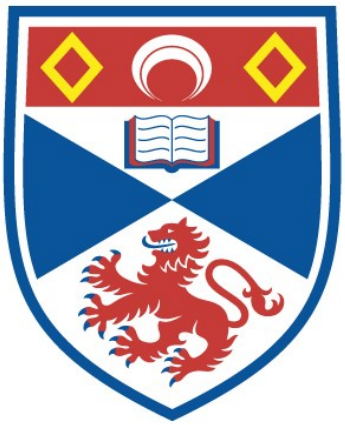
STUDIES OF SOME ANTIPARASITIC AGENTS

Louise Margaret Renton

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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Studies of Some Antiparasitic Agents

a thesis presented by

Louise Margaret Renton

to the

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in application for

The Degree of Doctor of Philosophy

St. Andrews

September 1996

Declaration

I, Louise Margaret Renton, hereby certify that this thesis, which is approximately 40,000 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

date 11 November 1996 signature of candidate

I was admitted as a research student in October 1993 and as a candidate for the degree of Ph.D. in September 1994; the higher study for which this is a record was carried out in the University of St. Andrews between 1993 and 1996.

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I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate for the degree of Ph.D. in the University of St. Andrews and that the candidate is qualified to submit this thesis in application for that degree.

date 11-11-96 signature of supervisor

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dedicated with love

to the memory of my father

Acknowledgements

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Finally, I would like to thank my mother for her unfailing support and encouragement throughout my academic career, and Terry who has put up with me being a student all these years.

Abbreviations and Symbols

A	absorbance
ABTS	[2,2'-azinobis (3-ethyl-2,3-dihydrobenzothiazole-6-sulphonate)]
ATP	adenosine triphosphate
BH_4	(6R)-tetrahydro-L-biopterin
BSA	bovine serum albumin
CM	cytoplasmic membrane
CW	cell wall
DBHG	dibenzylhydroxyguanidine
DBNBS	3,5-dibromo-4-nitrobenzene sulphonate
DMPO	5,5-dimethyl-1-pyrroline N-oxide
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DW	dry weight
E	electrode potential
EDRF	endothelium derived relaxing factor
eV	electron volt
FCS	foetal calf serum
GMP	guanosine monophosphate

GSNO	S-nitrosoglutathione
GTP	guanosine triphosphate
HCC	hepatocellular carcinoma
IC_{50}	50% inhibitory concentration
IFN	interferon
IUPAC	International Union of Pure and Applied Chemistry
K	rate constant
LPS	lipopolysaccharide
N	number of bacteria
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NMMA	N-monomethyl arginine
NOS	nitric oxide synthase
iNOS	inducible nitric oxide synthase
nNOS	neuronal nitric oxide synthase
OM	outer membrane
PBS	phosphate buffered saline
PG	peptidoglycan
QHS	qinghaosu
RBS	Roussin's black salt
RNA	ribonucleic acid

S	S-layer
SDM	Schneiders drosophila medium
SNAP	S-nitroso-N-acetylpenicillamine
SNP	sodium nitroprusside
Tj-9	Sho-saiko-to
TNF	tumour necrosis factor
λ	wavelength
ε	extinction coefficient

Abstract

Each year, between 300 and 500 million people develop malaria. Close to 3 million people die as a result. Due to resistance there are now few drugs left which are effective against malaria; one is a Chinese herbal remedy called qinghaosu (artemisinin).

A section of this thesis represents an attempt to understand further the mode of action of this antimalarial agent. Several mechanisms involving iron have been proposed and the main categories reviewed (chapter 3). Techniques including ESR spectrometry, fluorescence and absorbance measurements were used to further probe the mode of action of qinghaosu. The findings of these studies provide support for a mechanism proposed by Wu *et al.*, involving cleavage of the peroxy bridge in qinghaosu with the ferrous ion as opposed to antimalarial activity being due to the generation of high valent iron-oxo species.

The ether derivative of qinghaosu, arteether has been studied using 2D NMR in order to provide further insight into the unusual structure of this compound (chapter 4). A comparison of the sp^2 lactone functionality in qinghaosu with the sp^3 ether group in arteether was performed. Evidence is presented for different conformations and chemical shift values.

The immune system protects the human body from invasion by foreign substances or cells. A key part of this immune response comes from cellular components known as macrophages. The killing process of macrophages appears to involve nitric oxide. However, this natural defence mechanism is not very efficient as the body is susceptible to invasion by microbes. The term microbe includes both parasites and bacteria.

The toxicity of nitric oxide (NO) and NO donor compounds has been investigated using the bacterium *E. coli* (chapter 5). In addition, studies have been performed using parasites. Malarial parasites (*Plasmodia*) are difficult to culture, therefore *Leishmania* parasites which cause the tropical disease leishmaniasis were used as a model (chapter 6). Evidence is presented for the toxic nature of NO donor compounds towards both parasites and bacteria. However, qinghaosu and its derivatives did not prove effective against *Leishmania mexicana* parasites in contrast with their reported antimalarial activity. The conclusions of this study suggest that qinghaosu possesses a toxicity mechanism selective for malarial parasites.

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Chapter 1

Tropical Disease and the Immune System

1.1 Malaria

Malaria is one of the major infectious diseases in the world today despite years of effort first to eradicate it and when that failed, to reduce its impact on mortality and morbidity. The World Health Organisation estimate that there are 300-500 million people infected with malaria.[1]

Malaria is an infectious disease transmitted by mosquitoes. It is caused by minute parasitic protozoa of the genus *Plasmodium*. Only female mosquitoes suck blood and parasites infect a human while a mosquito of the genus *Anopheles* is feeding. In humans the parasite multiplies in the liver over 7-10 days, causing no symptoms. Parasites then burst from the liver cells to invade erythrocytes and multiply again. The new parasites invade more erythrocytes. The cycle is repeated, causing fever each time parasites break free and invade. If a female *Anopheles* feeds on this patient, parasites multiply in her stomach wall and thousands of new parasites migrate to the salivary glands. When the mosquito feeds again she injects saliva containing parasites into another human, hence infecting them.[2]

Humans are host to four species of malaria parasite: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malariae*. While there are considerable major differences between species and their pathogenicity and epidemiology as well as subtle but important differences in appearance, development and host-parasite relationships, all four species share a common basic life cycle.

The species *P. vivax*, *P. ovale* and *P. malariae* may cause severe illness but are rarely fatal. They destroy red blood cells in peripheral capillaries causing anaemia. These are the types of malaria which cause distinctive intermittent fevers. Bouts of fever correspond to the reproductive cycle of the parasite. The species *P. falciparum* causes much more serious and progressive illness. In this case red blood cells become sticky and form clumps in the capillaries of the deep organs of the body causing microcirculatory arrest. If this happens in the brain, delirium, coma, convulsions and

death may result. Cerebral malaria is by far the most serious form of the infection.[1]

Each year severe falciparum malaria causes up to 2 million deaths. *P. falciparum* accounts for most of the infections in Africa and for over one third of infections in the rest of the world. More than two billion people, nearly 40% of the world's population are at risk.[3]

1.1.1 Disease Control

The strength of malaria is in the enormous reproductive capacity of the parasite and mosquito; its weakness is in the long development time before an infected mosquito becomes infectious to man.

