20 min; ii. aqueous NH$_4$Cl, 85%.

The first step was to cyclize S-(-)-citronellal to R-(+)-isopulegol by the Prins reaction (scheme 5) in which an electrophilic addition of the aldehyde side chain to the alkene side chain followed by capture of a nucleophile (scheme 5). S-(-)-citronellal was stirred in dichloromethane at 0 °C under nitrogen. Tin (IV) chloride (1 M) was added catalytically as a solution of dichloromethane. After 20 min stirring and quenching with ammonium chloride and usual workup, it gave the alcohol 64 in 85% yield.

This reaction involves a highly stereoselective concerted ene reaction which is proposed to involve a six-membered transition state (figure 22). However, different stereoisomers of isopulegol can be obtained if different Lewis acids are involved. The formation of different isomers can be explained by the intermediacy of a tertiary carbocation. It has been established that the 'carbocation mechanism' is more likely to be involved in a strongly acidic condition. However, tin (IV) chloride is a good Lewis acid for effecting the concerted reaction.

**Figure 22**: Proposed basis for stereoselective cyclization of (-)-citronellal 63 to (+)-isopulegol 64 by the ene reaction.
Alcohol 64 was easily identified by $^1$H NMR spectroscopy. Its precursor, citronellal, contains signals due to aldehyde (δ 9.41, t, 1H) and olefinic (δ 5.08, m, 1H) protons and two allylic methyl groups (δ 1.61, s, 1H and 1.68, s, 1H). In contrast, isopulegol 64 contains signals due to diastereotopic terminal olefinic protons (δ 4.90, d, 1H, $J = 20$ Hz, δ 4.86, d, 1H, $J = 28$ Hz) and only one allylic methyl group (δ 1.71, s, 3H).

![Scheme 6](image)

**Scheme 6**: Hydroboration-oxidation of isopulegol 64; BH$_3$-THF, THF, N$_2$, 0 °C, then r.t., 6 hr; ii NaOH, H$_2$O$_2$ (30%), 0 °C then r.t., 1 hr, 38%

Next, isopulegol 64 was hydroborated followed by oxidative workup to give a diol consisting of two epimers 65 and 66 in a ratio of 7:1 and in an overall yield of 38%. Isopulegol 64 was first stirred in tetrahydrofuran at 0 °C under nitrogen. BH$_3$-THF (1M) complex was introduced dropwise. After 6 hours stirring at ambient temperature, oxidative workup in which sodium hydroxide was added at 0 °C followed by hydrogen peroxide, quenching with ammonium chloride and usual workup, it yielded the pair of epimers.

Hydroboration-oxidation is a common synthetic practice which converts an alkene into an alcohol by the net addition of water across the double bond. The two groups are added in a syn fashion leading to cis-stereochemistry. The first step, addition of borane, is a concerted process in which boron will attach to a less substituted side.
The second step, oxidative workup in which the boron atom is attacked by nucleophilic hydroperoxide ion and oxygen atom migrates after the attack, affords the alcohol 65 or 66 that are illustrated in figure 24.

Hydroboration followed by oxidative workup of compound 64 gave a mixture of epimers 65 and 66. The alcohol 65, as reported previously, is the major product. The preference of the methyl group to be axial is explained by the fact that the hydroxyl group in the cyclohexane ring forms a boron-oxide complex with a boron compound in which an unusual intermolecular complexation with different boron compound might be involved. A stereoisomer of diol 65, depicted in figure 25, directs the syn-addition in such a way that results in formation of a bicyclic ring.
intermediate which the methyl group in the propanol side chain must be in an equatorial position. After the attack by hydroxide ion, it yields the isomer.

![Figure 25: Proposed mechanism of formation of the stereoisomer of compound 65](image)

**Scheme 7**: Selective protection of the diol 65; TIPSiCl, NEt3, DMAP (0.1 eq), N2, CH2Cl2, r.t., 72 hr; brine, 95%

The following step is to selectively protect the primary alcohol in the propanol side chain. Diol 65 was added to a flask consisting DMAP, triethylamine and triisopropylsilyl chloride in dichloromethane under nitrogen. After stirring for three days it afforded the protected isopulegol in 95% yield.

Protection of the primary alcohol is necessary because the secondary alcohol in the cyclohexane ring has to be oxidized to prepare the precursor for Robinson annulation that is described below. The primary alcohol, that is generally more susceptible to oxidation than a secondary one, was selectively protected with a
triisopropylsilyl (TIPS) group by using triisopropylsilyl chloride and 4-(N,N-dimethylamino)pyridine (DMAP). Regardless of the extensive usage in organic synthesis, the mechanism of DMAP is not thoroughly studied.\textsuperscript{6} However, the electron donating N,N-dimethylamino group in DMAP enhances its basicity. The likely pathway is nucleophilic substitution of the alcohol on the silicon with displacement of chloride.\textsuperscript{7}

After the silylation of the primary alcohol, the secondary alcohol was cleanly oxidized to the ketone 68 by using the Swern oxidation.

\textbf{Scheme 6}: Swern oxidation; i. DMSO, (COCl)\textsubscript{2}, -78 °C, CH\textsubscript{2}Cl\textsubscript{2}, N\textsubscript{2}, 1.5 hr; ii. NEt\textsubscript{3}, 0 °C then r.t., 1 hr; iii. brine, 94%

The protected alcohol 67 was added to a solution mixture containing dimethyl sulfoxide and oxalyl chloride in dichloromethane at -78 °C. After treating with triethylamine, the mixture was allowed to warm temperature to give the ketone 68 in 94% yield. Introduced in 1978,\textsuperscript{8} the Swern oxidation works well with primary, secondary, allylic, benzylic, and hindered alcohols. The key of the oxidation lies in the fact that dimethyl sulfoxide (DMSO) is activated by the electrophile, oxalyl chloride which is the most effective of the various activators used for DMSO. The activated dimethyl sulfoxide reacts with alcohol 67 to give the key intermediate alkoxy sulfonium ion. Base has to be added to initiate proton abstraction so that the intermediate can collapse to the ketone 68.
The next step is to alkylate the compound 68 in order to obtain the Robinson annelation precursor. Robinson annelation is a combination of conjugate addition and an aldol reaction which usually uses methyl vinyl ketone to react with a thermodynamically generated ketone enolate, that is, the ketone is treated with a weak base under protic conditions in the presence of the methyl vinyl ketone (MVK). The reaction has been widely used to synthesize different polycyclic compounds such as steroids.\(^\text{14}\) MVK has also been used under aprotic conditions. The metalated lactim 89 offers conjugate addition product 90 with methyl vinyl ketone in a yield of 31% while an organolithium reagent 91 treated with methyl vinyl ketone gave a product 92 in 41% yield.\(^\text{9, 10}\)
The ketone, (+)-dihydrocarvone 86, was treated with the strong base lithium \(N,N\)-di-iso-propylamide (LDA) in the aprotic solvent tetrahydrofuran (THF) at low temperature. The adduct 87 and 88 was obtained in acceptable yield (53% for 87 and 12% for 88).\(^\text{11}\) (scheme 9)

![Scheme 9](image)

**Scheme 9**: i. LDA, THF, \(N_2\), -78 °C then 25 °C, 2 hr; ii. MVK, -78 °C then 25 °C, 4 hr; iii. HCl, (53% for 87 and 12% for 88)

Treatment of the ketone 68 with LDA in THF at – 78 °C gives the kinetic enolate 84 of 68. Methyl vinyl ketone was introduced as a solution in THF. After *in situ* hydrolysis of the \(\alpha\)-silyl group by adding dilute hydrochloric acid such that the pH of the final solution was about 3, the diketone 69 was able to be isolated, but only in a very small yield (< 1%). About 50% of starting material 68 was recovered.

![Scheme 10](image)

**Scheme 10**: Alkylation of 68 with methyl vinyl ketone; i. LDA, -78 °C, THF, \(N_2\), 1.5 hr; ii. methyl vinyl ketone, -78 °C, 2 hr; iii. 0 °C, 3.5 hr, then pH 2; iv. NaHCO\(_3\) (5%), < 1%
Although use of aprotic conditions – usually THF at low temperature enables the specific kinetic enolate to be generated by treatment of a ketone with a strong base such as LDA, MVK is highly reactive and has a tendency to polymerize under aprotic basic conditions, either because traces of base will cause the MVK to polymerize, or the enolate that is produced in the conjugate addition adds to more methyl vinyl ketone to give eventually a polymer. This is probably what happened in the reaction above. α-Trimethylsilyl methyl vinyl ketone is an MVK equivalent which was developed to overcome the problem of trying to use MVK under aprotic conditions. The use of a bulky α-silyl group to inhibit polymerization of MVK under aprotic conditions was first introduced by Stork and coworkers. The trimethylsilyl group provides a steric blockade to anionic polymerization, and it may also partially stabilize the negatively charged enolate produced by the conjugate addition. It is generally accepted that silicon, with its vacant 3d orbitals, can stabilize an adjacent negative charge. Once the desired enolate is generated, then it is treated with α-trimethylsilyl methyl vinyl ketone. The adduct can be treated with aqueous acid or base to generate the normal MVK diketone, which is then subjected to intramolecular aldol reaction to give the annelation product.

Therefore, it was decided to use 3-trimethylsilyl-3-buten-2-one. Although this was commercially available in the past, it is no longer available, and therefore it has to be prepared according to the literature method.

\[
\text{Scheme 11: Preparation of 3-trimethylsilyl-3-buten-2-ol; i. Mg, 1,2-dibromoethane (0.04 eq), THF, Ar, reflux, 1 hr; ii. CH}_3\text{CHO, reflux, 1 hr; iii. aqueous NH}_4\text{Cl}
\]
The precursor of the product is 3-trimethylsilyl-3-buten-2-ol. This was prepared by first converting (1-bromoethyl)trimethylsilane into the Grignard reagent. A mixture of magnesium and small amount of 1,2-dibromoethane were stirred in THF at reflux temperature, and it was then treated with (1-bromoethyl)trimethylsilane followed by acetaldehyde. After quenching with ammonium chloride, the product 3-trimethylsilyl-3-buten-2-ol was obtained which was used without purification.

\[
\text{OH} \quad \text{SiMe}_3 \quad \text{i, ii} \quad \text{O} \quad \text{SiMe}_3
\]

**Scheme 12**: Jones Oxidation; i. H$_2$CrO$_4$ in H$_2$SO$_4$, acetone, 0 °C; ii. brine, 45% in two steps

The next step involved Jones oxidation, in which a solution of chromium trioxide in diluted sulfuric acid was added to a solution of 3-trimethyl-3-buten-2-ol in acetone at 0 °C. The ketone was isolated in an overall yield of 45% for the two steps. It is difficult to carry out the oxidation, because of the relatively small molecular weight of the starting alcohol. It can only be purified by distillation. Because of the low efficiency of the literature method, a new method for synthesizing 3-trimethylsilyl-3-buten-2-ol has been developed,\(^\text{16}\) which is claimed as a so-called 'one-pot' preparation from vinylsilanes.

\[
\text{SiMe}_3 \quad \text{i, ii} \quad \text{O} \quad \text{SiMe}_3
\]

**Scheme 13**: One-pot synthesis of α-trimethylsilyl enones from vinylsilanes,\(^\text{16}\) i. O$_2$, hv, TPP; ii. Ac$_2$O, py, DMAP, MeCO$_2$Me, -5 °C, 4.5 hr, 49%
The synthesis is based on regioselective photooxygenation of vinylsilanes, but the method has not been tried here.

**Scheme 14:** Alkylation of compound 68; i. LDA, -78 °C, THF, N₂, 1.5 hr; ii. 3-TMS-3-buten-2-one, -78 °C, 2 hr; iii. 0 °C, 3.5 hr, then pH 2; iv. aqueous NaHCO₃, 5%

According to scheme 14, the ketone 68 was treated with LDA at low temperature (-78 °C) to give the kinetic enolate 84 of 68. The solution was then treated with 3-(trimethylsilyl)-3-buten-2-one to give the diketone 68 in a yield of 5% after hydrolysis of the α-silyl group.

![Diagram of Scheme 14](image)

**Figure 28: In situ desilylation**

The carbonyl group is protonated at the low pH, which enables a nucleophilic attack by water at the silicon to take place. After desilylation, an enol is formed which will be rearranged to yield a ketone. Trimethylsilyl hydroxide is unstable, and dimerizes to hexamethyldisiloxane by elimination of water.
The result of the alkylation of the ketone 68 by using \(\alpha\)-trimethylsilyl methyl vinyl ketone is disappointing (5% yield), and the disparity with literature is very great.\(^3\) Nevertheless, adjustments such as increasing the temperature and time course or the concentration of salt (by adding one equivalent amount of lithium bromide) of the reaction were made but no improvement was observed. It was anticipated that the size of the triisopropylsilyl group, the primary alcohol protecting group, might hinder approach of the \(\alpha\)-trimethylsilyl methyl vinyl ketone to the and the disparity has not been resolved.

Therefore, the plan was made to replace the triisopropylsilyl group with a less bulky protecting group, a benzyl group.

\[
\begin{align*}
\text{Scheme 15: Protection of diol 65 with benzyl chloride; NaH (60%), BnCl, DMF,} \\
\text{N}_2, 10^\circ\text{C, 3 hr; ii. D.I. water, 47% for 74, 15% for 75}
\end{align*}
\]

The diol 65 in \(N,N\)-dimethylformamide (DMF) was added to a suspension of sodium hydride in DMF at 10 °C. Benzyl chloride was introduced and after three hours reaction time, the regioisomers 74 and 75 were isolated in yields of 47% and 15% respectively. Sodium hydride, as a strong base, deprotonates the diol 65 generating an alkoxide ion for nucleophilic attack on the benzyl chloride. However, the deprotonation is not selective; however, the primary alkoxide is less hindered, and it will be more favoured to react with the benzyl chloride. The ratio of 74 to 75 is about 5:1 of 74 to 75 and they are separable products.
Scheme 16: Oxidation with pyridinium dichromate; i. PDC, DMF, r.t., 24hr; ii. D.I. water, 94%

Immediately after the separation, compound 74 was oxidized to the ketone 76 with pyridinium dichromate (PDC) in dimethylformamide during 24 hours in 94% yield. Pyridinium dichromate, an efficient oxidant that transforms alcohols into ketones or aldehydes, is obtained by addition of pyridine to a solution of chromium trioxide in water.\(^\text{17}\)

Scheme 17: Alkylation of ketone 76; i. LDA, -78 °C, THF, N\(_2\), 30 min; ii. 3-TMS-3-buten-2-one, -78 °C, 1 hr; iii. 0 °C, 2.5 hr, then pH 3; iv. aqueous NaHCO\(_3\) (5%), 13%

Next, the alkylation of ketone 76 with α-trimethylsilyl methyl vinyl ketone was carried out. The LDA solution was prepared at 0 °C in THF, and ketone 76 was introduced into the LDA solution at -78 °C followed by the 3-trimethylsilyl-3-buten-2-one. After stirring at 0 °C for 2.5 hours, the mixture was treated with dilute hydrochloric acid to give the diketone 77 in 13% yield. Although the yield is improved (13%) compared to the previous result (5%), it is still not satisfying. The low yield of alkylation is discouraging, as this reaction is crucial for the total
synthesis. A possible reason is that the kinetic enolate \textsuperscript{85} generated from LDA is too inert to react at such a low temperature.

A different idea for alkylating the protected pulegone \textsuperscript{76} was examined. An alkylating agent, 4-iodo-2-methyl-1-butene, was made\textsuperscript{18} in an attempt to replace \( \alpha \)-trimethylsilyl methyl vinyl ketone and alkyate compound \textsuperscript{76} by nucleophilic substitution. 4-Iodo-2-methyl-1-butene has the potential for alkylating the ketone \textsuperscript{76} because these kinds of reagents may be capable of trapping regiospecifically generated enolate ions under aprotic, non-equilibrating conditions.\textsuperscript{14} If the nucleophilic substitution works, the methylene group can be removed by oxidative cleavage using ruthenium chloride hydrate and sodium periodate\textsuperscript{19} which then yields the same product \textsuperscript{77} as using methyl vinyl ketone.

![Scheme 18: Preparation of alkyl iodide from alcohol; i. \( \text{Ph}_3\text{P, imidazole, I}_2, \text{CH}_2\text{Cl}_2, 25 ^\circ\text{C, N}_2, 3 \text{ hr, 50\%} \)](image)

4-Iodo-2-methyl-1-butene was prepared from 3-methyl-3-buten-1-ol and iodine in the presence of imidazole and triphenylphosphine in a yield of 50\% (Scheme 16). Next the ketone \textsuperscript{76} was deprotonated with LDA at -78 \(^\circ\text{C}\) in tetrahydrofuran, and treated with 4-iodo-2-methyl-1-butene. However, no alkylated product could be isolated. Attempts to improve this by using the method of transmetallating the enolate with zinc\textsuperscript{21} was not successful at all.
**Scheme 19:** Alkylation of ketone 76 with 4-iodo-2-methyl-1-butene; alkylation of compound 68: i. LDA, -78 °C, THF, N₂, 1.5 hr; ii. 4-iodo-2-methyl-1-butene, -78 °C, 2 hr; iii. 0 °C, 3.5 hr, then pH 2; iv. aqueous NaHCO₃, 5%

**Future Work**

It is necessary to use more active equivalents of the enolate. The nitrogen analogs of enols and enolates – enamines and imine anions are to be considered. In the preparation of enamines from ketones and secondary amines, there is a tendency for formation of the less-substituted enamine to predominate. Subsequently, the regiochemistry of the alkylation would be unambiguous. These reactions usually work with those ketones that do not give addition products with methyl vinyl ketone.

Surprisingly, it has been reported recently that difficulties in the alkylation of the protected isopulegone 78 can be overcome by using Noyori's zincate method in the high yield of 89%; the method is depicted in scheme 20.

**Scheme 20:** Noyori's zincate alkylation: i. LDA, -78 °C, THF, Ar, 40 min; ii. 0 °C, 30 min then -78 °C; iii. HMPA, Et₂Zn; iv. ((E)-3-Iodo-1-methylpropenyl)trimethylsilane, r.t, 24 hr; v. aqueous NH₄Cl, 89%
This example does highlight the possibility of alkylating a kinetic enolate with a halide compound by simple nucleophilic substitution in a high yield. How a smooth alkylation can take place in the presence of diethyl zinc and hexamethyldisilazane (HMPA) is not clear, but the method will be tried in our group.

In addition, another plan based on enolate equivalents is to use the more reactive enolate equivalent derived from the N,N-dimethylhydrazone (DMH) derivative 80 of the protected isopulegol derivative 68 in an alkylation reaction. After the synthesis of intermediate 80, the planned path is shown in scheme 21.

Scheme 21: Proposal for conversion of protected pulegol derivative 68 into the annelation precursor 69 via the DMH derivative 80

Other alkylating agents will also be considered. Mannich base precursor of methyl vinyl ketone generates methyl vinyl ketone \textit{in situ} in the presence of bases. \(\beta\)-Haloketone, and 4-chlorobutan-2-one can also release methyl vinyl ketone under base treatment, although it is quite difficult to handle. Therefore, a controlled release of MVK under the reaction conditions might be tried.
2.2 To probe potential biomimetic chemistry of the artemisinins:

As mentioned in the Introduction, it is necessary to examine the reactions with cysteine and other natural thiols, other hydride donors such as NAD(P)H and FADH, and other oxidizable substrates in the presence of electron-deficient substrates such as methylene blue, other dyes, and naphthoquinones such as menadione.

![Scheme 22: Redox reactions of NAD⁺ and NADH](image)

The proposed work is based on the hypothesis that artemisinins may exert their activity by accelerating decomposition of crucial biomolecules such as cofactors involved in various redox or other cellular processes, and which thereby may affect PfATP6, and hence calcium homeostasis in the parasite. Ms. Wing Yan Ho who worked previously in the Haynes group in this area, claimed that she found artemisinin is somehow catalyzing electron transfer from cysteine to Fe³⁺ complexed in ferrioxamine to generate Fe²⁺. If this happens to be true, then it means that artemisinins should be capable of enhancing oxidation of other substrates, e.g. NADPH or FADH in the presence of an electron acceptor, e.g. Fe³⁺, or one or more of the pro-oxidants. In this way, a proposal for mechanism of action of the
artemisinins will emerge. The parallel with the redox cycling involving GSH, NADPH and glutathione reductase described in the Introduction becomes obvious.

2.2.1 Effect of artemisinin on the reduction of desferrioxamine-Fe (III)

The first redox system will be that already examined by Ms. Ho, namely cysteine with desferrioxamine (DFO) complexed with Fe$^{3+}$, where the Fe$^{2+}$ complexing agent, bathophenanthroline disulfonic acid disodium salt (BPS), is also present.

![BPS](image)

Desferrioxamine B (figure 29) is a very strong natural iron chelator, which was first isolated from the micro-organism *Streptomyces pilosus*.\(^{22}\) It is commercially available as methanesulfonate salt of desferrioxamine, Desferal\(^{\text{®}}\). This hexadentate chelating ligand has excellent affinity and selectivity for ferric iron with stability constant of $10^{31}$, and forms the extremely stable ferric iron complex which is so called ferrioxamine B (figure 29).\(^{23}\) Desferrioxamine also shows affinity to ferrous iron but with much smaller stability constant of $10^7$. Desferrioxamine has a $pK_a$ 9.2.\(^{24, 25}\)
Figure 29: Structures of desferrioxamine B and its Fe$^{III}$ complex, ferrioxamine B.

Ferrioxamine B is not commercially available. Because of its excellent solubility in water and the specialized conditions required to obtain crystals,$^{26}$ it is difficult to generate pure ferrioxamine crystals with no extra iron or other metal ion incorporated into it. Therefore, ferrioxamine solution was prepared with atomic absorption standard iron solution in 1% HCl, the same manner as was carried out by Ms Ho. It was found later that the pH of the solution plays a crucial role in the formation of ferrioxamine B complex and this caused a substantial problem in the early stages of the project. This is discussed below.

The reaction was carried out by mixing the stock solutions in the following order: desferrioxamine-Fe (III), BPS, cysteine, and finally artemisinin under air. All stock solutions are at 0.5 mM before mixing. The spectra were recorded at designated time intervals. The UV-visible absorption spectra are presented in figures 102~104.
**Figure 102:** Desferrioxamine-Fe (III) (5 mM, 1 mL), BPS (5 mM, 1 mL), cysteine (5 M, 1 mL) and artemisinin (5 mM, 1 mL) under air. The last spectrum was scanned 10 min after the solution was mixed.

**Figure 103:** Desferrioxamine-Fe (III) (5 mM, 1 mL), BPS (5 mM, 1 mL) and cysteine (5 mM, 1 mL) under air. Solution was made up to 4 mL with 1:1 acetonitrile/ pH 7.4 phosphate buffer. The last spectrum was scanned 10 min after the solution was mixed.

The $\lambda_{\text{max}}$ absorption at 537nm shows the formation of Fe$^{\text{II}}$-BPS complex, which is in agreement with data recorded in the literature. However, the results of the UV study are not satisfying and have a very big difference from what is expected. In figure 102 and 103, although the ferrous iron-BPS complex did form...
during the course of the reaction, the artemisinins did not seem to enhance the reduction of Fe\(^{3+}\) to Fe\(^{2+}\). This is because the absorption at \(\lambda_{\text{max}}\) 537nm, demonstrating the formation of Fe\(^{2+}\)-BPS complex, in figure 102 did not exceed the absorption in figure 103, which is inconsistent with Ho’s findings. In order to solve the problem, different control experiments were carried out. One of the key findings is shown in figure 104.

**Figure 104**: Desferrioxamine-Fe (III) (5 mM, 1 mL) and BPS (5 mM, 1 mL) under air. Solution was made up to 4 mL with 1:1 acetonitrile/ pH 7.4 phosphate buffer. The last spectrum was scanned 10 min after the solution was mixed.

From figure 104, it is clear that the ferrous iron-BPS complex has formed in the absence of L-cysteine. It is believed that extremely low pH of the solutions is the source of the problem in these experiments. At this stage, the pH of the stock ferrioxamine B solution is found to be 0.83, based on the fact that the desferrioxamine-Fe(III) was prepared from 1% aqueous hydrogen chloride solution. A low pH of the solutions might weaken the binding of ferric iron to desferrioxamine, and cause dissociation, such that the "free" ferric iron ended up being trapped by BPS, with the appearance of the absorption at 537 nm. The measured molar
absorption coefficient of ferrioxamine B at pH 0.83 is 0.38 mM$^{-1}$cm$^{-1}$. This is very different to the literature value of 2.48 mM$^{-1}$cm$^{-1}$. Therefore, the pH of the ferrioxamine B solution was increased to 7.12 by titrating the ferrioxamine B solution with dilute ammonia solution. The ferrioxamine B solution at pH 7.12 shows a much more promising molar absorption coefficient 2.56 mM$^{-1}$cm$^{-1}$ which is consistent with the literature value. Anaerobic conditions were used for the pH 7.1 environment, and other thiols, N-methyl-L-cysteine methyl ester and glutathione were examined.

**Figure 98**: Desferrioxamine-Fe(III) (50 µM, 0.5 mL), BPS (50 µM, 0.5 mL), N-Acetyl-L-cysteine methyl ester (50 µM, 0.5 mL), and artemisinin (50 µM, 0.5 mL). The last spectrum was scanned 25 min after the solution was mixed.
**Figure 99**: Desferrioxamine-Fe(III) (50 µM, 0.5 mL), BPS (50 µM, 0.5 mL), and N-Acetyl-L-cysteine methyl ester (50 µM, 0.5 mL). The last spectrum was scanned 25 min after the solution was mixed.

**Figure 100**: Desferrioxamine-Fe(III) (50 µM, 0.5 mL), BPS (50 µM, 0.5 mL), glutathione (50 µM, 0.5 mL), and artemisinin (50 µM, 0.5 mL). The last spectrum was scanned 25 min after the solution was mixed.
Figure 101: Desferrioxamine-Fe(III) (50 µM, 0.5 mL), BPS (50 µM, 0.5 mL), and glutathione (50 µM, 0.5 mL). The last spectrum was scanned 25 min after the solution was mixed. Solution was made up to 2 mL with phosphate buffer.

At pH 7.1 under anaerobic conditions, it is much more difficult to obtain ferrous iron-BPS complex based on the observation that use of N-acetyl cysteine (NAC) methyl ester or glutathione did not induce formation of the absorption at 537 nm which represents the formation of BPS-ferrous iron complex. The results are unsatisfying. There is no evidence that reduction of ferric iron or release of ferrous iron from desferrioxamine-Fe (III) takes place. Thus, no conclusion as to the role of artemisinin in the redox system of desferrioxamine-Fe (III) can be made.

Figure 30: Structures of common thiol compounds.

There is no detectable interaction of BPS and artemisinin. When BPS and ferrioxamine are mixed at low pH, the absorption at \( \lambda_{\text{max}} \) 537 nm appears, indicating
the formation of a ferric iron-BPS complex. Therefore, this obscures any role which L-cysteine may be playing as a reducing agent in the reaction at that pH. In contrast, at approximately physiological pH (pH 7.1), none of thiols examined can reduce the FO complex, and release ferrous iron from the desferrioxamine. Binding of desferrioxamine-Fe (III) at a high pH is believed to stronger than that at low pH. Consequently, the ferrous iron mediated decomposition induced by artemisinin and claimed by Ms. Ho could not be verified.

Next, desferrioxamine-Fe (III) was replaced by ferrioxamine E, a cyclic form of ferrioxamine B, in an attempt to eradicate the problem of forming the siderophore-iron complex and maintain the integrity of the redox system in question. Stock solutions were prepared with pH 7.4 phosphate buffer in order to obtain a physiological pH. Experiments were carried out either under air or under argon to evaluate the effect of molecular oxygen on the redox system. However, the results of using ferrioxamine E were no different to those obtained using desferrioxamine-Fe (III). No ferrous iron-BPS complex was detected based on the results indicated in figure 105~108. None of these figures display an absorption peak at λ 537 nm due to the ferrous iron-BPS complex. An experiment with the artemisinin derivative, 10-deoxoartemisinin, which is tested to be less sensitive to ferrous iron mediated decomposition, also failed to deliver a positive result (figure 109 and 110). It is believed that at pH 7.4, one equivalent amount of L-cysteine was unable to reduce ferrioxamine E such that ferrous iron could be liberated. Use of excess amounts of L-cysteine (100 folds) did produce an absorption at 537 nm, indicating the presence of the ferrous iron-BPS complex. However, artemisinin had little, if any, effect on the redox system.
**Figure 105**: Ferrioxamine E (50 µM, 0.5 mL), BPS (50 µM, 0.5 mL), cysteine (50 µM, 0.5 mL) and artemisinin (50 µM, 0.5 mL) under argon. The last spectrum was scanned 20 min after the solution was mixed.

**Figure 106**: Ferrioxamine E (50 µM, 0.5 mL), BPS (50 µM, 0.5 mL), cysteine (50 µM, 0.5 mL) and artemisinin (50 µM, 0.5 mL) under air. The last spectrum was scanned 15 min after the solution was mixed.
Figure 107: Ferrioxamine E (50 µM, 0.5 mL), BPS (50 µM, 0.5 mL), and cysteine (50 µM, 0.5 mL) under argon. The last spectrum was scanned 20 min after the solution was mixed. Solution was made up to 2 mL with phosphate buffer.

Figure 108: Ferrioxamine E (50 µM, 0.5 mL), BPS (50 µM, 0.5 mL), and cysteine (50 µM, 0.5 mL) under air. The last spectrum was scanned 15 min after the solution was mixed. Solution was made up to 2 mL with phosphate buffer.
**Figure 109:** Ferrioxamine E (50 µM, 0.5 mL), BPS (50 µM, 0.5 mL), cysteine (50 µM, 0.5 mL) and 10-deoxoartemisinin (50 µM, 0.5 mL) under argon. The last spectrum was scanned 20 min after the solution was mixed.

**Figure 110:** Ferrioxamine E (50 µM, 0.5 mL), BPS (50 µM, 0.5 mL), and cysteine (50 µM, 0.5 mL) under argon. The last spectrum was scanned 20 min after the solution was mixed. Solution was made up to 2 mL with pH 7.4 phosphate buffer.
2.2.2 Effect of artemisinins on reduction of methylene blue

An investigation based on the redox indicator properties of the couple, methylene blue and leuco-methylene blue, was carried out in order to evaluate the effect of artemisinins on the reduction of methylene blue by means of UV spectrophotometry. Methylene blue is a leuco dye, which is a dye whose molecules can acquire two forms, one of which is colorless. This distinctive property makes methylene blue a common redox indicator in analytical chemistry and a suitable candidate to monitor the redox system of interest. Leuco-methylene blue can be generated by using a number of reductants such as dithionite, NADH, L-cysteine, glucose and others.  

![Graph](image)

**Figure 41:** Methylene blue (40.6 µM, 1 mL) and cysteine (2.72 mM, 1 mL, ~ 60 fold excess) under argon; recording interval between successive spectra was about 1 min.
Figure 42: Methylene blue (46.9 µM, 1 mL) and cysteine (0.47 mM, 1 mL, ~10 fold excess); under argon; recording interval between successive spectra was about 1.5 min.

Figure 43: Methylene blue (40.6 µM, 1 mL) and cysteine (0.136 mM, 1 mL, ~ 3 fold excess) under argon; recording interval between successive spectra was 1 min.