Vaccination

The malaria parasite has a complex life cycle in the human host and presents a number of potential targets for vaccine induced immune attack. Development of effective synthetic or recombinant peptide vaccines against malaria has been slower than some had anticipated. A complication peculiar to parasites is that protective immune responses to these organisms are usually stage-specific: the parasite can escape from a protective immune response when it transforms from one stage of its life cycle to the next. Therefore, a universally effective malaria vaccine will probably have to include antigens from different stages of the parasite life cycle.[4]

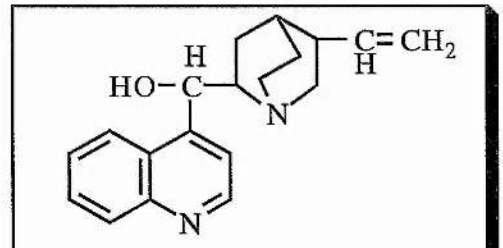
In addition each species has many different strains, and surface proteins vary from one to the next, so it is essential to use portions of protein that are common to as many strains as possible. Another risk is that the vaccine will allow those rare strains which can evade it to become suddenly successful in the population.[5]

The controversial Colombian malaria vaccine SPF 66 was tested in clinical trials in The Gambia and Tanzania. From these studies it was concluded that the effectivness of SPF 66 was between 8 and 30%. At present this vaccine is undergoing further clinical trials and has the potential to become another weapon in the fight against malaria.[6]

Antimalarial Drugs

Quinine

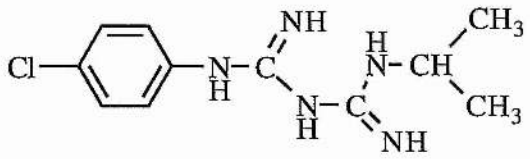
In the early 17th century Jesuit missionaries in South America learned of the medicinal value of *Cinchona* bark to treat and cure fevers. Two centuries later Pelletier and Caventou isolated the two main alkaloids contained within the bark; quinine and cinchonine. The chemical structure of quinine was elucidated in 1908 and key steps in its total synthesis were achieved by Woodward and Doering in 1944. Quinine was the first chemotherapeutic compound and the original antimalarial drug. Mass administration of quinine assisted early malaria control projects in Italy and other countries.



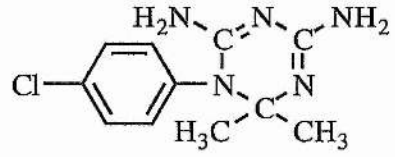
(1) quinine

Proguanil

A drug produced in the British programme to find new antimalarials during the second world war. It is a biguanidine compound which is converted to the active compound cycloguanil by metabolism in the body. This was a very useful drug but its value is now limited by resistance.



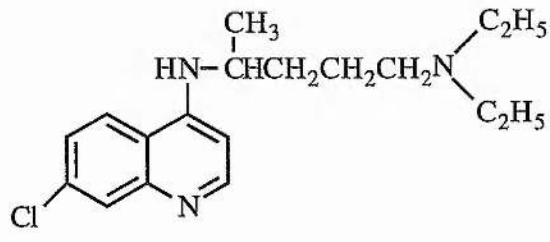
(2) proguanil



(3) cycloguanil

Chloroquine

Unlike the botanical quinine, chloroquine is a synthetically manufactured product. It belongs to a class of compounds known as the 4-aminoquinolines, antimalarials first developed in 1934 by the German pharmaceutical company I.G. Farben. The ideal antimalarial would have to cure quickly and effectively, it would have to be free of untoward side effects, it would have to work against all species and strains of malaria parasites and it would have to be cheap. Chloroquine came very close to that ideal. However, during the sixties it was confirmed that chloroquine resistant strains of *P. falciparum* had arisen under pressure of overuse and probably underdosage.[2]



(4) Chloroquine

Medicinal Plants

Because of the lack of availability of effective and affordable drugs, many of the people in disease endemic areas rely on traditional medical systems for treatment. In most cases the traditional therapy consists of the administration of plant extracts orally or as topical preparations. Scientific evaluation of medicinal plants used in the preparation of traditional remedies has in the past provided modern medicine with very effective drugs for the treatment of parasitic diseases.

Initially, two approaches are used in the search for new drugs from plants;

- the evaluation of ethnomedical information obtained from traditional medical practitioners.

- the random screening of plant species against target parasites.

The first approach is considered more productive since it relies on the cumulative wisdom of many generations of medical practitioners and any serious toxicity would have been detected during the long period of human use of the drug. The random screening of plants is employed in highly automated systems with high throughput and it is hoped that the very large number of species screened by this method will increase the chances of identifying new antiparasitic agents that are unknown even in traditional medicine.[7]

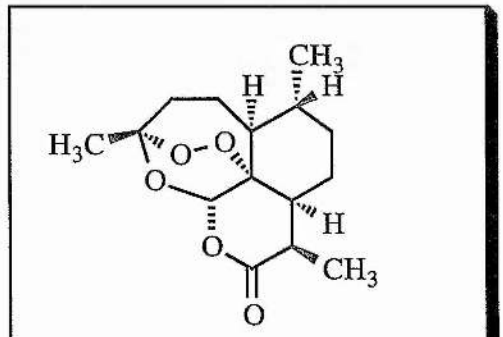
1.1.2 The Current Situation

The world could easily face a turning point in the next decade wherein multidrug-resistant malarial parasites emerge for which there is no effective drug. The spread of multidrug-resistant parasites which now exist in Southeast Asia, to a high transmission area of Africa would be a disaster of immense proportions. Few pharmaceutical companies have programmes in antimalarial drug development and many of these are being curtailed or discontinued, one of the reasons being that tropical diseases are not given the profile of AIDS or cancer. We need to recognise malaria as an emerging or re-emerging disease and take steps now to deal with the threat of untreatable malaria within the next decade.[8] Due to resistance there are now few drugs left which are effective against malaria. One effective drug is a Chinese herbal remedy called qinghaosu (artemisinin) and its derivatives. The artemisinins in particular have not attracted much funding because traditional herbal medicine cannot be patented and thus cannot generate big profits.

At present the French company Rhône-Poulenc Rorer manufactures the ether derivative of qinghaosu called artemether from shrub wormwood extracts. A course of artemether, consisting of six 80-milligram doses will cost about £9. This price is already subsidised under a deal between Rhône-Poulenc Rorer and the TDR (Tropical Diseases Research Programme). However, in Africa the cost could be a real problem. Another ether derivative of qinghaosu called arteether is currently being developed by the TDR and a Dutch company called Artecef. It is hoped that this drug will be registered as soon as 1998 and that its cost will be low.

1.1.3 Qinghaosu

Qinghaosu is the principal component in the antimalarial effect of the traditional Chinese herb *Artemisia annua L.* or *qinghao* . It is effective *in vivo* against chloroquine-resistant strains of *Plasmodium falciparum* [9] and is associated with low toxicity.[10] Qinghaosu, a representative of the cadinane class of sesquiterpene is now used successfully in patients in China with malaria. Derivatives of qinghaosu, such as the oil soluble artemether and arteether and the water soluble sodium artesunate appear to be more potent than qinghaosu itself.