Methylene blue was able to be reduced by L-cysteine, as shown in figures 41~43. In contrast to the colorless leuco form, methylene blue is highly colored with an intense absorption in the region 200-700 nm with $\lambda_{\text{max}}$ at 662 nm. The absorbance at $\lambda_{\text{max}}$ 664 nm was monitored. The gradual decrease in absorbance in
the region 500 to 700 nm shows the progress of reduction. The rate constant of the above reduction was found to be constant in the pH range 7.0 ~ 10.6. Reduction of methylene blue highly depends on the amount of cysteine used. In figure 41 and 42, methylene blue is shown to be smoothly and completely reduced to leuco-methylene blue. In contrast, a slight excess of L-cysteine do not entirely reduce all of the methylene blue. The absorption at $\lambda_{\text{max}}$ 664 nm re-emerges, showing that leuco-methylene blue is re-oxidized back to methylene blue, most likely by molecular oxygen dissolved in the buffer. Leuco-methylene blue is very sensitive to oxygen. Excess reducing agent is necessary in order to suppress the re-oxidation of leuco-methylene blue under the conditions examined.

**Figure 44:** A control experiment: methylene blue (65 $\mu$M, 1 ml) + cysteine (65 $\mu$M, 1 ml) + blank (buffer solution). Final volume of the solution was 2.05 mL. Wavelength 666 nm was monitored against time under air.
Figure 45: Methylene blue (65 µM, 1 mL) + cysteine (65 µM, 1 mL) + artemisone (0.1 eq.). Wavelength 666 nm was monitored against time under air. Final volume of the solution was 2.05 mL.

The effect of the new artemisinin antimalarial drug, artemisone 56 on the reduction of methylene blue by cysteine was studied.

Experiments were carried out under air and the absorption at \( \lambda_{\text{max}} \) 666 nm was monitored against time. Only an equivalent amount of reducing agent was used. As indicated in figure 44 and 45, no sign of reduction took place. In fact, as demonstrated above, cysteine is capable of reducing methylene blue. The result here can be explained by the fact that oxidation of leuco-methylene blue is faster.
than reduction of methylene blue by cysteine. The role of artemisone cannot be verified. Therefore, the conditions have to be changed to be anaerobic. The results are depicted here.

**Figure 46**: Methylene blue (65 μM, 1 mL) + cysteine (65 μM, 1 mL, 1 eq.) + artemisone (0.1 eq.) under Ar. Wavelength 666 nm was monitored against time. Final volume of the solution was 2.07 mL.

**Figure 47**: A control experiment: methylene blue (65 μM, 1 mL) + cysteine (65 μM, 1 mL, 1 eq.) + blank (phosphate buffer) under Ar. Wavelength 666 nm was monitored against time. Final volume of the solution was 2.07 mL.
No significant effect of artemisone can be observed from figure 46 and 47. Leuco-methylene blue is quickly re-oxidized which may be due to the dissolved molecular oxygen. In the presence of one equivalent artemisone, there was no change in the rate of reaction. No conclusions can be drawn on the role of artemisone on the redox system involving methylene blue and cysteine.

Next, the reducing agent is switched to NADH, an important coenzyme found in all living cells. The malaria parasite has been shown to possess a whole range of antioxidant defence mechanisms, including a complete glutathione system that involves NAD(P)H. It is of interest as a drug target for redox active drugs. NAD(P)H is crucial to biochemical analysis since many enzyme and substrate assays are coupled to the formation or consumption of NADH which is usually followed by spectrophotometry, fluorescence or chemiluminescence methods.

NADH, like cysteine, has been found to reduce methylene blue. It is found here as well by using a 10-fold excess of NADH, the methylene blue can be reduced (figure 48 and 49).
Figure 48: Methylene blue (46.9 µM, 1 mL) + NADH (0.47 mM, 1 mL, ~10-fold excess). Final volume was 2 mL. Recording interval between each successive spectrum was about 1.5 min. The last spectrum was recorded 40 min after the solutions were mixed.

Figure 49: Methylene blue (56.28 µM, 1 mL) + NADH (2.82 mM, 1 mL, ~50 fold excess). Final volume was 2 mL and time interval between each successive spectrum was about 30 sec. The last spectrum was recorded 17 min after the solutions were mixed.

Interference with redox-active enzymes, such as NADH, might explain the way that artemisinins work to kill the parasites. The artemisinin may serve as a mediator
to transfer a pair of electron from NADH to methylene blue, it could reveal a higher rate of reduction of methylene blue by electron transfer in which artemisinin would be regenerated (Scheme 23). The depletion of NADH would result in loss of enzyme function.

![Scheme 23: Putative reduction of methylene blue](image)

However, it is shown that artemisinin and its derivatives fail to participate in electron transfer from NADH to methylene blue under anaerobic conditions (figure 52).
Figure 52: Methylene blue (47 µM, 1 mL), artemisone (3.47 mg/ mL, 0.3 mL) or acetonitrile (0.3 mL) and NADH (235 µM, 1 mL). Final volume of the solution was 2.3 mL. The solid line represents the experiment using artemisone while the dotted line represents experiment using acetonitrile (control).

The absorption at $\lambda_{\text{max}}$ 664 nm of methylene blue was monitored against time. From figure 52, it shows that NADH does reduce methylene blue under anaerobic conditions, but the presence of artemisone makes no contribution to the redox cycle. Similar experiments were carried out by replacing NADH with cysteine; the same results were obtained, except that the reduction of methylene blue was faster using cysteine.

NADH, and its oxidized form NAD$^+$ also absorb in the UV region. Their strong absorption is due to the adenine base. NAD$^+$ has $\lambda_{\text{max}}$ at 260 nm while NADH is at 340 nm. The distinctive absorption at 340 nm of NADH has been utilized for further investigation on the redox system.
Figure 53: Methylene blue (47 µM, 1 mL) and NADH (470 µM, 1 mL). Solutions were made up to 2.1 mL by pH 7.4 phosphate buffer. The last spectrum was recorded 1 hour after the solutions were mixed. Peak at 340 nm represents absorption maximum of NADH.

Figure 54: NADH (470 µM, 1 mL). Solution was made up to 2.1 mL with pH 7.4 phosphate buffer. The last spectrum was recorded 30 min later than the first recorded.
Figure 55: Methylene blue (47 µM, 1 mL), artemisinin (470 µM, 0.1 mL) and NADH (470 µM, 1 mL). The last spectrum was recorded 1 hour after the solutions were mixed.

Experiments were carried out under air. It has been demonstrated that NADH is not oxidized during the whole time course of the experiment (figure 54). This is evidence that the oxidation of NADH is purely caused by methylene blue (figure 53 and 55). No leuco-methylene blue is detected as it would be immediately oxidized back to methylene blue by oxygen. Nevertheless, the rate of oxidation of NADH does not show any difference in the presence of artemisinin.

In the presence of ferrous iron

As discussed in the Introduction, an enormous amount of debate has centred on the idea that ferrous iron could 'activate' artemisinins by converting the peroxide via Fenton type cleavage into oxygen centred radicals which then generate carbon centred radicals by abstraction of H atoms from the periphery of the molecule. The products arising via the decomposition have been examined and are confirmed to be antimalarial inactive. Although it is very unlikely that the C-centred radicals will
react with biomolecules, the precursor O-centred radical are much more active, and these might interfere with the co-factors involved in redox-active enzymes, and thus lead to the death of parasites (figure 32).

**Figure 32:** Idea of ferrous iron generating O-centred radicals by Fenton cleavage (see figure 18, Introduction). These may interact with NADH cofactor, although intramolecular decomposition to C radicals as explained in the Introduction must be fast.

The idea was tested by using methylene blue, NADH, ferrous sulfate and ferrocyanide, and artemisinin.

**Figure 58:** Methylene blue (47 µM, 1 mL), artemisinin (470 µM, 0.1 mL), FeSO₄ (470 µM, 0.1 mL) and NADH (940 µM, 0.5 mL). Solution was made up to 2.2 mL with pH 7.4 phosphate buffer. The last spectrum was recorded 50 min after the solutions were mixed.
Figure 59: Methylene blue (47 \( \mu \)M, 1 mL), artemisinin (470 \( \mu \)M, 0.1 mL), FeSO\(_4\) (47 \( \mu \)M, 0.1 mL) and NADH (940 \( \mu \)M, 0.5 mL). Solution was made up to 2.2 mL with pH 7.4 phosphate buffer. The last spectrum was recorded 50 min after the solutions were mixed.

The absorption maximum of NADH (340 nm) decreases gradually (figure 58 and 59) demonstrating that the oxidation of NADH is occurring. Experiment replacing ferrous sulfate with ferricyanide failed to give any changes. The putative radical intermediates do not take part in that particular system involving NADH and methylene blue.

In the biological system, depletion of reduced glutathione has been attributed to competition of methylene blue with GSSG for NAD(P)H thereby inhibiting the conversion of GSSG to GSH and to the oxidation of reduced glutathione by hydroperoxide that is generated from NAD(P)H, methylene blue and molecular oxygen. Leuco methylene blue and hydroperoxide are produced at the expense of oxygen and NAD(P)H in each cycle which provides an explanation for methylene blue acting as a subversive substrate. It indicates that methylene blue changes the physiological function of the enzyme to the opposite. The antioxidant disulfide reductases reported here are thiol-producing enzymes guarding the reducing
environment of cellular spaces. In the presence of MB, they turn into pro-oxidant 
H$_2$O$_2$-producing enzymes and challenge the reducing environment that they are 
meant to protect.$^{33}$ The synergistic effect of artemisinins, however, has not been 
detected. Essential steps or elements in the studies might be missed which could be 
necessary to account for the role of artemisinins.

![Scheme 24: Depletion of Glutathione](image)

In order to fully understand the idea of synergistic effect of artemisinins with 
methylene blue, another possibility is now explored. Focus has been switched that 
artemisinins might has the potential to accelerate the auto-oxidation of 
leuco-methylene blue and consequently stimulates consumption of important 
co-enzymes such as NAD(P)H (scheme 24). Thus, the effect of artemisinin on 
oxidation of leuco-methylene blue was examined.

![Scheme 25: Proposed oxidation of leuco methylene blue](image)
**Figure 63:** *Leuco*-MB (1 mL of dichloromethane) and artemisinin (1 eq.) in acetonitrile under nitrogen.

**Figure 64:** *Leuco*-MB (in 1 mL of dichloromethane) and artemisone (5.5 mM, 1 mL) under nitrogen.
**Figure 65:** Leuco-MB (17.5 mg in 5 mL of degassed water, 0.2 mL) and artemisinin (5.4 mM, 0.2 mL) under air. Solutions were made up to 2 mL with dichloromethane.

**Figure 66:** Leuco-MB (16.7 mg in 5 mL of degassed water, 0.1 mL) and artemisinin (5.2 mM, 20 µL, ~catalytic) under air. Solutions were made up to 2 mL with dichloromethane.
The experiments were first conducted under nitrogen in order to maximize the effect of artemisinin on leuco methylene blue. However, the spontaneous oxidation of leuco methylene blue is dramatic. A trace amount of oxygen dissolved in the buffer would readily oxidize the leuco back to methylene blue.\(^6\) It is difficult to trap the intact leuco methylene blue at pH 7.4 although it is possible to stabilize this colorless compound by enzymes at pH 5.\(^{33}\)

The absorbance of methylene blue at \(\lambda_{\text{max}}\) 664 nm gradually increases, implying that leuco-methylene blue is oxidizing back to methylene blue by oxygen. It indicates that it is not possible to completely remove all the oxygen from the system. However, regardless the presence of oxygen, there is a definite effect of artemisinin on the oxidation of leuco-methylene blue. In the presence of artemisinin, the rate of oxidation is much higher than that of without artemisinin (figure 63). This finding may confirm the possibility of which artemisinins serve as a mediator in the electron transfer cycle from oxygen to leuco-methylene blue. In the electron transport chain, artemisinin could operate as a reversible electron acceptor-donor which results in the transfer of one hydride equivalent from leuco-methylene blue to oxygen.

The same enhancing effect can also be demonstrated under air and with artemisinin derivative, artemisone although the enhancing effect of artemisone is weaker than that of artemisinin (figure 64). However, the idea of the artemisinin acting as a reversible electron transfer agent becomes difficult to prove, as it is shown here that artemisinins do not act catalytically (figure 66). Search for an explanation of the enhancing effect is still in progress.

Concurrently, a variety of redox active groups have been examined against artemisinins. In many cases, the mediation of electron transfer in environmental systems has been attributed to redox-active groups which could involve quinones.\(^{34}\)
5-Hydroxy-1, 4-naphthoquinone (juglone) and 2-hydroxy-1,4-naphthoquinone (lawsone) are of particular interested quinones.

![Figure 33: Structures of juglone 96 and lawsone 97](image)

With the capability of mediating electron transfer, it was hoped that 5-hydroxy-1,4-naphthoquinone or 2-hydroxy-1,4-naphthoquinone could behave the same way as methylene blue, and the reduced naphthoquinones should not have the same problem of the rapid aerial oxidation of leuco methylene blue.

Nevertheless, results prove otherwise. Neither reduced 2-hydroxy-1,4-naphtha-quinone nor the reduced 5-hydroxy-1,4-naphthoquinone could show any response with artemisinins (figures 68~71). Other redox systems, for instance 1, 2, 4-naphthalenetriol, reduced lumiflavin and glutathione reductase were also examined, but these do not give any hints on the enhancing effect of artemisinins on oxidation of leuco-methylene blue.
Figure 68: Reduced 5-hydroxy-1,4-naphthoquinone (2 mL) and artemisinin (0.05 mL, 2.86 mM). The last spectrum was recorded 50 min after the solutions were mixed.

Figure 69: Reduced 5-hydroxy-1,4-naphthoquinone (2 mL). Solution was made up to 2.05 mL with dichloromethane. The last spectrum was recorded 50 min after the solutions were mixed.
**Figure 70:** Dichloromethane extracts of reduced 2-hydroxy-1,4-naphthoquinone (1 mL) and artemisinin (11.5 mM, 1 mL). Recording interval between each successive spectrum was 9 min.

**Figure 71:** Dichloromethane extracts of reduced 2-hydroxy-1,4-naphthoquinone (1 mL). Solution was made up to 2 mL with dichloromethane. Recording interval between each successive spectrum was 9 min.
1-Benzyl-1,4-dihydronicotinamide (BNAH) is a partial structural analogue of NADH. In order to improve understandings of the role of NADH in many biochemical processes, it is useful to use relatively simple models. Thus, NADH was substituted by BNAH and a series of studies was carried out for BNAH. As discussed above, methylene blue is reduced by NADH, thereby removing this co-factor from its proper function. The catalytic cycle is maintained by oxidation of the resulting leuco-methylene blue by oxygen. In this way, methylene blue is a non-competitive inhibitor and subversive substrate of GR and TrxR (thioredoxin reductase). In addition, it has been shown that methylene blue is capable of oxidizing BNAH (scheme 26) which is verified (figure 112 and 113).

Scheme 26: Reduction of methylene blue with BNAH
**Figure 112:** Methylene blue (100 µM, 0.5 mL), artemisinin or tert-butyl hydroperoxide (500 µM, 0.1 mL), and BNAH (500 µM, 0.5 mL) under argon. Interval between each successive spectra was 8 min. Solution was made up to 2 mL with 1:1 deionized water/ acetonitrile mixture.

**Figure 113:** Methylene blue (100 µM, 0.5 mL), artemisinin or tert-butyl hydroperoxide (500 µM, 0.1 mL), and BNAH (500 µM, 0.5 mL) under air. Interval between each successive spectra was 8 min. Solution was made up to 2 mL with 1:1 deionized water/ acetonitrile mixture. The last spectrum was recorded 1 hr 30 min after the solutions were mixed.
It is not surprising that the rate of oxidation of BNAH under argon is much slower than that of under air (figure 112 and 113) due to the spontaneous oxidation of leuco-methylene blue. Consequently, oxygen enhances the consumption of BNAH by stimulating the autoxidation. The low rate of oxidation of BNAH under argon has been utilized for monitoring the effect, if any, of artemisinins or tert-butyl hydroperoxide on the redox cycle of methylene blue.