(5) qinghaosu

It was to this traditional herbal remedy that Chinese scientists turned in an effort to fight the resurgence of malaria. Extraction of the dried leaves of *qinghao* with petroleum ether at low temperatures and chromatography on silica gel with subsequent recrystallisation gave fine, colourless crystals, named qinghaosu (extract of *qinghao*) in Chinese, but also given the Western name artemisinin. The yield was variable ranging from negligible quantities to almost 1% depending on the area from which the plant was collected. The formula $C_{15}H_{22}O_5$ suggested the compound to be a sesquiterpene and reaction with triphenylphosphine to give the phosphine oxide was consistent with the presence of a peroxide group. The structure of qinghaosu as well as its absolute configuration was determined by X-ray diffraction. The lactone ring has a trans configuration. The most unusual feature of the chemical structure is the 1,2,4-trioxane ring which may also be viewed as a bridging peroxide group. Qinghaosu is the only known 1,2,4-trioxane occurring in nature, although compounds with peroxide bridges are common, particularly in marine organisms. In extensive clinical trials in China,[10] qinghaosu showed promise in the treatment of otherwise drug-resistant forms of malaria, notably that caused by *P. falciparum*.

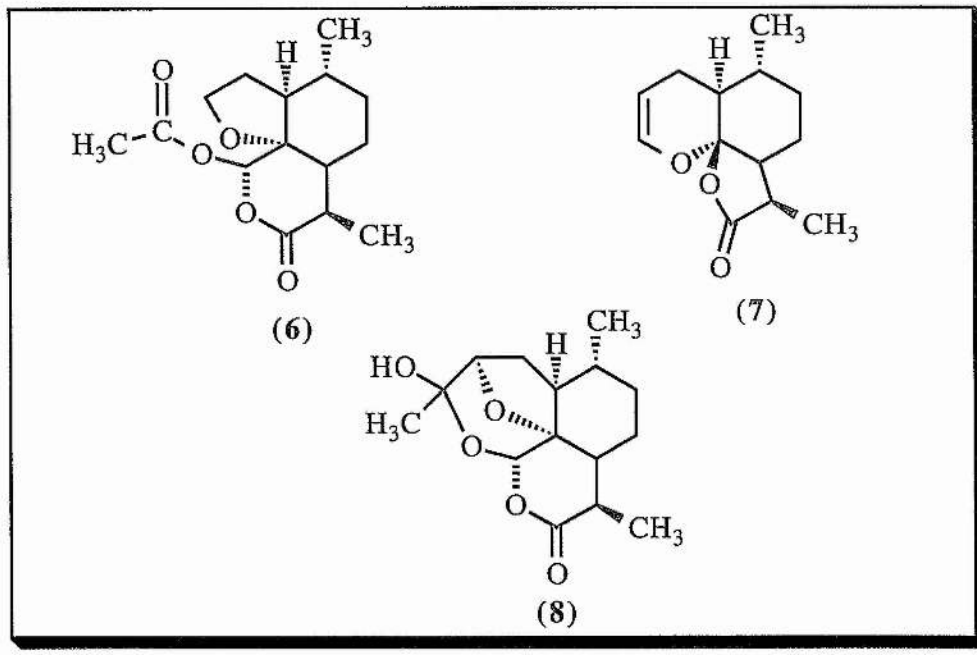
This discovery has occasioned a considerable amount of research in both China and the West into the synthesis, biosynthesis and biological action of qinghaosu.

Nomenclature

Although the Chinese name qinghaosu, meaning extract of green plant, is attractive it is frequently misspelt. In qinghaosu the 'q' is pronounced like 'ch' in 'cheat'. The systematic name is 3,6,9-trimethyl-9,10b-epidioxyperhydropyrano[4,3,2-*jk*] benzoxepin-2-one, but this is hardly convenient for regular use. The name adopted by *Chemical Abstracts* is artemisinin derived from the plant which is its source.[11]

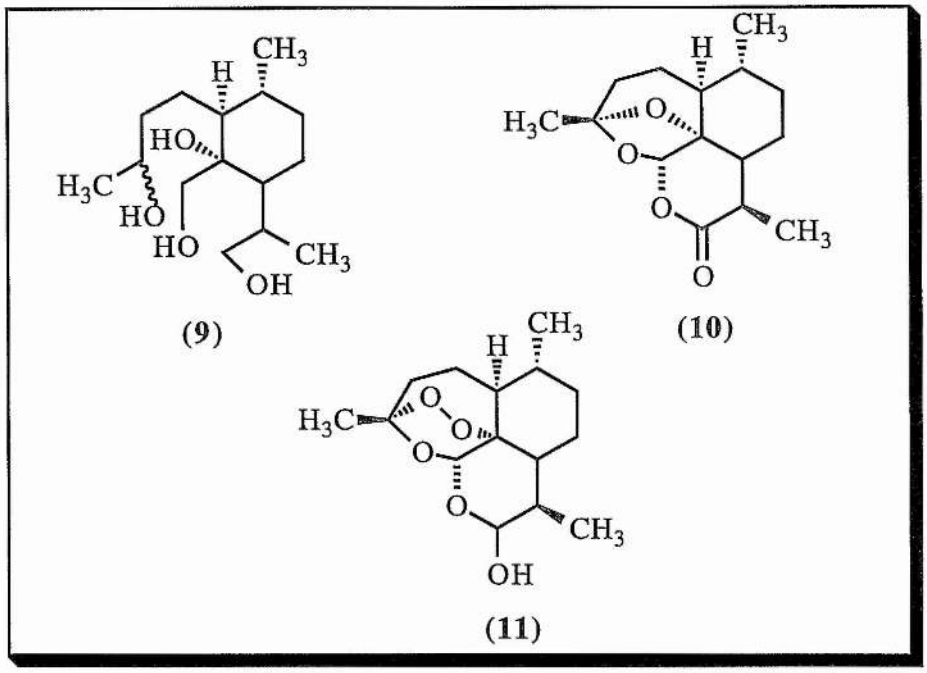
Chemical Reactions of Qinghaosu

The most interesting aspect of the chemistry of qinghaosu is the stability of the peroxide bridge. Indeed, qinghaosu can be recovered unchanged from neutral solvents after several days at temperatures up to 150°C. At higher temperatures the peroxide bridge is destroyed and (**6**), (**7**) and (**8**) are formed in low yields.[12]



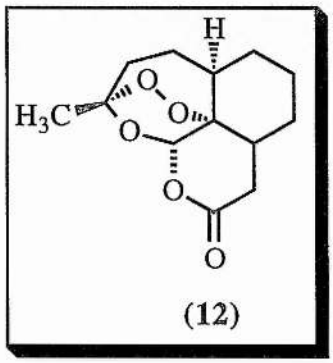
Reduction

Lithium aluminium hydride exhaustive reduction gives the tetrahydroxy compound (**9**), while reaction with hydrogen over palladium results in loss of the peroxide bridge to give 11-deoxyartemisinin (**10**). Reduction with borohydride leaves the peroxide bridge intact, giving the lactol 2-hydroxy-2-deoxoartemisinin (**11**). This is an important intermediate in the synthesis of qinghaosu analogues.[13]