Surprisingly, tert-butyl hydroperoxide was not capable of oxidizing leuco-methylene blue back to methylene blue (figure 111). The absorption maximum of BNAH at $\lambda_{\text{max}}$ 352 nm was monitored against time. The trend of the curves shows that BNAH in these three cases is oxidized. The one with tert-butyl hydroperoxide or artemisinin does not demonstrate a significant difference with the one that only contains methylene blue as an oxidant. Leuco-methylene blue might bypass the path of involving any peroxide compounds and is directly oxidized back to methylene blue by molecular oxygen. Further investigations are needed to confirm the effect of artemisinin, if any, on the redox system involving methylene blue and BNAH or other NADH models.
Figure 111: Methylene blue (100 µM, 0.5 mL), artemisinin or tert-butyl hydroperoxide (500 µM, 0.1 mL), and BNAH (500 µM, 0.5 mL) under argon. Solution was made up to 2 mL with 1:1 deionized water/ acetonitrile.

The reduction of unsaturated organic compounds by substituted dihydro-nicotinamide models has been widely examined as model compounds for the study of reduction by nicotinamide nucleotides. The reduction is most frequently described as involving an electron transfer followed by hydrogen transfer.

Scheme 27: Mechanism for oxidation of 1,4-dihydropyridines

The possibility of artemisinins or tert-butyl hydroperoxide stimulating the reduction therefore is now evaluated.
**Figure 79:** $K_3Fe(CN)_6$ (1.5 mM, 1 mL), artemisinin (1.5 mM, 1 mL), and BNAH (30 $\mu$M, 1 mL). Peak maximum of BNAH (352 nm) was monitored. Solution was made up to 3 mL with 1:1 acetonitrile/water mixture in the control.

**Figure 82:** $K_3Fe(CN)_6$ (3 mM, 0.2 mL), *tert*-butyl hydroperoxide (3 mM, 0.2 mL), and BNAH (3 mM, 0.2 mL). Peak maximum of BNAH (352 nm) was monitored. Solution was made up to 3 mL with 1:1 acetonitrile/water mixture.
According to figure 79 and 82, tert-butyl hydroperoxide and artemisinin do not seem to play a significant role in the reduction of BNAH. Experiments substituting BNAH with NADH show similar results. In contrast, an intriguing result of the effect of tert-butyl hydroperoxide on the redox system involving BNAH and ferrous sulfate is demonstrated (figure 89).

**Figure 89:** Tert-butyl hydroperoxide (3 mM, 0.2 mL), BNAH (3 mM, 0.2 mL) and FeSO₄ (3 mM, 0.2 mL). Peak maximum of BNAH (352 nm) was monitored. Solution was made up to 3 mL with 1:1 acetonitrile/water mixture.

It is shown that BNAH is efficiently oxidized by the combination of tert-butyl hydroperoxide and ferrous sulfate. The rate of oxidation depends on both the concentration of tert-butyl hydroperoxide and ferrous sulfate. Neither tert-butyl hydroperoxide nor ferrous sulfate alone has any effect on the BNAH. The capability of tert-butyl hydroperoxide oxidizing BNAH with the help of ferrous iron could be explained by Fenton reaction.
According to Fenton reaction, alkoxy radicals are produced from tert-butyl hydroperoxide by reductive cleavage. The resulting alkoxy radical undergoes proton abstraction from the BNAH molecules. The same effect was observed with NADH (figure 91 and 92).

**Figure 90**: Tert-butyl hydroperoxide (3 mM, 0.2 mL), BNAH (3 mM, 0.2 mL) and FeSO$_4$ (3 mM, 0.2 mL). Peak maximum of BNAH (352 nm) was monitored. Solution was made up to 3 mL with 1:1 acetonitrile/water mixture. The last spectrum was scanned 25 min after the solution was mixed.
Figure 91: Tert-butyl hydroperoxide (3 mM, 0.2 mL), NADH (3 mM, 0.2 mL) and FeSO$_4$ (3 mM, 0.2 mL). Peak maximum of NADH (330 nm) was monitored. Solution was made up to 3 mL with 1:1 acetonitrile/ water mixture.

Figure 92: Tert-butyl hydroperoxide (3 mM, 0.2 mL), NADH (3 mM, 0.2 mL) and FeSO$_4$ (3 mM, 0.2 mL). Peak maximum of NADH (330 nm) was monitored. Solution was made up to 3 mL with 1:1 acetonitrile/ water mixture. The last spectrum was scanned 30 min after the solution was mixed.

The rate of oxidation of BNAH under argon was dramatically enhanced by the presence of tert-butyl hydroperoxide and ferrous sulfate. Because of the peroxide group in artemisinin, a connection has been sought between tert-butyl hydroperoxide and artemisinin. If tert-butyl hydroperoxide or artemisinin could
enhance the oxidation of BNAH, one possible mode of action, which is to upset the redox balance in intracellular reduced glutathione, might be discovered and the synergism of methylene blue with artemisinin may be explained. However, artemisinin does not behave in the same way as tert-butyl hydroperoxide under the same conditions towards BNAH or NADH (Figure 93 and 94).

**Figure 93**: Artemisinin (3 mM, 0.2 mL), BNAH (3 mM, 0.2 mL) and FeSO₄ (3 mM, 0.2 mL). Peak maximum of BNAH (352 nm) was monitored. Solution was made up to 3 mL with 1:1 acetonitrile/water mixture.
The result is disappointing. What may be happening is that the artemisinins are first required to undergo ring opening to generate *free hydroperoxide*. As explained in the Introduction (pp. 18, figure 7), artemisinin and DHA do undergo ring opening under mild conditions to generate free hydroperoxide. This 'unzipping' of the artemisinin 16 and dihydroartemisinin 17 nuclei are facile processes. For artemisinins such as artemisone which do not bear a group at C-10 which drives the ring opening, it is possible that the role of the third non-peroxidic oxygen atom is to stabilize the cation which would form on ring opening of the peroxide bridge (figure 34).

**Figure 94**: Artemisinin (3 mM, 0.2 mL), NADH (3 mM, 0.2 mL) and FeSO₄ (3 mM, 0.2 mL). Peak maximum of NADH (330 nm) was monitored. Solution was made up to 3 mL with 1:1 acetonitrile/water mixture.
**Summary and Future Work**

It has been difficult to unambiguously pin down how the artemisinins are likely to contribute to destruction of the malaria parasite. What has been shown is that artemisinins are capable of accelerating the oxidation of the leuco methylene blue back to methylene blue. As methylene blue is demonstrated to act as a subversive substrate (Scheme 29) in destroying NAD(P)H cofactor which is normally required in the operation of essential parasite enzymes (Scheme 1, p. 39), this will nicely...
account for the synergism that artemisinins display as antimalarials when mixed with methylene blue.

\[
\begin{align*}
\text{NADPH} + 2X & \rightarrow \text{NADP}^+ + 2X^- + H^+ \\
2X^- + 2O_2 & \rightarrow 2X + 2O_2^-
\end{align*}
\]

**Scheme 29**: Consumption of NADPH by subversive substrate\(^ {38}\)

Other members of the research group are engaged in pinpointing the chemistry which takes place when artemisinin reacts with leuco methylene blue. It is shown convincingly that the artemisinin is reduced (Scheme 30) as the leumethylene blue is oxidized back to methylene blue.

**Scheme 30**: Reduction of artemisinin by leuco methylene blue; work conducted by Ms. Wing-Chi Chan. The intermediate diol from artemisinin is detected by \(^1\)H NMR spectroscopy. This slowly ring closes to the reduced 2-deoxyartemisinin.

Methylene blue is capable of oxidizing NADH. We have shown here that artemisinin is not capable of oxidizing NADH or the model compound BNAH. However, the model system tert-butyl hydroperoxide in the presence of ferrous iron is capable of oxidizing NADH and BNAH.
It does however require some thought into the setting of a laboratory system which can mimic the biological system, and induce the artemisinins to oxidize the NADH. Therefore, the future work will probe more deeply the nature of the acceleration of the oxidation of methylene blue, and whether hydroperoxide analogues also work for this reaction, and then to use the results in hybrid systems containing methylene blue or leucomethylene blue, NADH and the artemisinin derivative.

Therefore, if artemisinins are capable of oxidizing the NADH then this will be a better explanation of their antimalarial activities. As explained in the Introduction, artemisinins and diverse peroxides are antimalarial active. This means that their binding to the PfATP6 is unlikely, and their effect on this calcium transporter is likely to be a secondary effect mediated by destruction of the co-factor essential for the operation of this transporter. The underlying effect is that the artemisinins are greatly enhancing oxidative stress.
References


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Chapter 3 Experimental

3.1 General

Artemisinin and dihydroartemisinin were obtained from the Kunming Pharmaceutical Corporation, Kunming, Yunnan Province, China. Other chemicals were purchased from commercial sources and used without further purification. All reactions solvents were purchased as HPLC grade and where appropriate, distilled prior to use. Tetrahydrofuran was dried over sodium wire and distilled from sodium benzophenone ketyl. Dichloromethane was distilled from calcium hydride. Acetonitrile was dried over phosphorus pentoxide, fractionally distilled and stored over type 4 Å molecular sieves under nitrogen.

Analytical thin layer chromatography was performed on pre-coated aluminum plates (Merck Kieselgel 60 F_{254}). The plates were visualized by ultraviolet light (λ=254nm) and/ or by treating with 5% ammonium molybdate in 10% concentrated sulphuric acid followed by heating with a hot gun. Column chromatography was performed with silica gel (Merck Kieselgel 60, 230-400 mesh) under optimum pressure.

$^1$H and $^13$C NMR spectra were recorded on a Varian Mercury 300 or a Bruker AV 400, with the former referenced at δ7.26 and the latter at δ77.00 in CDCl₃ respectively. Infrared spectra of solid samples were recorded on a FT-IR PerkinElmer Spectrum One spectrometer. Mass spectra were recorded on a Finnigan TSQ 7000 Mass Spectrometer operating in CI mode. Melting points were recorded on a Leica Microscope Heating Stage 350. All UV-vis absorption spectra were measured in a PerkinElmer Lambda 900 UV/VIS/NIR spectrophotometer using a 3 mL screw-capped quartz cuvette. The temperature was 298 ± 1K during the whole course of
the experiment. Test solutions in anaerobic conditions were deoxygenated before measurements by nitrogen or argon bubbling at 30 min. Spectrophotometric experiments were performed in pH 7.4 phosphate buffer or 1:1 acetonitrile/water mixtures.

3.2 Towards the preparation of ent-Artemisone

![Scheme 31](image)

**Scheme 31:** (a) SnCl₄, DCM, 0 °C; (b) BH₃-THF, then H₂O₂, NaOH; (c) i-Pr₃Cl, DMAP, Et₃N; (d) (COCl)₂, DMSO, Et₃N; (e) LDA, 3-(trimethylsilyl)-3-buten-2-one, then pH 2.

**R- (+)-Isopulegol 64**

A 100 mL single-neck round bottom flask was dried in high temperature oven, added some molecular sieve beads and flushed with nitrogen of S-(−)-citronellal 63 (5 mL, 4.28 g, 27.72 mmol) was added into the flask followed by dichloromethane (40 mL). The flask was cooled to 0 °C by immersing in an ice-water bath and the reaction mixture was stirred for 20 min before the addition of aqueous ammonium chloride (10%, 40 mL). The aqueous layer was separated and extracted with three 20 mL-portions of dichloromethane. The organic layer was combined and washed with brine (30 mL). The organic layer was separated and dried with magnesium sulfate. The filtrate was concentrated under reduced pressure. Then residual amounts of
solvents were removed under vacuum using a mechanical pump to leave the product as a colorless oil (3.6 g, 85%). $^1$H NMR: δ 4.90 (d, 1H, $J = 20$ Hz), 4.86 (d, 1H, $J = 28$ Hz), 3.99 (s, 1H), 3.47 (td, 1H, $J = 5.6$, 13.9 Hz), 1.71 (s, 3H), 1.1-2.1 (m, 8H), 0.95 (d, 3H, $J = 8.68$ Hz). $^{13}$C NMR: δ 146.38, 112.62, 70.11, 53.89, 42.39, 34.07, 31.20, 29.38, 22.52, 18.93. The data was in a good agreement with the literature.

(1S, 3S, 4R, 8S)-9-[(Triisopropylsilyl)oxy]-p-menthol 67

(+)- Isopulegol 64 (4.15 g, 26.9 mmol) was added to a round-bottomed flask (150 mL) secured with a septum containing fleshly distilled tetrahydrofuran (30 mL), and the solution was flushed with nitrogen at 0 °C. A solution of borane in tetrahydrofuran (30 mL, 30 mmol, 1 M in THF) was added dropwise to the flask via cannula. The reaction mixture was allowed to stir at ambient temperature for 6 hours. Aqueous solution of sodium hydroxide (3 M, 10 mL) was added to the flask slowly at 0 °C by syringe followed by aqueous hydrogen peroxide (10 mL, 30 %). After the addition was complete, the mixture was stirred for further 30 min at ambient temperature before pouring into saturated aqueous ammonium chloride (30 mL) and diluting with ethyl acetate (30 mL). The organic layer was separated and the aqueous layer was extracted with three-20 mL portions of ethyl acetate. The organic layer was combined and then washed with saturated aqueous ammonium chloride (20 mL) and brine (20 mL x 2), dried with anhydrous magnesium sulfate, and concentrated by rotary evaporation giving white semisolid. Recrystallization from 5 % ethyl acetate/hexane mixture gave the known diol in a form of white crystalline solid (1.76g, 38 %): mp 105-106.4 °C (lit mp 107 °C). $^1$H NMR: δ 3.67 (dd, 1H, $J = 5.4$, 10.6 Hz), 3.60 (dd, 1H, $J = 3.5$, 10.7 Hz), 3.47 (td, 1H, $J = 4.2$, 10.3 Hz), 1.95 (m, 1H), 1.86 (m, 1H), 1.58 (m, 2H), 1.34 (m, 2H), 1.28 (dd, 1H, $J = 3.3$, 12.6 Hz),
0.95 (d, 3H, $J = 7.3$ Hz), 0.91 (d, 3H, $J = 6.6$ Hz), 0.8-1.0 (m, 2H). $^{13}$C NMR: $\delta$

69.68, 66.58, 48.46, 44.25, 38.41, 34.52, 31.33, 29.42, 21.99, 11.95.

The diol (2.31 g, 13.4 mmol), triisopropylsilyl chloride (2.58 g, 13.4 mmol), triethylamine (2.1 mL, 15.1 mmol), and DMAP (0.164 g, 1.34 mmol) were mixed in a round-bottom flask (100 mL) flushed with nitrogen and filled with dichloromethane (30 mL). The mixture was stirred at ambient temperature for three days. After the reaction was complete, the mixture was poured into brine (25 mL) and diluted with dichloromethane (25 mL). The aqueous layer was separated and extracted with three 20 mL portions of dichloromethane. The combined organic layer was dried with magnesium sulfate, filtered, and concentrated by rotary evaporator. Flash chromatography with ethyl acetate-hexane (10:90) on silica gel gave the product as a clear oil (4.17 g, 95%). $^1$H NMR: $\delta$ 4.06 (br s, 1H), 3.74 (dd, 1H, $J = 7.8, 13.1$ Hz), 3.64 (dd, 1H, $J = 4, 13.1$ Hz), 3.43 (td, 1H, $J = 4.2, 10.3$ Hz), 1.92 (m, 1H), 1.87 (m, 1H), 1.60 (m, 1H), 1.52 (m, 1H), 1.12-1.47 (m, 3H), 1.05 (m, 21H), 0.95 (d, 3H, $J = 9.7$ Hz), 0.8-1.0 (m, 2H), 0.91 (d, 3H, $J = 8.8$ Hz). $^{13}$C NMR: $\delta$ 69.96, 67.77, 49.03, 43.90, 38.20, 34.73, 31.33, 28.69, 22.06, 17.89, 12.73, 11.82 (SiCH).

$(1S,4R,8S)$-9-$

{(Triisopropylsilyl)oxy}$-p-menthone 68

Dimethyl sulfoxide (2.2 mL, 31 mmol) and then oxalyl chloride (1.35 mL, 15.5 mmol) were added to dichloromethane (40 mL) in a nitrogen-purged round-bottom flask (150 mL) immersed in a dry-ice acetone cooling bath at -78 °C. The mixture was stirred at -78 °C for 20 min. Alcohol 67 (4.32 g, 13.2 mmol) as a solution of dichloromethane (20 mL) was added to the flask via cannula. The resultant solution was stirred at -78 °C for 1.5 hours. Triethylamine (5.4 mL, 38.8 mmol) was
introduced, and the reaction mixture was allowed to stir at ambient temperature for an additional hour. The mixture was poured into brine (50 mL). The organic layer was separated and the aqueous layer was extracted with three 30 mL portions of dichloromethane. The combined organic layer was washed with brine (30 mL), dried with magnesium sulfate, and concentrated by rotary evaporator. Flash chromatography with ethyl acetate/ hexane (4:96) was employed to purify the crude product and gave a yellow oil 68 in a yield of 94 % (4.04 g). \(^1\)H NMR: \(\delta 3.68 \text{ (dd}, 1\text{H}, J = 5.2, 9.6 \text{ Hz}), 3.61 \text{ (dd}, 1\text{H}, J = 5.6, 9.6 \text{ Hz}), 2.33 \text{ (m}, 1\text{H}), 2.30 \text{ (m}, 1\text{H}), 2.01 \text{ (m}, 1\text{H}), 1.95-2.04 \text{ (m}, 2\text{H}), 1.75-1.92 \text{ (m}, 2\text{H}), 1.29-1.51 \text{ (m}, 2\text{H}), 1.04 \text{ (m}, 21\text{H}), 1.00 \text{ (d}, 3\text{H}, J = 6.4 \text{ Hz}), 0.98 \text{ (d}, 3\text{H}, J = 6.8 \text{ Hz}). \(^13\)C NMR: \(\delta 212.15, 65.70, 52.11, 51.08, 35.68, 35.03, 34.23, 29.83, 22.26, 18.00, 15.27, 11.97.

\((1S,2R,4R,8S)-2-(3'-\text{Oxobutyl})-9-\{(\text{triisopropylsilyl})\text{oxy}\}-\text{p-menthone} \ 69\)

An LDA solution was prepared in the following manner: To a round bottom flask (100 mL) purged with nitrogen and filled with tetrahydrofuran (7 mL), diisopropylamine (0.26 mL, 1.86 mmol) was added. The flask was immersed in a dry-ice/ acetone bath and cooled to -78 °C. n-Butyl lithium (0.77 mL, 1.93 mmol, 2.3 M in hexane) was introduced dropwise to the flask. The resultant mixture was stirred for 20 min at -78 °C.

The ketone 68 (500 mg 1.53 mmol), as a solution of tetrahydrofuran (3 mL) was added slowly via cannula to the LDA solution and the reaction mixture was stirred at 1.5 hours at -78 °C before the addition of 3-(trimethylsilyl)-3-buten-2-one (224 mg, 1.58 mmol). The buten-2-one, dissolved in tetrahydrofuran (3 mL), was added dropwise to the flask and stirred for 2 hours at -78 °C, and for 3.5 hours at 0 °C by replacing the dry-ice/ acetone with an ice-water bath. After the reaction was complete, the mixture was treated with hydrogen chloride (10 %) to achieve a pH 3
solution and continued to stir for an additional hour before pouring into aqueous solution of sodium bicarbonate (5 mL, 5 %). The mixture was diluted with ethyl acetate (10 mL). The aqueous layer was separated and extracted with three 15 mL portions of ethyl acetate. The combined organic layer was washed with two 10 mL portions of brine, dried with magnesium sulfate and concentrated by rotary evaporator. Flash chromatography with ethyl acetate/ hexane (10:90) afforded diketone 69 as a yellow oil (30 mg, 5 %). ¹H NMR: δ 3.45 (dd, 1H, J = 6, 9.8 Hz), 3.39 (dd, 1H, J = 6.8, 10 Hz), 2.42 (m, 1H), 2.31 (m, 2H), 2.09 (s, 3H), 2.03-2.2 (m, 1H), 1.75 (m, 2H), 1.64-2.06 (m, 4H), 1.2-1.6 (m, 3H), 1.03 (m, 23H), 0.90 (d, 3H, J = 6.4 Hz). ¹³C NMR: δ 213.48, 208.98, 65.53, 57.20, 53.12, 41.45, 40.87, 35.02, 34.95, 31.14, 29.76, 20.52, 20.21, 17.98, 15.58, 11.93.

Scheme 32: Preparation of 3-trimethylsilyl-3-buten-2-one 98.

3-Trimethylsilyl-3-buten-2-ol 97
To a three necked round bottomed flask (250 mL) equipped with a condenser, an additional funnel and a magnetic stirrer and flushed with argon, magnesium turnings (1.84 g, 75.7 mmol) and tetrahydrofuran (40 mL) were added. 1,2-Dibromoethane (0.18 mL) was added by syringe to initiate the formation of Grignard reaction. A solution of silane 96 (10 g, 56 mmol) in tetrahydrofuran (15 mL) was added dropwise to the flask when the reaction mixture was warm and begun to reflux. The mixture was maintained at reflux for an additional hour after the addition was complete. Then acetaldehyde was introduced as a solution of tetrahydrofuran (15 mL) at reflux temperature and continued to reflux for one hour after the addition. A
distillation head was set up and distillate (40 mL) was collected. The flask was allowed to cool down in an ice water bath, diluted with diethyl ether (30 mL), and dissolved into saturated ammonium chloride (30 mL). The salts were filtered and washed with diethyl ether. The aqueous layer was separated and extracted with three 20 mL portions of diethyl ether. The combined organic layer was washed with brine, dried with magnesium sulfate, and concentrated by distillation at atmospheric pressure affording the crude butenol 97 (11 g). The butenol 97 was used for the next step without further purification.

3-Trimethylsilyl-3-buten-2-one 98

To a round bottom flask (100 mL) charged with acetone (20 mL), the crude butenol 97 (11 g) was added. The flask was cooled to 0 °C. An aqueous solution of chromium trioxide and sulfuric acid (19 mL) was introduced to the flask dropwise. After the addition was complete, the resultant solution was poured into diethyl ether (90 mL) followed by deionized water (60 mL). Then the aqueous layer was saturated with sodium chloride. The aqueous layer was separated and extracted with five 50 mL portions of diethyl ether. The combined organic layer was washed with two 30 mL portions of brine, dried with magnesium sulfate, and concentrated by distillation at atmospheric pressure affording a pale yellow liquid of butenone 98 (44.8% in two steps, 4.92 g). 1H NMR: δ 6.50 (d, J = 1.6 Hz, 1H), 6.17 (d, J = 2 Hz, 1H), 2.29 (s, 3H), 0.14 (s, 9H).
Scheme 33: a. BH$_3$-THF, then H$_2$O$_2$, NaOH; NaH, BnCl; b. PDC; c. LDA, 3-(trimethylsilyl)-3-buten-2-one, then pH 3

(1S, 3S, 4R, 8S)-9-(phenylmethoxy)-p-menthol 74

The known diol (white crystalline solid) was obtained from 65 as described above. A solution of the known diol 65 (2.11 g, 12.3 mmol) in dimethylformaide (20 mL) was added to a suspension of 60%NaH (1.08 g, 27 mmol) at 10 °C immersed in a water bath. The mixture was stirred for 45 min before introducing a solution of benzyl chloride (1.72 g, 13.5 mmol) in dimethylformamide (6 mL). The resultant mixture was stirred at 10 °C for three hours. After that, cold deionized water (20 mL) was poured into the flask and diethyl ether (30 mL) was employed to dilute the resultant solution. The separated organic layer was washed with three 20 mL portions of deionized water, dried with magnesium sulfate, and concentrated by reduced pressure. Flash chromatography with hexane/ ethyl acetate/ acetone (14:3:3) of the crude product gave compound 74 in a yield of 47% (1.47 g) and compound 75 in a yield of 15% (0.47 g). Compound 74: $^1$H NMR (CDCl$_3$): δ 7.37 (m, 5H), 4.52 (dd, $J = 12, 22.4$ Hz, 2H), 3.38-3.59 (m, 3H), ), 2.17 (m, 1H), 2.04 (m, 1H), 1.58 (m, 2H), 1.34 (m, 2H), 1.12 (m, 1H), 0.96 (d, $J = 7.6$ Hz, 3H), 0.91 (d, $J = 6.4$ Hz, 3H); Compound 75: $^1$H NMR (CDCl$_3$): δ 7.37 (m, 5H), 4.70-4.41 (dd, $J = 11.2$ Hz,
2H), 3.61-3.40 (m, 3H), 2.17-1.12 (m, 7H), 0.98 (d, J = 7.2 Hz, 3H), 0.93 (d, J = 6.4 Hz, 3H).

\[(1S, 3S, 4R, 8S)-9-(phenylmethoxy)-p-menthone 76\]

To a round bottom flask (100 mL) charged with pyridinium dichromate (5.12 g, 13.6 mmol) and dimethylformamide (10 mL), compound 74 (1.47 g, 5.72 mmol) was added and the reaction mixture was stirred at room temperature for 24 hours. After that, the resultant mixture was poured into deionized water (20 mL) and diluted with diethyl ether (20 mL). The separated organic layer was washed with ten 10 mL portions of deionized water, dried with magnesium sulfate, and concentrated under reduced pressure. Flash chromatography with petroleum ether/ethyl acetate/acetone (9:0.5:0.5) afforded a crude product as a colorless liquid of compound 76 in a yield of 94% (1.41 g). \(^1\)H NMR (CDCl\(_3\)): \(\delta 7.33\) (m, 5H), 4.47 (d, J = 2.4 Hz, 2H), 3.47 (dd, J = 7.6, 12.2 Hz, 1H), 3.38 (dd, J = 8, 12 Hz, 1H), 2.17-1.12 (m, 7H), 1.01 (d, J = 2.2 Hz, 3H), 1.00 (d, J = 3.2 Hz, 3H).

\[(1S, 2R, 4R, 8S)-2-(3'-Oxobutyl)-9-phenylmethoxy-p-menthone 77\]

To a round bottom flask (50 mL) purged with argon and filled with tetrahydrofuran (17 mL), diisopropylamine (1.15 mL, 8.3 mmol) was added. The flask was cooled to 0 °C in an ice-water bath. n-Butyl lithium (5.84 mL, 7.5 mmol, 1.4 M in hexane) was introduced dropwise to the flask. After the resultant mixture was stirred for 15 min at 0 °C, the flask was immersed in a dry-ice/acetone bath. The ketone 77 (1.41 g, 5.42 mmol) as a solution of tetrahydrofuran (1 mL) was added slowly via cannula to the LDA solution and the reaction mixture was stirred at 30 min at -78 °C before the addition of 3-(trimethylsilyl)-3-buten-2-one. The buten-2-one (1.46 g, 10 mmol), dissolved in tetrahydrofuran (1 mL), was added dropwise to the flask via cannula and
the resultant solution was stirred for one hour at -78 °C, and for 2.5 hours at 0 °C. After the reaction was complete, the mixture was treated with hydrogen chloride (3.6 %) to achieve a pH 3 solution and continued to stir for an additional 15 min before pouring into aqueous solution of sodium bicarbonate (10 mL, 5 %). The mixture was diluted with ethyl acetate (20 mL). The aqueous layer was separated and extracted with three 20 mL portions of ethyl acetate. The combined organic layer was washed with two 15 mL portions of brine, dried with magnesium sulfate and concentrated by rotary evaporator. Flash chromatography with 12 % ethyl acetate in hexane afforded the diketone 77 as pale yellow oil (0.24 g, 13.4 %). $^1$H NMR: $\delta$ 7.36 (m, 5H), 4.50 (d, $J = 2.8$ Hz, 2H), 3.48 (dd, $J = 5.2$, 9.2 Hz, 1H), 3.40 (dd, $J = 5.6$, 9 Hz, 1H), 2.14 (s, 3H), 1.08 (d, $J = 6$ Hz, 3H), 1.2 (d, $J = 6.8$ Hz, 3H); $^{13}$C NMR: $\delta$ 213.0, 209.0, 138.5, 128.1, 127.2, 72.7, 57.0, 53.2, 41.2, 40.3, 34.6, 32.4, 30.4, 29.6, 20.3, 20.0, 15.7.

![Scheme 18: Preparation 4-iodo-2-methyl-1-butene](image)

**Scheme 18**: Preparation 4-iodo-2-methyl-1-butene

**4-Iodo-2-methyl-1-butene 12**

To a round bottom flask (250 mL) charged with dichloromethane (140 mL) and of Ph$_3$P (15.72 g, 60 mmol), was added imidazole (4.08 g, 60 mmol) followed by iodine (15.24 g, 60 mmol). The resultant mixture was stirred at room temperature for 5 min. Then, 3-methyl-3-buten-1-ol (4.3 g, 50 mmol) as a solution of dichloromethane (30 mL) was introduced to the flask through cannula. The resultant mixture was stirred at ambient temperature for 3 hours. Most of the solvent was removed by rotavapor and the residue was diluted with pentane (40 mL). The resulting residue was
extracted with five 30 mL portions of pentane. The organic layer was combined and concentrated by rotavapor. Flash chromatography with pentane afforded iodide 99 (4.82 g, 50 %) that became colorless on storage over Cu powder. $^1$H NMR (CDCl$_3$): $\delta$ 4.86 (br s, 1H), 4.75 (br s, 1H), 3.26 (t, $J = 7.6$ Hz, 2H), 2.59 (t, $J = 7.6$ Hz, 2H), 1.74 (s, 3H).
3.3 Potential Biomimetic Chemistry and Redox reactions

Background

In order to probe the role played by each of glutathione and DFO in suppressing antimalarial activity of artemisinin, either by interfering with production of ROS, or scavenging the iron required for activation of artemisinins, experiments were carried out by Wing Yan Ho. Instead of using glutathione, cysteine was used, together with artemisinin and FO in aqueous acetonitrile buffered to pH 7. Control experiments with artemisinin and each of FO, DFO and cysteine alone indicated no reaction took place. Thiols such as cysteine have been reported to reduce the Fe$^{3+}$ in FO, thus releasing a large local concentration of Fe$^{2+}$ which may be capable of destroying artemisinin. However, mixtures of cysteine with FO had no effect on artemisinin in the control experiments. Surprisingly, however, it was reported by Ho that the cysteine reduces Fe$^{3+}$ in FO, when a Fe$^{2+}$ complexing agent in present. This was followed by UV-VIS spectroscopy. Desferrioxamine B is colorless while its Fe$^{3+}$ complex, ferrioxamine B, is brownish red in color. The absorption spectrum of ferrioxamine B in 1:1 acetonitrile and pH 7.4 phosphate buffer shows the $\lambda_{max}$ absorption at 448 nm with molar absorption coefficient $\varepsilon$ 2.48 mm$^{-1}$cm$^{-1}$ (figure 35).
The course of the reaction of artemisinin with FO in the presence of l-cysteine was monitored by Ho at the $\lambda_{\text{max}}$ absorbance at 537 nm. In order to elucidate the nature of this interaction, bathophenanthroline sulfonate disodium salt hydrate (BPS) was used in the measurement. BPS is a ferrous iron chelating agent, which can be used for intercepting Fe$^{2+}$ produced during reduction of FO by l-cysteine. The absorption spectrum of Fe$^{2+}$-BPS complex in 1:1 acetonitrile and a pH 7.4 phosphate buffer shows the $\lambda_{\text{max}}$ absorption at 537 nm with $\varepsilon$ 22140 L eq$^{-1}$ cm$^{-1}$ (figure 36).\textsuperscript{4}
Figure 36: UV-VIS absorption spectrum of Fe$^{2+}$-BPS complex.