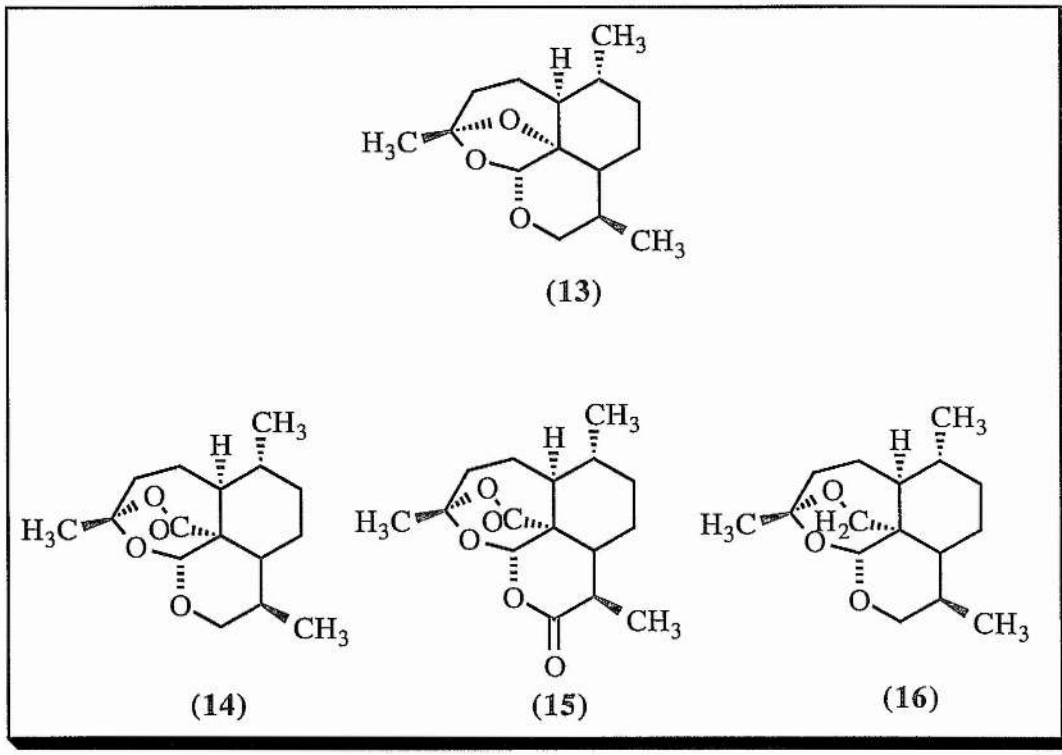
Antimalarial Activity of Qinghaosu

It is almost certain that the crucial structure in qinghaosu which gives it its antimalarial activity is the peroxide bridge. Other parts of the molecule may be modified without loss of antimalarial activity. Removal of one or two of the methyl groups at positions 3 and 6 (**12**) leaves a molecule which is still active against *P. falciparum*.

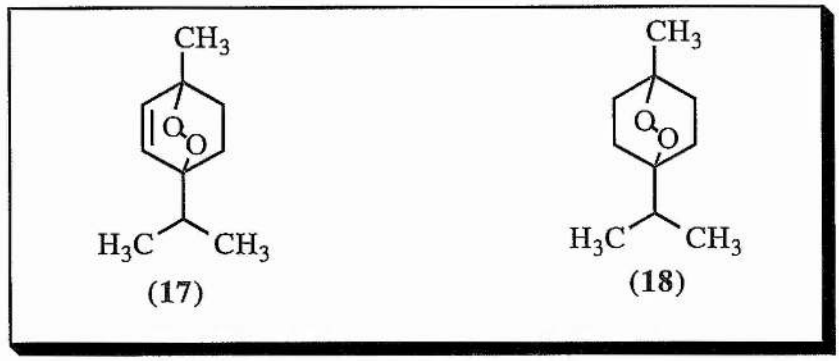


The lactol (**11**) obtained by reduction of qinghaosu, although chemically unstable is a better antimalarial agent than qinghaosu itself, indicating clearly that the carbonyl group is not essential for biological activity.[14]

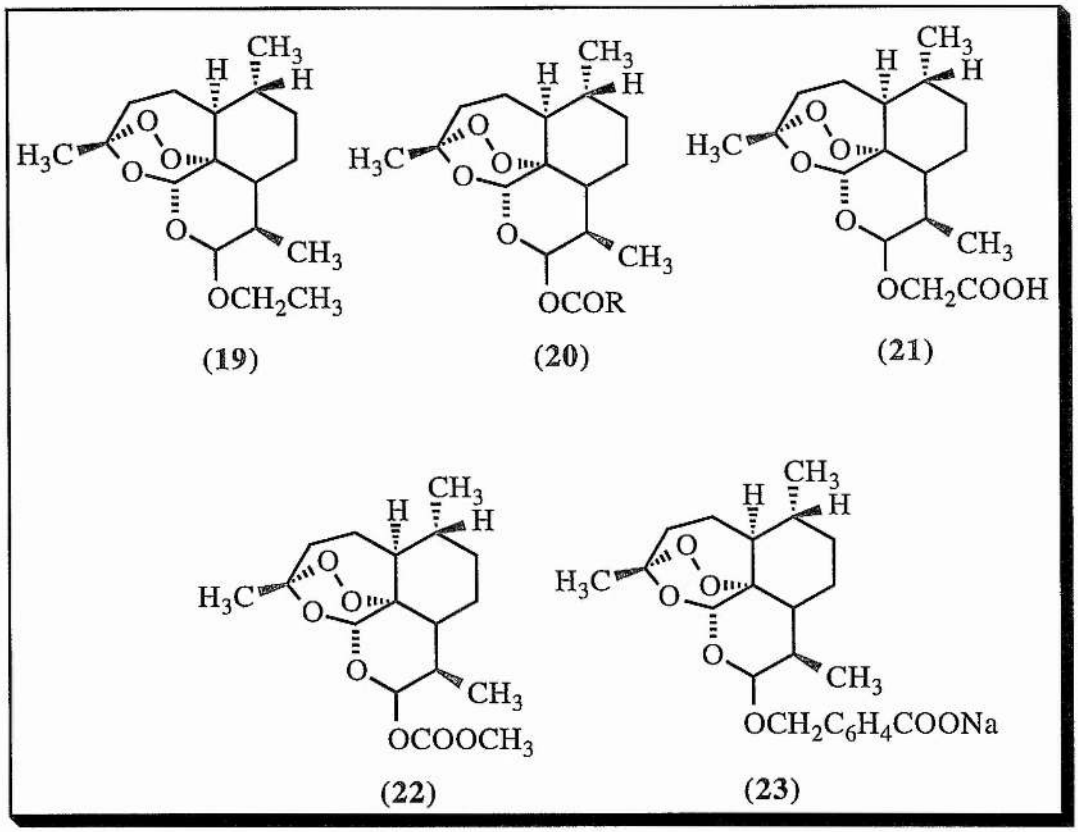
On the other hand, 11-deoxoartemisinin[15] (**10**) and 2-deoxo-11-deoxyartemisinin[14] (**13**) are not active against the malarial parasite and neither are the carba-analogues (**14**), (**15**) and (**16**).[16] All these compounds lack the peroxide bridge.



The converse appears also to be true, many compounds not obviously related to qinghaosu but which contain a peroxide group are antimalarial agents. Vennerstrom *et al.*[17] examined 23 peroxides of diverse chemical structures including di-t-butyl peroxide, ascaridole (**17**) and dihydroascaridole (**18**). Several were found to be active *in vitro* against *P. falciparum* although none were active *in vivo*. This suggests that the remainder of the qinghaosu molecule is responsible for the delivery of the drug to the infected erythrocyte in an active form where it can exercise its toxicity to the parasite.



Although the peroxide bridge may be the crucial structure in qinghaosu the rest of the molecule has a profound effect on the *in vitro* and *in vivo* antimalarial activity of qinghaosu and related compounds. The ether derivative of 2-hydroxy-2-deoxoartemisinin (**19**) is a better antimalarial than qinghaosu itself[18] and is the form of qinghaosu which is currently under commercial development. It is generally called arteether. Esters (**20**) are generally as effective as qinghaosu but the corresponding acids (**21**) are much less so. This may be a consequence of their lower solubility in lipids. Carbonates (**22**) are the least effective of this group of compounds. However, sodium artelinate (**23**) is only slightly less effective than qinghaosu *in vitro* and has the advantage of water solubility, and therefore can be administered orally.[19]



Quantum mechanical calculations give excellent correlation between calculation and experiment in cases where the structure has been determined by X-ray crystallography.[20] Thus it may be possible to use quantum pharmacology to explore as yet unsynthesised compounds in the search for an even better antimalarial drug.

Mode of Action

The solubility of qinghaosu and its derivatives is a very important factor. For clinical applications the drug must be administered in oil as an intramuscular injection. However, since the 4th century (when a specific use of *qinghao* for the treatment of malaria was first recorded) the administration of the active species was in the form of an aqueous decoction. Preparation involved soaking a handful of herbs in one litre of water, and the patient would drink all of the strained liquor.[21] The action of qinghaosu

towards the malarial parasite appears to be different from that of chloroquine and this may well be why qinghaosu is effective against parasites which have become chloroquine resistant.

1.2 Leishmaniasis

Malaria parasites are difficult to culture therefore leishmania parasites are often used in parasitic studies. In effect the disease leishmaniasis is used as a model for malaria.

Leishmaniasis is a group of diseases with very different clinical manifestations and public health consequences, ranging from self-healing disfiguring lesions in a minority of cases to severe epidemics with high fatality rates. It is endemic in the tropical regions of America, Africa and the Indian sub-continent and in the sub-tropics of South-West Asia and the Mediterranean. The disease is caused by species of the intracellular protozoan parasite belonging to the genus *Leishmania*.[22]

Leishmaniasis is transmitted by the bite of female sandflies. These belong to the genus *Phlebotomus* in the 'old world' (Southern Europe, Africa, Middle East, India and South-East Asia) and *Lutzomyia* in the new world (central and South America).[23] The reservoir of infection is the amastigote form of the parasite. The amastigote is usually intracellular and is a roughly spherical structure (2-5 μm long and 1.5-2.5 μm broad). The sandfly becomes infected by taking up amastigotes during a blood meal, the amastigotes being in the blood or skin of the infecting host. The amastigotes are liberated from the cells in which they are found (in the stomach of the sandfly) and begin to multiply by fission. The amastigote becomes elongated (15-20 μm long and 1.5-3 μm broad), this organism is known as the promastigote. The mobile promastigotes migrate from the gut to the mouth parts of the sandfly and are injected during feeding.[23]

The leishmaniasis can be classified into four groups:

Cutaneous leishmaniasis, caused by *Leishmania major* and *Leishmania tropica* , is the most prevalent form of the disease and is characterised by a skin ulcer which heals spontaneously leaving an unsightly scar. Diffuse cutaneous leishmaniasis, caused by *Leishmania aethiopica* and *Leishmania mexicana*, causes widespread thickening of the

skin with lesions resembling those of lepromatous leprosy which do not heal spontaneously. Mucocutaneous leishmaniasis is caused by *Leishmania braziliensis* and results in highly disfiguring leprosy-like tissue destruction and swelling. Visceral leishmaniasis or kala-azar is caused by *Leishmania donovani* or *Leishmania chagasi*, common symptoms include fever, malaise, weight loss and diarrhoea accompanied by anaemia, skin darkening and hepatosplenomegaly. Visceral leishmaniasis is fatal if untreated due to the failure of the host to mount an effective, protective immune response.[24]

1.2.1 Disease Control

Leishmaniasis, like most diseases due to protozoan infection is largely a problem of developing countries and therefore offers little commercial incentive for pharmaceutical companies to develop cheap but effective antileishmanial drugs.

Vaccination

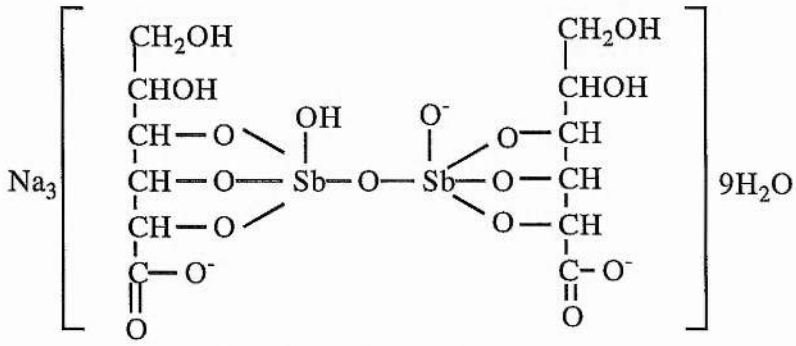
Vaccination may be the best answer to the control of leishmaniasis as individuals who have recovered from the disease remain immune to further infection. Therefore, vaccination is feasible in principle. However, so far the only immunisation strategy used with any success in humans has been restricted to the cutaneous disease.[7]

Antileishmanial Drugs

Antimonials

It is well known that compositions containing antimony and naturally occurring compounds have long been used in folk medicine and in cosmetics throughout much of the Middle East, though without recognition of the potential toxic effects. In the Middle Ages there was a marked enthusiasm for exhibition of antimonials as panaceas. An interesting aspect of that lay in the formation of a therapeutic wine by allowing white wine to stand in vessels made of antimony, or by placing antimony-containing substances in wine for varying times. Eventually not only potassium antimonyl tartrate was isolated from such a source, but it was learned from experience that antimonials were far from safe remedies. As a result antimonial therapy fell into disuse for several centuries. In 1908 it was shown that potassium antimonyl tartrate was successful in treating mucocutaneous leishmaniasis. Consequently antimonials were reintroduced into therapy and led to a broader base for investigations into antileishmanial agents.[23]

Most of the antimonial drugs employed in the chemotherapy of parasitic diseases are derivatives of acids of the element. These are usually of complex structure, either as derivatives of the hydrated oxides or types having a carbon-antimony bond. Sodium antimonyl (V) gluconate (sodium stibogluconate) was the drug of choice for therapy.