Ho carried out reactions by mixing FO, then BPS, cysteine, and finally artemisinin stock solutions and recording the spectra at certain time intervals (figures 37~39). In order to show that there was no interaction between BPS and artemisinin, the order of mixing the reagents was changed - BPS, then artemisinin, cysteine, and finally FO stock solutions were mixed in that order to the final concentrations of 0.5 mm (figure 40).

Figure 37: UV-VIS absorption spectrum of reaction of FO with l-cysteine in presence of BPS at final concentration of 5.0 μm.
Figure 38: UV-VIS absorption spectrum of reaction of FO with l-cysteine in presence of BPS after adding artemisinin at final concentration of 5.0 μm.

Figure 39: All-in-one UV-VIS absorption spectrum of FO reaction of artemisinin with l-cysteine at final concentration of 5.0 μm.
**Figure 40:** UV-VIS absorption spectrum of ferrioxamine reaction of artemisinin 17 with l-cysteine at final concentration of 0.5 mm.

From the spectra, it is clear that with the $\lambda_{\text{max}}$ absorption at 537 nm, the Fe$^{2+}$-BPS complex is formed within 2 minutes of mixing. The Fe$^{3+}$ in ferrioxamine is reduced to Fe$^{2+}$ by l-cysteine and is released and captured by BPS. Remarkably, in figures 39 and 40, the absorbance at $\lambda_{\text{max}}$ 537 nm is nearly three times those without artemisinin in the reaction mixture at the same concentration. Thus, the presence of artemisinin enhanced the reduction of Fe$^{3+}$ to Fe$^{2+}$ and release from FO. It was expected that the Fe$^{2+}$ released from FO would react with artemisinin in the reaction mixture. However, this did not take place. Possibly the BPS-Fe$^{2+}$ complex may not react with artemisinin, but this was not tested by Ho. However, BPS alone did not interact with artemisinin. This is a remarkable result. It infers that somehow, artemisinin is catalyzing electron transfer from cysteine to Fe$^{3+}$ in FO to generate Fe$^{2+}$. If this is the case, then it means that artemisinins should be capable of enhancing oxidation of other substrates, e.g. NADPH or FADH in the presence of an electron acceptor, e.g. Fe$^{3+}$, or one or more of the pro-oxidants. In this way, a convincing proposal for mechanism of action of the artemisinins will emerge. The
parallel with the redox cycling involving GSH, NADPH and glutathione reductase
described above becomes obvious.

Therefore, my task is to reproduce the above results obtained by Dr. Ho, and
if they are reproducible, to apply them to other redox systems.
3.3.1.1 Reduction of Methylene Blue with Cysteine

A stock solution of methylene blue (40.64 µM) was obtained by dissolving methylene blue (1.3 mg) in a pH 7.4 phosphate buffer (100 mL) under argon in a volumetric flask (100 mL). The flask was secured with a septum. Three stock solutions of cysteine (2.72 mM, 0.47 mM, and 0.136 mM) were prepared in the same manner. The methylene blue solution (1 mL) was transferred to an argon-purged UV cuvette by a syringe followed by the cysteine solution (1 mL), to give a total volume of 2 mL. Spectrophotometric measurements were taken as quickly as possible after injection of the cysteine into the quartz cuvette. The wavelength range from 500 nm to 800 nm was monitored against time and the results were plotted in figures 41~43.

![Absorbance vs Wavelength](image)

**Figure 41**: Methylene blue (40.6 µM, 1 mL) and cysteine (2.72 mM, 1 mL, ~ 60 fold excess) under Ar; recording interval between successive spectra was about 1 min.
**Figure 42:** Methylene blue (46.9 µM, 1 mL) and cysteine (0.47 mM, 1 mL, ~10 fold excess); under Ar; recording interval between successive spectra was about 1.5 min.

**Figure 43:** Methylene blue (40.6 µM, 1 mL) and cysteine (0.136 mM, 1 mL, ~ 3 fold excess) under Ar; recording interval between successive spectra was 1 min.
3.3.1.2 Effect of artemisone on reduction of Methylene Blue with Cysteine

Stock solutions of methylene blue (65 µM), cysteine (65 µM), and artemisone (1.39 mM) were prepared as in 3.3.1.1 except the case of artemisone which acetonitrile was chosen as a solvent. 1 mL of methylene blue was injected into a cuvette followed by cysteine. 10 mol % of artemisone was injected into the cuvette by a micro-syringe. Spectrophotometric experiments were taken as quickly as possible after artemisone was injected into the quartz cuvette. Peak maximum of methylene blue (666 nm) was monitored against time. Experiments under argon and under air were performed and results were plotted in figures 44~47.

![Graph showing absorbance at 666 nm against time](image)

**Figure 44:** A control experiment: methylene blue (65 µM, 1 ml) + cysteine (65 µM, 1 ml) + blank (buffer solution). Final volume of the solution was 2.05 mL. Wavelength 666 nm was monitored against time under air.
Figure 45: Methylene blue (65 μM, 1 mL) + Cysteine (65 μM, 1 mL) + Artemisone (0.1 eq.). Wavelength 666 nm was monitored against time under air. Final volume of the solution was 2.05 mL.

Figure 46: Methylene blue (65 μM, 1 mL) + cysteine (65 μM, 1 mL, 1 eq.) + artemisone (0.1 eq.) under Ar. Wavelength 666 nm was monitored against time. Final volume of the solution was 2.07 mL.
**Figure 47**: A control experiment: methylene blue (65 µM, 1 mL) + Cysteine (65 µM, 1 mL, 1 eq.) + blank (phosphate buffer) under Ar. Wavelength 666 nm was monitored against time. Final volume of the solution was 2.07 mL.
3.3.1.3 Reduction of Methylene blue with NADH

Stock solutions of methylene blue (46.9 µM and 56.3 µM) were prepared as described above. Stock solutions of NADH (0.47 mM and 2.82 mM) were prepared by adding appropriate amount of pH 7.4 phosphate buffer into vials containing known amount of NADH (1 mg or 5 mg) and then secured with a septum. Experiments were done as the same in 3.3.1.1 that NADH was used instead. Wavelength range from 500 nm to 800 nm was monitored and the results were plotted in figures 48 and 49.

![Figure 48](image_url)

**Figure 48**: Methylene blue (46.9 µM, 1 mL) + NADH (0.47 mM, 1 mL, ~10 fold excess). Final volume was 2 mL. Recording interval between each successive spectrum was about 1.5 min. The last spectrum was recorded 40 min after the solutions were mixed.
**Figure 49**: Methylene blue (56.28 µM, 1 mL) + NADH (2.82 mM, 1 mL, ~50 folds excess). Final volume was 2 mL and time interval between each successive spectrum was about 30 sec. The last spectrum was recorded 17 min after the solutions were mixed.

3.3.1.4 Effect of 10α-sulfamido-DHA on reduction of Methylene blue with NADH

The redox system of methylene blue and NADH was used to test against 10α-sulfamido-DHA. Stocks solutions of methylene (31 µM) and NADH (1.88 mM) were prepared according to 3.3.1.3. A stock solution of 10α-sulfamido-DHA (5 mg/mL in acetonitrile) was prepared. Solutions were injected into a UV cuvette in the following sequence: methylene blue (1 mL), NADH (1 mL), and 10α-sulfamido-DHA (0.3 mL). Spectrophotometric measurements were taken as quickly as possible after 10α-sulfamido-DHA was injected into the cuvette. A control experiment was done in the same manner that pure acetonitrile was used instead of 10α-sulfamido-DHA solution. Results were plotted in figures 50 and 51.
**Figure 50:** Methylene blue (31 µM, 1 mL) + NADH (1.88 mM, 1 mL, ~60 fold excess) + 10α-sulfamido-DHA (5 mg/mL, 0.3 mL). Time interval between each successive was 30 sec. The last two spectra were recorded after 10α-sulfamido-DHA was injected. Final volume of the solution was 2.3 mL.

**Figure 51:** A control experiment: Methylene blue (56.28 µM, 1 mL) + NADH (2.82 mM, 1 mL, ~50 folds excess) + 0.3 mL acetonitrile. The last spectrum was recorded after acetonitrile was injected. Final volume of the solution was 2.3 mL.
3.3.1.5 Effect of artemisone on reduction of Methylene blue with NADH

A similar experiment to 3.3.1.4 was done. 10α-Sulfamido-DHA was replaced by artemisone. Stock solutions of methylene blue (47 µM), NADH (235 µM), and artemisone (3.47 mg/mL) were prepared according to 3.3.1.4. Wavelength 666 nm, peak maximum of methylene blue, was monitored against time. Results were plotted in figure 52.

**Figure 52:** Methylene blue (47 µM, 1 mL), artemisone (3.47 mg/ mL, 0.3 mL) or acetonitrile (0.3 mL) and NADH (235 µM, 1 mL). Final volume of the solution was 2.3 mL. The solid line represents the experiment using artemisone while the dotted line represents experiment using acetonitrile.
3.3.1.6 Effect of artemisinin on reduction of Methylene blue with NADH

A variant of experiment 3.3.1.5 was done by replacing artemisone with artemisinin. Stocks solution of methylene blue (47 μM), NADH (470 μM), and artemisinin (470 μM) were prepared as described above. Solution of artemisinin was prepared by using acetonitrile. The solutions were injected into a UV cuvette in the following order under argon: methylene blue (1 mL), artemisinin (0.1 mL or 10 μL), and NADH (1 mL). Spectrophotometric measurements from range 200 nm to 800 nm were taken as quickly as possible after NADH was injected under air. Wavelength from 200 nm to 800 nm was recorded. Result were depicted in figures 53~56.

![Graph](image)

**Figure 53:** Methylene blue (47 μM, 1 mL) and NADH (470 μM, 1 mL). Solutions were made up to 2.1 mL by pH 7.4 phosphate buffer. The last spectrum was recorded 1 hour after the solutions were mixed. Peak at 340 nm represents absorption maximum of NADH.
**Figure 54:** NADH (470 µM, 1 mL). Solution was made up to 2.1 mL with pH 7.4 phosphate buffer. The last spectrum was recorded 30 min later than the first recorded.

**Figure 55:** Methylene blue (47 µM, 1 mL), artemisinin (470 µM, 0.1 mL) and NADH (470 µM, 1 mL). The last spectrum was recorded 1 hour after the solutions were mixed.
**Figure 56**: Methylene blue (47 µM, 1 mL), artemisinin (470 µM, 10 µL, ~catalytic amount) and NADH (470 µM, 1 mL). The last spectrum was recorded 1 hour after the solutions were mixed.

The same experiment was reported while spectrophotometric measurement at 340 nm was taken which was depicted in figure 57.

**Figure 57**: Methylene blue (47 µM, 0.5 mL), Artemisinin (470 µM, 0.5 mL) and NADH (747 µM, 0.5 mL) under air. Peak maximum 340 nm was monitored. The solutions were made up to 2 mL with pH 7.4 phosphate buffer.
3.3.1.7 Effect of artemisinin on reduction of Methylene blue with NADH, and ferrous sulfate

Stock solutions of methylene blue (47 µM), NADH (940 µM), artemisinin (470 µM) and ferrous sulfate (470 µM and 47 µM) were prepared as above. Solutions were injected into a UV cuvette in following sequence: methylene blue (47 µM, 1 mL), artemisinin (470 µM, 0.1 mL), ferrous sulfate (470 µM or 47 µM, 0.1 mL) and NADH (940 µM, 0.5 mL). Spectrophotometric measurements under air from range 200 nm to 800 nm were taken as quickly as possible after NADH was injected. Results were plotted in figure 58 and 59.
**Figure 58**: Methylene blue (47 µM, 1 mL), Artemisinin (470 µM, 0.1 mL), FeSO₄ (470 µM, 0.1 mL) and NADH (940 µM, 0.5 mL). Solution was made up to 2.2 mL with pH 7.4 phosphate buffer. The last spectrum was recorded 50 min after the solutions were mixed.

**Figure 59**: Methylene blue (47 µM, 1 mL), Artemisin (470 µM, 0.1 mL), FeSO₄ (47 µM, 0.1 mL) and NADH (940 µM, 0.5 mL). Solution was made up to 2.2 mL with pH 7.4 phosphate buffer. The last spectrum was recorded 50 min after the solutions were mixed.
3.3.1.8 Effect of artemisinin reduction of Methylene blue with BNAH and ferrous sulphate

Stock solutions of methylene blue (50 µM), BNAH (500 µM), artemisinin (500 µM), and ferrous sulphate (500 µM) were prepared as above except that 1:1 acetonitrile/water mixture was used as solvent. Solutions were injected under air into a UV cuvette in the following sequence: methylene blue (50 µM, 1 mL), artemisinin (500 µM, 0.1 mL), FeSO₄ (500 µM, 0.1 mL), and BNAH (500 µM, 1 mL). Spectrophotometric measurements under air from 200 nm to 800 nm were taken as quickly as possible after BNAH was injected. Several control experiments were performed using the same stock solutions. Results were plotted in figure 60. The experiment was repeated in an inert gas (argon) atmosphere and the result was plotted in figure 61.
**Figure 60:** Methylene blue (50 µM, 1 mL), artemisinin (500 µM, 0.1 mL), FeSO₄ (500 µM, 0.1 mL) and BNAH (500µM, 1 mL) under air. Solutions were made up to 2.2 mL with pH 7.4 phosphate buffer in the control experiments.

**Figure 61:** Methylene blue (50 µM, 1 mL), artemisinin (500 µM, 0.1 mL), FeSO₄ (500 µM, 0.1 mL) and BNAH (500µM, 1 mL) under argon. Solutions were made up to 2.2 mL with pH 7.4 phosphate buffer in the control experiments.
3.3.2.1 *Effect of artemisinin on oxidation of leuco-MB*

To a N\textsubscript{2} purged Schenk flask containing methylene blue (18 mg) in degassed deionized water (5 mL), was added sodium dithionite (in excess). The mixture was kept stirring until the blue colored solution was gone and pale yellow precipitates were given out. Freshly distilled diethyl ether (10 mL) was used to extract *leuco*-methylene blue. Solutions turned into two layers after ether was added. *leuco*-methylene blue in diethyl ether (1 mL) was transferred into a screw-capped quartz cuvette purged with N\textsubscript{2} by a syringe. 0.1 eq. of artemisinin (6.3 mM) in acetonitrile prepared in N\textsubscript{2} was added to the cuvette by a syringe. The final volume of the mixture was made up to 2 mL with diethyl ether. The mixture was shaken well and wavelength range from 400 nm to 800 nm was monitored. Result was plotted in figure 62. The same experiment was repeated using dichloromethane to extract *leuco*-MB. Peak maximum at 654 nm was monitored. A control experiment without artemisinin was done in which the solution was made up to 2 mL with acetonitrile. Result was depicted in figure 63.
Figure 62: *Leuco-MB* (in 1 mL of diethyl ether) and artemisinin (1 eq.) in acetonitrile. Time interval of successive spectrum was 2 min. The last spectrum was recorded 1.5 hr after the solutions were mixed.

Figure 63: *Leuco-MB* (1 mL of dichloromethane) and artemisinin (1 eq.) in acetonitrile under nitrogen.
3.3.2.2 Effect of artemisone on leuco-MB

Solutions of leuco-MB (17.4 mg in 5 mL of degassed water) and artemisone (5.5 mM in dichloromethane) were prepared as above. 1 mL of leuco-MB was injected into a screw-capped UV cuvette purged with nitrogen before the addition of artemisone (1 mL). A control experiment of not using artemisone was done in which the solution was made up to 2 mL with dichloromethane. Peak maximum at 654 nm was monitored and results were illustrated in figure 64.

![Graph](image)

**Figure 64:** Leuco-MB (in 1 mL of dichloromethane) and artemisone (5.5 mM, 1 mL) under nitrogen.

3.3.2.3 Effect of catalytic amount of artemisinin on oxidation of leuco-MB

A similar experiment to 3.3.2.1 was done. A less concentrated leuco-MB was used instead. Solutions of leuco-MB (17.4 mg in 5 mL of degassed water) and artemisinin (5.4 mM) dichloromethane were prepared as above. 0.2 mL of leuco-MB was injected into a screw-capped UV cuvette purged with nitrogen before the addition of
artemisinin (0.2 mL). A control experiment without artemisinin was done in which solution was made up to 2 mL with dichloromethane. Peak maximum at 654 nm was monitored and results were illustrated in figure 65.

![Figure 65: Leuco-MB (17.5 mg in 5 mL of degassed water, 0.2 mL) and artemisinin (5.4 mM, 0.2 mL) under air. Solutions were made up to 2 mL with dichloromethane.](image)

A variant of 3.3.2.3 was done with reducing the amount of artemisinin used. Solutions of leuco-MB (16.7 mg in 5 mL of degassed water) and artemisinin (5.2 mM) dichloromethane were prepared as above. 0.1 mL of leuco-MB was injected into a screw-capped UV cuvette purged with nitrogen before the addition of artemisinin (20 µL). Solutions were made up to 2 mL with dichloromethane. Change of absorbance at 654 nm under air was recorded. Result was illustrated in figure 66.
3.3.3.1 Oxidation of 5-hydroxy-1,4-naphthoquinone with artemisinin

Solutions of 5-hydroxy-1,4-naphthoquinone (115 μM) (figure 67) in pH 7.4 phosphate buffer and artemisinin (2.86 mM) in dichloromethane were prepared as described in 3.3.2.1. To a N₂ purged Schenk flask containing the 5 quinone (115 μM, 5 mL) was added sodium dithionite (in excess). Dichloromethane (10 mL) was used to extract the reduced quinone. Dichloromethane extracted (2 mL) was injected to a nitrogen-purged UV cuvette. Artemisinin (2.86 mM, 50 μL) was injected after the dichloromethane extract. Wavelength range from 250 nm to 700 nm was monitored. A control experiment without artemisinin was done in which solution was made up to 2.05 mL with dichloromethane. Result was illustrated in figures 68 and 69.
Figure 67: 5-hydroxy-1,4-naphthoquinone (115µM) in pH 7.4 phosphate buffer.

Figure 68: Reduced 5-hydroxy-1, 4-naphthoquinone (2 mL) and artemisinin (0.05 mL, 2.86 mM). The last spectrum was recorded 50 min after the solutions were mixed.
Figure 69: Reduced 5-hydroxy-1, 4-naphthoquinone (2 mL). Solution was made up to 2.05 mL with DCM. The last spectrum was recorded 50 min after the solutions were mixed.

3.3.3.2 Effect of artemisinin on oxidation of 2-hydroxy-1,4-naphthoquinone

To a nitrogen purged Schenk flask containing 2-hydroxy-1,4-naphthoquinone (20 mg) in degassed deionized water (5 mL) was added sodium dithionite (in excess). Dichloromethane (10 mL) was used to extract the reduced quinone. Dichloromethane extracted (1 mL) was injected to a nitrogen-purged UV cuvette. Artemisinin (11.5 mM, 1 mL) was injected after the dichloromethane extract. Wavelength range from 250 nm to 700 nm was monitored. A control experiment without artemisinin was done in which solution was made up to 2 mL with dichloromethane. Results were illustrated in figures 70 and 71.
Figure 70: DCM extracted of reduced 2-hydroxy-1,4-naphthoquinone (1 mL) and artemisinin (11.5 mM, 1 mL). Recording interval between each successive spectrum was 9 min.

Figure 71: DCM extracted of reduced 2-hydroxy-1,4-naphthoquinone (1 mL). Solution was made up to 2 mL with DCM. Recording interval between each successive spectrum was 9 min.
3.3.3.3 Effect of artemisinin on oxidation of 1,2,4-naphthalenetriol with artemisinin

From a nitrogen purged Schenk flask containing 10 mL of 1,2,4-naphthalenetriol (20 mg) in degassed deionized water, 1 mL of solution was extracted. The triol was injected to a N₂-purged UV cuvette. Artemisinin (10 mM in acetonitrile, 1 mL) was injected after the addition of the triol. Wavelength range from 250 nm to 600 nm was monitored. Results were illustrated in figure 72.

![Absorbance vs Wavelength](image)

**Figure 72**: 1,2,4-Naphthalenetriol (11.35 mM, 1 mL) and artemisinin (10 mM, 1 mL) under nitrogen. Final volume of the mixture was 2 mL. Time interval between each successive spectrum was 9 min.

A similar experiment was done under air. Stock solutions of 1,2,4-naphthalenetriol (3 mM) in phosphate buffer and artemisinin (3 mM) in acetonitrile were prepared. 0.4 mL of 1,2,4-naphthalenetriol was mixed with 0.4 mL of artemisinin in a UV cuvette. A control was done without artemisinin. Solutions were made up to 2 mL with pH 7.4 phosphate buffer. Results were characterized in figures 73 and 74.
Figure 73: 1,2,4-Naphthalenetriol (3 mM, 0.4 mL) and artemisinin (3 mM, 0.4 mL) under air. Solution was made up to 2 mL with pH 7.4 phosphate buffer. The last spectrum was recorded 1 hour after the solutions were mixed.

Figure 74: 1,2,4-Naphthalenetriol (3 mM, 0.4 mL) under air. Solution was made up to 2 mL with pH 7.4 phosphate buffer. The last spectrum was recorded 1 hour after the solutions were mixed.
3.3.3.3 Effect of artemisinin oxidation of reduced lumiflavin

To a nitrogen purged Schenk flask containing 10 mL of lumiflavin (5.8 mg) in degassed deionized water was added sodium dithionite (in excess). The mixture was stirred for 10 min. Dichloromethane (10 mL) was added to extract the reduced lumiflavin. Dichloromethane extracted (2 mL) was injected to a nitrogen purged UV cuvette. Artemisinin (1.84 mM, 100 µL) was injected after the dichloromethane extract. Wavelength range from 250 nm to 700 nm was monitored. A control experiment without artemisinin was done. Results were illustrated in figure 75 and 76.

Figure 75: Reduced lumiflavin extract (2 mL) with artemisinin (1.84 mM, 100 µL). The last spectrum was scanned 35 min after the solutions were mixed.
**Figure 76**: Reduced lumiflavin extract (2 mL). The last spectrum was scanned 1 hour after the solutions were mixed.

### 3.3.4.1 Effect of artemisinin oxidation of NADH with Potassium Ferricyanide

Stock solutions of artemisinin (500 µM), K₃Fe(CN)₆ (5 mM), and NADH (500 µM) were prepared in volumetric flasks using 1:1 acetonitrile/water as a solvent. The solutions were added into a UV cuvette in following sequence: Artemisinin (1 mL), K₃Fe(CN)₆ (0.1 mL), and NADH (1 mL). A control was done without adding artemisinin and made the solution up to 2.1 mL with 1:1 acetonitrile/water mixture. Spectrophotometric measurements were taken as quickly as possible after NADH was mixed. Wavelength range from 200 nm to 800 nm was monitored. Results were illustrated in figure 77 and 79.
Figure 77: Artemisinin (500 μM, 1 mL), K₃Fe(CN)₆ (500 μM, 0.1 mL), and NADH (500 μM, 1 mL). The last spectrum was scanned 1 hr 45 min after the NADH was mixed.

Figure 78: K₃Fe(CN)₆ (500 μM, 0.1 mL), and NADH (500 μM, 1 mL). The solution was made up to 2.1 mL with 1:1 acetonitrile/water mixture. The last spectrum was scanned 1hr 45 min after the NADH was mixed.
3.3.4.2 Effect of artemisinin on oxidation of BNAH with Potassium Ferricyanide

Stock solutions of artemisinin (1.5 mM), K₃Fe(CN)₆ (1.5 mM), and BNAH (30 µM) were prepared in volumetric flasks using 1:1 acetonitrile/water as a solvent. The solutions were added into a UV cuvette in following sequence: K₃Fe(CN)₆ (1 mL), Artemisinin (1 mL) and BNAH (1 mL). A control was done without adding artemisinin and made the solution up to 3 mL with 1:1 acetonitrile/water mixture. Spectrophotometric measurements were taken as quickly as possible after BNAH was mixed. Peak maximum 352 nm (BNAH) was monitored against time. Results were illustrated in figure 79. A similar experiment was done with various concentrations of the solutions. Stock solutions of artemisinin (3 mM), K₃Fe(CN)₆ (3 mM), and BNAH (3 mM) were prepared. Experiment was repeated as described above and results were illustrated in figure 80 and 81.

![Figure 79](image-url)

**Figure 79:** K₃Fe(CN)₆ (1.5 mM, 1 mL), artemisinin (1.5 mM, 1 mL), and BNAH (30 µM, 1 mL). Peak maximum of BNAH (352 nm) was monitored. Solution was made up to 3 mL with 1:1 acetonitrile/water mixture in the control.
**Figure 80:** $\text{K}_3\text{Fe(CN)}_6$ (3 mM, 0.2 mL), artemisinin (3 mM, 0.2 mL), and BNAH (3 mM, 0.2 mL). Peak maximum of BNAH (352 nm) was monitored. Solution was made up to 3 mL with 1:1 acetonitrile/ water mixture.

**Figure 81:** $\text{K}_3\text{Fe(CN)}_6$ (3 mM, 1 mL), artemisinin (3 mM, 1 mL), and BNAH (3 mM, 0.2 mL). Peak maximum of BNAH (352 nm) was monitored. Solution was made up to 3 mL with 1:1 acetonitrile/ water mixture.
3.3.4.3 Effect of tert-butyl hydroperoxide on oxidation of BNAH with Potassium Ferricyanide

Stock solutions of tert-butyl hydroperoxide (3 mM), K$_3$Fe(CN)$_6$ (3 mM), and BNAH (3 mM) were prepared in volumetric flasks using 1:1 acetonitrile/water as a solvent. The solutions were added into a UV cuvette in following sequence: K$_3$Fe(CN)$_6$ (0.2 mL or 1 mL), tert-butyl hydroperoxide(0.2 mL or 1 mL) and BNAH (0.2 mL). A control was done without adding tert-butyl hydroperoxide and made the solution up to 3 mL with 1:1 acetonitrile/water mixture. Spectrophotometric measurements were taken as quickly as possible after BNAH was mixed. Peak maximum 352 nm (BNAH) was monitored against time. Results were illustrated in figures 82 ~ 84.

![Graph](image_url)

**Figure 82:** K$_3$Fe(CN)$_6$ (3 mM, 0.2 mL), tert-butyl hydroperoxide (3 mM, 0.2 mL), and BNAH (3 mM, 0.2 mL). Peak maximum of BNAH (352 nm) was monitored. Solution was made up to 3 mL with 1:1 acetonitrile/water mixture.
Figure 83: K$_3$Fe(CN)$_6$ (3 mM, 1 mL), tert-butyl hydroperoxide (3 mM, 1 mL), and BNAH (3 mM, 0.2 mL). Peak maximum of BNAH (352 nm) was monitored. Solution was made up to 3 mL with 1:1 acetonitrile/water mixture.

Figure 84: K$_3$Fe(CN)$_6$ (3 mM, 20 μL), tert-butyl hydroperoxide (3 mM, 0.2 mL), and BNAH (3 mM, 0.2 mL). Peak maximum of BNAH (352 nm) was monitored. Solution was made up to 3 mL with 1:1 acetonitrile/water mixture.
3.3.4.4 Effect of artemisone oxidation of BNAH with Potassium Ferricyanide

Stock solutions of artemisone (3 mM), K₃Fe(CN)₆ (3 mM), and BNAH (3 mM) were prepared in volumetric flasks using pH 7.4 phosphate buffer as a solvent. The solutions were added into a UV cuvette under nitrogen in following sequence: BNAH (0.2 mL), K₃Fe(CN)₆ (0.2 mL) and artemisone (0.2 mL). A control was done without adding artemisone and the solution was made up to 3 mL with the buffer. Spectrophotometric measurements were taken as quickly as possible after K₃Fe(CN)₆ was mixed. Peak maximum 352 nm (BNAH) was monitored against time. Results were illustrated in figure 85. A similar experiment was repeated with catalytic potassium ferricyanide and artemisinin. Result was characterized in figure 86.

Figure 85: BNAH (3 mM, 0.2 mL), K₃Fe(CN)₆ (3 mM, 0.2 mL), and artemisone (3 mM, 0.2 mL) mixed under nitrogen. Peak maximum of BNAH (352 nm) was monitored. Solution was made up to 3 mL with pH 7.4 phosphate buffer.
Figure 86: BNAH (3 mM, 0.2 mL), artemisone (3 mM, 0.2 mL) or artemisinin (3 mM, 0.2 mL) and K₃Fe(CN)₆ (0.6 mM, 0.2 mL) mixed under nitrogen. Peak maximum of BNAH (352 nm) was monitored. Solution was made up to 3 mL with pH 7.4 phosphate buffer.

3.3.4.5 Effect of artemisinin oxidation of NADH with Potassium Ferricyanide

Stock solutions of artemisinin (3 mM), K₃Fe(CN)₆ (0.6 mM), and NADH (3 mM) were prepared in volumetric flasks using pH 7.4 phosphate buffer as a solvent. The solutions were added into a UV cuvette under nitrogen in following sequence: NADH (0.2 mL), K₃Fe(CN)₆ (0.2 mL) and artemisinin (0.2 mL). A control was done without adding artemisinin and the solution was made up to 3 mL with the buffer. Spectrophotometric measurements were taken as quickly as possible after K₃Fe(CN)₆ was mixed. Peak maximum 340 nm (NADH) was monitored against time. Results were illustrated in figure 87.
Figure 87: NADH (3 mM, 0.2 mL), K₃Fe(CN)₆ (0.6 mM, 0.2 mL), and artemisinin (3 mM, 0.2 mL) mixed under nitrogen. Peak maximum of NADH (340 nm) was monitored. Solution was made up to 3 mL with pH 7.4 phosphate buffer.

3.3.4.6 Effect of tert-butyl hydroperoxide on oxidation of BNAH with ferrous sulfate

Stock solutions of tert-butyl hydroperoxide (3 mM), FeSO₄ (3 mM), and BNAH (3 mM) were prepared in volumetric flasks using 1:1 acetonitrile/ water mixture as a solvent. The solutions were added into a UV cuvette under air in following sequence: t-BuOOH (0.2 mL), BNAH (0.2 mL) and FeSO₄ (0.2 mL or 20 μL). Control experiments were done without adding t-BuOOH (set 2) or FeSO₄ (set 3) and the solution was made up to 3 mL with the 1:1 acetonitrile/ water mixture. Spectrophotometric measurements were taken as quickly as possible after FeSO₄ was mixed. Peak maximum 352 nm (BNAH) was monitored against time. Results were illustrated in figures 88–90.
Figure 88: Tert-butyl hydroperoxide (3 mM, 0.2 mL), BNAH (3 mM, 0.2 mL) and FeSO$_4$ (3 mM, 20 μL). Peak maximum of BNAH (352 nm) was monitored. Solution was made up to 3 mL with 1:1 acetonitrile/water mixture.