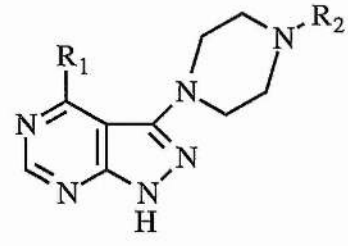
sodium antimonyl (V) gluconate - sodium stibogluconate

Sodium stibogluconate is among the more potent and well tolerated of antimonial drugs used in the treatment of leishmaniasis. However, it must be administered daily in intramuscular doses for several weeks. Sodium stibogluconate has serious toxic side effects including cardiac and/or renal failure and is not very effective against cutaneous leishmania.[24]

Pyrazolo[3,4-d]pyrimidines

Adenine and hypoxanthine are indispensable purine bases essential for the growth of various parasites such as *L. donovani* and *L. brazeliensis* [25] These parasites being incapable of synthesising purines on their own are totally dependent on the host for their availability. Hypoxanthine after several enzymic transformations by hypoxanthine-guanine phosphoribosyl transferase is converted into ATP and incorporated into parasite RNA which is vital for its existence. The knowledge of host-parasite biochemical differences provide insight into the design and synthesis of effective antileishmanial agents.

Pyrazolo[3,4-d]pyrimidines, the isosteres of purines impair the biosynthesis of RNA in leishmania parasites.[26] This inhibitory property has led to the design of various pyrazolo[3,4-d]pyrimidines with a high lipophilic substituent for effective antileishmanial activity.



substituted pyrazolo[3,4-d]pyrimidine

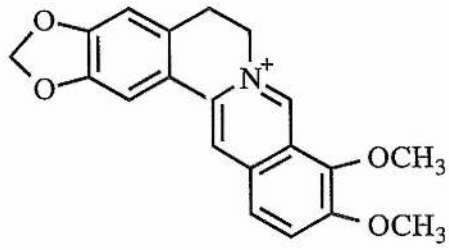
Experimental drugs such as the pyrazolopyrimidines, allopurinol ribonucleotide the 8-aminoquinoline WR6026 and the steroid synthetase inhibitor itraconazole show promise and are currently being evaluated for the treatment of various forms of leishmaniasis.[27]

Medicinal Plants

In the case of leishmaniasis more than 20 plant species from as many taxonomic families have been shown to contain compounds with significant activity against various species of *leishmania*. The active constituents of the plants have been isolated and their activity confirmed. The activity of the antileishmanial plant extracts has been attributed to compounds belonging to a diverse chemical group.[7]

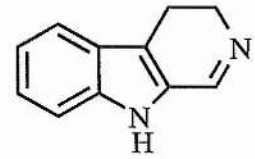
i) Isoquinolines

e.g. berberine



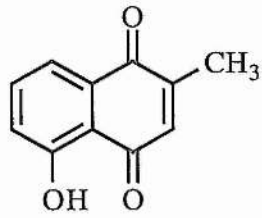
ii) Indole alkaloids

e.g. harmaline



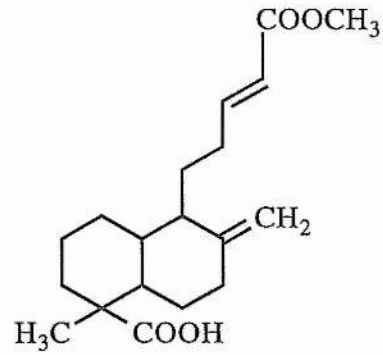
iii) Quinones

e.g. plumbagin



iv) Terpenes

e.g. polyalthialabdane



1.2.2 Qinghaosu

Qinghaosu and its derivatives have been shown to be effective against *Schistosoma mansoni* and *Clonorchis sinensis*.[28] Thus, this group of compounds have broad antiparasitic potential. The effectiveness of qinghaosu and its derivatives has been investigated against *L. major in vitro* and *in vivo* and these compounds were found to be active.[29]

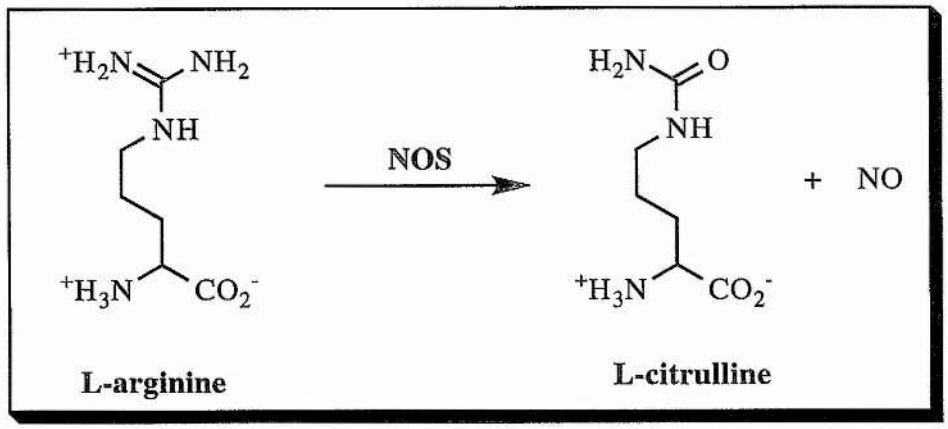
1.3 The Immune System

The immune system is the body's reaction by which foreign matter both living and non-living is neutralised or destroyed. The non-specific immune response non-selectively protects against foreign substances or cells without having to recognise their specific identities: a key part of that response comes from cellular components known as macrophages which are found in virtually all organs and tissues, their structure varying somewhat from location to location. For macrophages to respond they have to be activated by substances known as cytokines. The role of macrophages is quite extensive and includes engulfing foreign matter (phagocytosis) and if necessary, killing it by injection of cytotoxic substances. Macrophages can also kill invading microbes by contact without phagocytosis. It is the killing process which appears to involve nitric oxide (NO).[30]

1.3.1 The Physiological Role of Nitric Oxide

In 1980 Furchgott and Zawadski[31] demonstrated that the vascular relaxation induced by acetylcholine was dependent on the presence of the endothelium and provided evidence that this effect was mediated by a second messenger, later to be known as the EDRF[32] (Endothelium Derived Relaxing Factor). The EDRF was identified as nitric oxide.

The mechanism by which vascular relaxation hence vasodilation takes place involves the binding of the agent to a receptor on the endothelial cell, which initiates the release of Ca^{2+} ions. Calcium is one of the agents required for the stimulation and action of the enzyme NO-synthase which is present in endothelial cells. NO-synthase (NOS) converts L-arginine to L-citrulline and NO. NO then diffuses from the endothelial cell wall into the smooth muscle where it stimulates guanylate cyclase to catalyse the conversion of guanosine triphosphate (GTP) into cyclic guanosine monophosphate (cGMP). This process triggers a phosphorylation cascade which eventually brings about relaxation of the smooth muscle.