Figure 89: Tert-butyl hydroperoxide (3 mM, 0.2 mL), BNAH (3 mM, 0.2 mL) and FeSO$_4$ (3 mM, 0.2 mL). Peak maximum of BNAH (352 nm) was monitored. Solution was made up to 3 mL with 1:1 acetonitrile/water mixture.
Figure 90: Tert-butyl hydroperoxide (3 mM, 0.2 mL), BNAH (3 mM, 0.2 mL) and FeSO₄ (3 mM, 0.2 mL). Peak maximum of BNAH (352 nm) was monitored. Solution was made up to 3 mL with 1:1 acetonitrile/water mixture. The last spectrum was scanned 25 min after the solution was mixed.

A similar experiment was done by replacing BNAH with NADH (3 mM). Results were shown in figure 91 and 92.

Figure 91: Tert-butyl hydroperoxide (3 mM, 0.2 mL), NADH (3 mM, 0.2 mL) and FeSO₄ (3 mM, 0.2 mL). Peak maximum of NADH (330 nm) was monitored. Solution was made up to 3 mL with 1:1 acetonitrile/water mixture.
Figure 92: Tert-butyl hydroperoxide (3 mM, 0.2 mL), NADH (3 mM, 0.2 mL) and FeSO\textsubscript{4} (3 mM, 0.2 mL). Peak maximum of NADH (330 nm) was monitored. Solution was made up to 3 mL with 1:1 acetonitrile/ water mixture. The last spectrum was scanned 30 min after the solution was mixed.

3.3.4.7 Effect of artemisinin on oxidation of BNAH with ferrous sulphate

Stock solutions of artemisinin (3 mM), FeSO\textsubscript{4} (3 mM), and BNAH (3 mM) were prepared in volumetric flasks using 1:1 acetonitrile/ water mixture as a solvent. The solutions were added into a UV cuvette under air in following sequence: artemisinin (0.2 mL), BNAH (0.2 mL) and FeSO\textsubscript{4} (0.2 mL or 20 μL). Control experiments were done without adding artemisinin and the solution was made up to 3 mL with the 1:1 acetonitrile/ water mixture. Spectrophotometric measurements were taken as quickly as possible after FeSO\textsubscript{4} was mixed. Peak maximum 352 nm (BNAH) was monitored against time. Results were illustrated in figure 93. A similar experiment was done by replacing BNAH with NADH (3 mM). Results were shown in figure 94.
**Figure 93:** Artemisinin (3 mM, 0.2 mL), BNAH (3 mM, 0.2 mL) and FeSO₄ (3 mM, 0.2 mL). Peak maximum of BNAH (352 nm) was monitored. Solution was made up to 3 mL with 1:1 acetonitrile/ water mixture.

**Figure 94:** Artemisinin (3 mM, 0.2 mL), NADH (3 mM, 0.2 mL) and FeSO₄ (3 mM, 0.2 mL). Peak maximum of NADH (330 nm) was monitored. Solution was made up to 3 mL with 1:1 acetonitrile/ water mixture.

3.3.4.8 *Effect of artemisinin/ tert-butyl hydroxide on oxidation of BNAH with* $K_4FeCN_6$
Stock solutions of artemisinin (3 mM), tert-butyl hydroperoxide (3 mM), K₄FeCN₆ (3 mM), and BNAH (3 mM) were prepared in volumetric flasks using a pH 7.4 phosphate buffer as a solvent. The solutions were added into a UV cuvette under air in following sequence: K₄FeCN₆ (0.2 mL), BNAH (0.2 mL) and artemisinin (0.2 mL) or tert-butyl hydroperoxide (0.2 mL). A control experiment was done without adding artemisinin or tert-butyl hydroperoxide and the solution was made up to 3 mL with the buffer. Spectrophotometric measurements were taken as quickly as possible after artemisinin or tert-butyl hydroperoxide was mixed. Peak maximum 352 nm (BNAH) was monitored against time. Results were illustrated in figure 95.

Figure 95: K₄FeCN₆ (3 mM, 0.2 mL), NADH (3 mM, 0.2 mL) and artemisinin (3 mM, 0.2 mL) or t-BuOOH (3 mM, 0.2 mL). Peak maximum of NADH (330 nm) was monitored. Solution was made up to 3 mL with pH 7.4 phosphate buffer.
3.3.5.1 Effect of artemisone on reduction of glutathione

An assay kit of glutathione reduction was bought from Sigma-Aldrich. Stock solutions of glutathione (2 mM), NADPH (2 mM), and glutathione reductase in assay buffer were prepared according to the description. And solution of artemisone (0.21 mM) in acetonitrile was also prepared. Solutions were mixed in a UV cuvette in the following order: Set 1: glutathione (1 mL), glutathione reductase (40 µL), and NADPH (100 µL). Set 2: glutathione (1 mL), artemisone (40 µL), and NADPH (100 µL). Set 3: glutathione (1 mL) and NADPH (100 µL). Set 4: glutathione (1 mL), artemisone (40 µL), glutathione reductase (40 µL) and NADPH (100 µL). Solutions were made up to 2 mL with assay buffer and wavelength at 340 nm (NADPH) was monitored against time. Result was illustrated in figure 96.
Set 1: Glutathione (2 mM, 1 mL), glutathione reductase (40 µL), and NADPH (2 mM, 100 µL). Set 2: Glutathione (2 mM, 1 mL), artemisone (0.21 mM, 40 µL), and NADPH (2 mM, 100 µL). Set 3: Glutathione (2 mM, 1 mL) and NADPH (2 mM, 100 µL). Set 4: Glutathione (2 mM, 1 mL), artemisone (0.21 mM, 40 µL), glutathione reductase (40 µL) and NADPH (2 mM, 100 µL).

A similar experiment was done with an addition of ferrooxamine. A stock solution of ferrooxamine (0.588 mM) in the assay buffer and artemisone (0.2 mM) in acetonitrile were prepared. The solution was mixed in the following order: Set 1: glutathione (1 mL), ferrooxamine (34 µL), glutathione reductase (40 µL), and NADPH (100 µL). Set 2: glutathione (1 mL), artemisone (100 µL), glutathione reductase (40 µL) and NADPH (100 µL). Set 3: glutathione (1 mL), ferrooxamine (34 µL), glutathione reductase (40 µL) and NADPH (100 µL). Result was illustrated in figure 97.
**Figure 97**: Set 1: Glutathione (2 mM, 1 mL), ferrioxamine (0.59 mM, 34 µL), glutathione reductase (40 µL), and NADPH (2 mM, 100 µL). Set 2: Glutathione (2 mM, 1 mL), artemisone (0.2 mM, 100 µL), glutathione reductase (40 µL) and NADPH (2 mM, 100 µL). Set 3: Glutathione (2 mM, 1 mL), ferrioxamine (0.59 mM, 34 µL), glutathione reductase (40 µL) and NADPH (2 mM, 100 µL).

### 3.3.6.1 Preparation of desferrioxamine-Fe (III) solution

The desferrioxamine-Fe (III) solution was prepared by dissolving desferrioxamine mesylate (165 mg, 0.251 mmol) into 14 mL of atomic absorption standard iron solution (1008 µg/mL in 1% HCl) in a 25 mL volumetric flask and then filled up to the mark with pH 7.4 phosphate buffer. The stock solution was titrated against NH₄OH (6.14 mL) to give a pH 7.08 desferrioxamine-Fe (III) solution (8.1 mM). The stock solution was stored at 0-5 °C and used within three months.
3.3.6.2 Effect of artemisinin reduction of desferrioxamine-Fe (III) with N-Acetyl-L-cysteine methyl ester

Stock solutions of N-acetyl-L-cysteine methyl ester (50 µM), desferrioxamine-Fe(III) (50 µM), artemisinin (50 µM), and BPS (50 µM) were prepared in volumetric flasks by dissolving in pH 7.4 phosphate buffer under argon. Solutions were injected into a UV cuvette in the following order: desferrioxamine-Fe (III) (0.5 mL), BPS (0.5 mL), N-Acetyl-L-cysteine methyl ester (0.5 mL), and artemisinin (0.5 mL). A control was done without adding artemisinin and the solution was made up to 2 mL with the buffer. Wavelength range from 300 nm to 700 nm was monitored. Results were shown in figure 98 and 99.

Figure 98: Desferrioxamine-Fe (III) (50 µM, 0.5 mL), BPS (50 µM, 0.5 mL), N-Acetyl-L-cysteine methyl ester (50 µM, 0.5 mL), and artemisinin (50 µM, 0.5 mL). The last spectrum was scanned 25 min after the solution was mixed.
Figure 99: Desferrioxamine-Fe (III) (50 µM, 0.5 mL), BPS (50 µM, 0.5 mL), and N-Acetyl-L-cysteine methyl ester (50 µM, 0.5 mL). The last spectrum was scanned 25 min after the solution was mixed.

3.3.6.3 Effect of artemisinin reduction of desferrioxamine-Fe (III) with glutathione

A similar experiment to 3.3.6.2 was done by replacing N-acetyl-L-cysteine methyl ester with glutathione. Solutions were injected into a UV cuvette in the following order: desferrioxamine-Fe (III) (0.5 mL), BPS (0.5 mL), glutathione (0.5 mL), and artemisinin (0.5 mL). A control was done without adding artemisinin and the solution was made up to 2 mL with the buffer. Wavelength from 300 nm to 700 nm was monitored. Results were shown in figure 100 and 101.
**Figure 100:** Desferrioxamine-Fe (III) (50 µM, 0.5 mL), BPS (50 µM, 0.5 mL), glutathione (50 µM, 0.5 mL), and artemisinin (50 µM, 0.5 mL). The last spectrum was scanned 25 min after the solution was mixed.

**Figure 101:** Desferrioxamine-Fe (III) (50 µM, 0.5 mL), BPS (50 µM, 0.5 mL), and glutathione (50 µM, 0.5 mL). The last spectrum was scanned 25 min after the solution was mixed. Solution was made up to 2 mL with phosphate buffer.

### 3.3.6.4 Effect of artemisinin reduction of desferrioxamine-Fe (III) with L-cysteine
Stock solutions of Desferrioxamine-Fe (III) (0.5 mM), L-cysteine (0.5 mM), artemisinin (5 mM), and BPS (5 mM) were prepared in volumetric flasks with a mixture of 1:1 acetonitrile/ pH 7.4 phosphate buffer under air. The solutions were injected into a UV cuvette in the following order: Desferrioxamine-Fe (III) (1 mL), BPS (1 mL), cysteine (1 mL), and artemisinin (1 mL). The experiments were done under air. Control experiments were done without adding artemisinin. Results were shown in figures 102~104. Solutions were made up to 4 mL with the buffer/acetonitrile mixture.

![Graph](image)

**Figure 102**: Desferrioxamine-Fe (III) (5 mM, 1 mL), BPS (5 mM, 1 mL), cysteine (5 M, 1 mL) and artemisinin (5 mM, 1 mL) under air. The last spectrum was scanned 10 min after the solution was mixed.
**Figure 103:** Desferrioxamine-Fe (III) (5 mM, 1 mL), BPS (5 mM, 1 mL) and cysteine (5 mM, 1 mL) under air. Solution was made up to 4 mL with 1:1 acetonitrile/ pH 7.4 phosphate buffer. The last spectrum was scanned 10 min after the solution was mixed.

**Figure 104:** Desferrioxamine-Fe (III) (5 mM, 1 mL) and BPS (5 mM, 1 mL) under air. Solution was made up to 4 mL with 1:1 acetonitrile/ pH 7.4 phosphate buffer. The last spectrum was scanned 10 min after the solution was mixed.
3.3.6.5 Effect of artemisinin reduction of ferrioxamine E with cysteine

Stock solutions of ferrioxamine E (50 µM), cyteine (50 µM), artemisinin (50 µM), and BPS (50 µM) were prepared in volumetric flasks with pH 7.4 phosphate buffer under argon. Minimum amount of methanol was used to dissolve artemisinin. The solutions were injected into a UV cuvette in the following order: ferrioxamine E (0.5 mL), BPS (0.5 mL), cysteine (0.5 mL), and artemisinin (0.5 mL). The experiments were done either under argon or under air. Results were shown in figure 105 and 106. Control experiments were done without adding artemisinin. Solutions were made up to 2 mL with the buffer. Results were illustrated in figure 107 and 108. A similar experiment was done by replacing artemisinin with 10-deoxoartemisinin of which results were shown in figure 109 and 110.

![Graph](image)

**Figure 105:** Ferrioxamine E (50 µM, 0.5 mL), BPS (50 µM, 0.5 mL), cysteine (50 µM, 0.5 mL) and artemisinin (50 µM, 0.5 mL) under argon. The last spectrum was scanned 20 min after the solution was mixed.
**Figure 106**: Ferrioxamine E (50 µM, 0.5 mL), BPS (50 µM, 0.5 mL), cysteine (50 µM, 0.5 mL) and artemisinin (50 µM, 0.5 mL) under air. The last spectrum was scanned 15 min after the solution was mixed.

**Figure 107**: Ferrioxamine E (50 µM, 0.5 mL), BPS (50 µM, 0.5 mL), and cysteine (50 µM, 0.5 mL) under argon. The last spectrum was scanned 20 min after the solution was mixed. Solution was made up to 2 mL with phosphate buffer.
Figure 108: Ferrioxamine E (50 µM, 0.5 mL), BPS (50 µM, 0.5 mL), and cysteine (50 µM, 0.5 mL) under air. The last spectrum was scanned 15 min after the solution was mixed. Solution was made up to 2 mL with phosphate buffer.

Figure 109: Ferrioxamine E (50 µM, 0.5 mL), BPS (50 µM, 0.5 mL), cysteine (50 µM, 0.5 mL) and 10-deoxoartemisinin (50 µM, 0.5 mL) under argon. The last spectrum was scanned 20 min after the solution was mixed.
Figure 110: Ferrioxamine E (50 µM, 0.5 mL), BPS (50 µM, 0.5 mL), and cysteine (50 µM, 0.5 mL) under argon. The last spectrum was scanned 20min after the solution was mixed. Solution was made up to 2 mL with pH 7.4 phosphate buffer.

3.3.6.6 Effect of artemisinin and tert-butyl hydro peroxide on reduction of BNAH with methylene blue

Stock solutions of methylene blue (100 µM), tert-butyl hydroperoxide (500 µM), artemisinin (500 µM), and BNAH (500 µM) were prepared in volumetric flasks with 1:1 deionized water/ acetonitrile under argon. The solutions were injected into a UV cuvette in the following order: methylene blue (0.5 mL), artemisinin or tert-butyl hydroperoxide (0.1 mL) and BNAH (0.5 mL). The experiments were done under argon. Control experiments were done without adding artemisinin or tert-butyl hydroperoxide. Solutions were made up to 2 mL with the 1:1 deionized water/ acetonitrile mixture. Results were shown in figure 111 and 113
**Figure 111**: Methylene blue (100 µM, 0.5 mL), artemisinin or tert-butyl hydroperoxide (500 µM, 0.1 mL), and BNAH (500 µM, 0.5 mL) under argon. Solution was made up to 2 mL with 1:1 deionized water/ acetonitrile.

**Figure 112**: Methylene blue (100 µM, 0.5 mL), artemisinin or tert-butyl hydroperoxide (500 µM, 0.1 mL), and BNAH (500 µM, 0.5 mL) under argon. Interval between each successive spectrum was 8 min. Solution was made up to 2 mL with 1:1 deionized water/ acetonitrile mixture.
Figure 113: Methylene blue (100 µM, 0.5 mL), artemisinin or tert-butyl hydroperoxide (500 µM, 0.1 mL), and BNAH (500 µM, 0.5 mL) under air. Interval between each successive spectrum was 8 min. Solution was made up to 2 mL with 1:1 deionized water/acetonitrile mixture.
References