1.3.2 Isoforms of NOS

The family of NOS isoforms generally falls into two categories: (i) a constitutive form, regulated by Ca^{2+} and calmodulin, activated by (6R)-tetrahydro-L-biopterin (BH_4) and (ii) a cytokine-inducible form not known to be regulated post-transcriptionally.[33] The categories can be divided further according to the tissue NOS is derived from. The following NOS isoforms have been cloned and sequenced: constitutive cerebellar[34], endothelial and inducible murine macrophages.[35] All NOS isoforms require NADPH and O_2 as co-substrates. Other known co-factors are flavin adenine dinucleotide and flavin mononucleotide which are believed to form a cycle with NADPH in the reductase domain of the enzyme.[36] Evidence is consistent with the direct formation and release of NO from the enzyme, but this has never been shown quantitatively with any of the purified NOS isoforms.

The isoform associated with macrophage function is the inducible form (referred to as iNOS); iNOS "knockout mice" have been bred. These animals do not express iNOS and provide information on the function of NO in the immune system. iNOS "knockout mice" appear to be more susceptible to microbial and parasitic invasion than wild type mice, but only slightly. With other infections the "knockout mice" are no more susceptible than the wild type control mice. This implies that NO may be only one agent of many involved in the control of infection. Male mice deficient in neuronal

NOS have been found to exhibit behavioural abnormalities; these animals tend to be more aggressive than the control mice. In contrast female nNOS-deficient mice exhibited no behavioural abnormalities compared with the control group.[37]

1.3.3 Macrophage cytotoxicity

Once the production of NO from L-arginine in endothelial cells had been established it became clear that a similar process occurs in activated macrophages and that both nitrite and nitrate come from the common precursor, NO. NO production was confirmed independently by three groups,[38] and it now seems certain that in activated macrophages there is a process occurring that parallels that taking place in endothelial cells. Nature appears to have been economical in using the same enzyme for two entirely different tasks but clearly the properties of NO are special enough to make this profitable. However, it does pose a problem. If any part of the body suffers from a massive infection there will be much macrophage activity. The NO produced as a consequence will have not only substantial cytotoxicity, *i.e.* it kills the invading cells, but will also bring about massive hypotension. This condition is known as septic shock and can be fatal. Now that the nature of the species responsible for septic shock has been identified much research is being carried out to find a treatment for this condition.

1.3.4 Mechanisms of Nitric Oxide Toxicity

As mentioned previously, the cytotoxic effects of nitric oxide are associated with the inducible form of the enzyme nitric oxide synthase (iNOS). Once synthesised in the cell this enzyme will release large amounts of nitric oxide over a long period of time. Under these conditions nitric oxide can exhibit toxicity in various ways:

Inhibition of DNA Synthesis

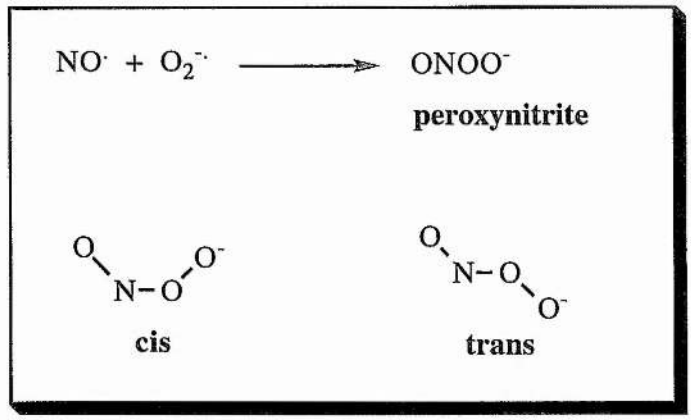
DNA represents a major target for NO. Under aerobic conditions NO is converted to a nitrosating agent (probably dinitrogen trioxide) which can react with DNA bases or intact DNA, to induce base deamination.[39] This reaction alters the structure of DNA and can result in genetic changes in the cell because of C-T mutations. NO can also initiate DNA strand breaks.[40] This may be due to the formation of peroxynitrous acid which can react with double stranded DNA to cause strand breaks.[41] NO can also inhibit DNA synthesis at concentrations which result in cell toxicity.[42]

This effect of NO may occur as a result of inhibition of enzymes involved in DNA synthesis. A target which has been investigated is ribonucleotide reductase which provides deoxyribonucleotides for synthesis of DNA.[43] NO donors inhibit this enzyme and the β_2 subunit of the enzyme contains a tyrosyl free radical which is effectively scavenged by NO under aerobic conditions.

NO has been implicated in diabetes.[44] NO and oxygen radicals inhibit functions of insulin containing cells and may contribute to cell destruction. In pre-diabetic insulitis, cytokines secreted from macrophages may inhibit insulin secretion, glucose oxidation, DNA synthesis and cause DNA damage.

Formation of Peroxynitrite

It has been proposed that NO reacts with superoxide (O_2^-) in many pathological states to form a secondary cytotoxic species; the peroxynitrite anion ($ONOO^-$).[45] Formation of peroxynitrite from activated phagocytic cells could mediate nitric oxide dependent microbial killing. Peroxynitrite is a potent bactericidal agent,[46] killing *E. coli* in direct proportion to its concentration.



Upon protonation peroxynitrous acid is formed. This is a strong oxidising agent, it can oxidise the bromide ion, the iodide ion, hydroxylamine and hydrogen peroxide.[47] The decomposition route of peroxynitrous acid at neutral pH is unknown and there is much controversy over the mechanism.

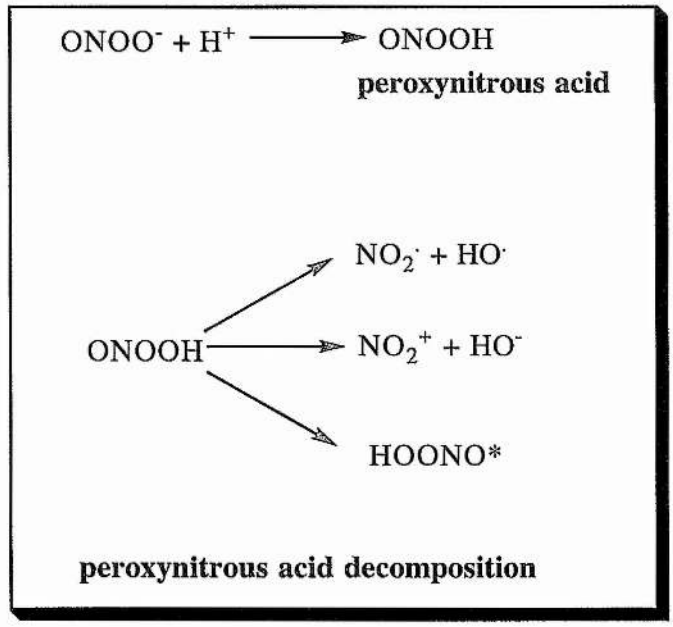
At present there are three proposed mechanisms for the decomposition of peroxynitrous acid;

Peroxynitrous acid may undergo homolytic decomposition to form NO_2 and hydroxyl radicals (HO·).[48] Beckman *et al.*[46] have shown that peroxynitrous acid spontaneously decomposes to form a potent oxidant with the reactivity of hydroxyl radical without a requirement for transition metal catalysis. This is a much favoured explanation as it has been shown that peroxynitrous acid can both hydroxylate and nitrate aromatic amino

acids such as tyrosine and tryptophan. Also, the polymerisation of methyl acrylate in the presence of peroxynitrous acid has been used as additional evidence for the formation of free radicals in its decomposition. Although HO· and NO_2· can recombine to form nitric acid, this reaction in solution is much slower than most reactions involving HO·

Peroxynitrous acid may also undergo heterolytic decomposition to form the nitronium cation (NO_2^+) and the hydroxide ion (HO^-).[49] This possibility has arisen due to the lack of effect of various hydroxyl radical scavengers on the rate of reaction of peroxynitrous acid.

The third alternative is the formation of an excited state peroxynitrous acid molecule (HOONO*) which is more reactive than the ground state peroxynitrous acid molecule but less reactive than the hydroxyl radical.[50] All these mechanisms may have a role in peroxynitrite mediated toxicity.



Inhibition of Mitochondrial Enzymes

This may be another factor in nitric oxide-mediated cytotoxicity. NO has a high affinity for iron-containing enzymes and forms paramagnetic mononuclear iron-dithiol-dinitrosyl complexes and haem nitrosyl complexes.[51] Therefore, the iron-sulphur and haem-containing enzymes in the mitochondrial respiratory chain are potential targets. The iron-sulphur centres in complexes I and II of the respiratory chain are inhibited by NO.[52] This inhibition of mitochondrial enzymes will cause a drop in ATP levels which results in cell death. NO is also able to reversibly de-energise mitochondria. At high concentrations, NO will mobilise mitochondrial Ca^{2+} which disturbs the Ca^{2+} homeostasis and results in cell death.[53]

The Toxicity of Reactive Intermediates

Iron catalysed generation of HO· from O_2^- and hydrogen peroxide (H_2O_2) results in severe and irreversible damage of both mammalian cells and pathogenic microbes. It has been shown[54] that the malarial parasite exerts an oxidant stress on infected red blood cells. Lipid peroxidation is used as the indicator of effect of oxidant stress, and lipid peroxidation in malaria infected erythrocytes is higher than normal cells. The increase in lipid peroxidation in parasitised cells may be due to reactive oxygen species generated by the living parasites. One possibility is that some of the oxidants are produced secondary to catabolism of host cell haemoglobin.

The effector functions of NO like those of $O_2^{-}\cdot$ involve iron dependent reactions. However the reactivity of NO is more selective and more specific than the reactivity of reactive oxygen species. Nitric oxide synthesised by the inducible nitric oxide synthase mobilises cellular iron and inhibits the catalytic activity of iron dependent enzymes.[55] This inhibits mitochondrial respiration and causes cytostasis which in most cases is reversible.

The experimental evidence suggests that the pattern of NO induced enzymatic inhibition is due to the formation of paramagnetic mononuclear iron-dithiol-dinitrosyl complexes of the general formula $Fe(RS)_2(NO)_2$ and paramagnetic nitrosyl haem complexes.[56] This targeting of intracellular iron explains the specificity as well as the reversibility of NO induced metabolic inhibition.

Nitrogen Dioxide: The Toxic Species?

Much research has focused on the toxicity of hydroxyl radicals from the decomposition of peroxynitrite, however an equal amount of $NO_2\cdot$ is expected to be formed. $NO_2\cdot$ contains an unpaired electron making it reactive with many free radicals. It is a highly toxic and potent oxidant capable of initiating fatty acid oxidation[57] and nitrosylation of aromatic amino acids.[58] As oxidation takes place over a period of time the slow action of macrophages in destroying bacteria could be attributed to the production of the toxic species $NO_2\cdot$.

1.3.5 The Antimicrobial Activity of Nitric Oxide

NO has been demonstrated to exert *in vitro* microbicidal or microbiostatic activity against a rapidly expanding list of helminths, protozoa, yeasts, mycobacteria, bacteria and viruses. In a smaller number of cases an *in vivo* role of NO in the host response to infection has been demonstrated in experimental models, providing evidence that it is part of the body's natural defence mechanism.[59]

Antileishmanial Activity

When mouse macrophages are cultured with inflammatory mediators interferon (IFNγ) and tumour necrosis factor (TNFα) in the presence of low doses of lipopolysaccharide

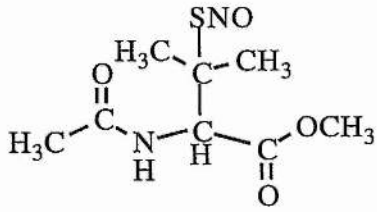
(LPS) they produce large amounts of NO measured as nitrite in culture supernatant compared with controls incubated in medium alone.[60] The production of NO correlates directly with the antileishmanial activity of the macrophages. Cells activated with IFNγ and TNFα are very efficient at killing the parasites compared with control cells incubated in medium alone. The antileishmanial activity of macrophages is not affected by D-NMMA but can be progressively and completely abolished by physiological concentrations of L-NMMA (a competitive inhibitor of NO from L-arginine).[61] These results demonstrate that NO is not only necessary but is sufficient to account for the antileishmanial activity in this system.

Antimalarial Activity

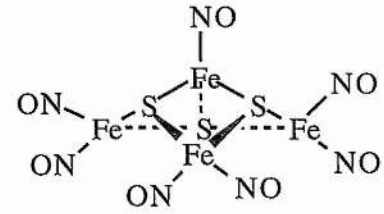
The role of nitric oxide has also been demonstrated in malaria infection.[62] Mice were infected with *Plasmodium chabaudi chabaudi* and developed the infection. NO was measured in the plasma as nitrate. When the mice were treated with L-NMMA , NO production was completely abolished and the mice were significantly more susceptible to infection. These results indicate that NO is at least partially responsible for the resistance against malaria infection.

The World Health Organisation have tested the compounds S-nitroso-N-acetylpenicillamine (SNAP), sodium nitroprusside (SNP) and Roussin's Black Salt (RBS) *in vitro* on clones of human malaria; *P. falciparum* D6 (Sierra Leone clone) and W2 (Indochina clone).[63] SNAP is a S-nitrosothiol which decomposes thermally and photochemically to form the disulphide and NO.[64] Roussin's Black Salt is an iron sulphur cluster nitrosyl which is a potent source of NO. Sodium nitroprusside (SNP) can act as a direct electrophilic nitrosating agent. It was found that the drugs had a very slight effect on the parasite with SNP being most active. In comparison to antimalarials such as chloroquine and qinghaosu their level of activity was not significant.

Sodium nitroprusside



S-nitroso-N-acetylpenicillamine



Roussin's Black Salt

The susceptibility of the human malaria parasite *P. falciparum* to killing by NO and related molecules has been investigated. It was found that saturated solutions of NO did not inhibit parasite growth, but the oxidation products, nitrite (NO_2^-) and nitrate (NO_3^-) were toxic to the parasite in millimolar concentrations. Pathogens reported to be inhibited by cell derived reactive nitrogen intermediates include *Leishmania major*, *Shistosoma mansoni* and *Plasmodium* liver stages.[65]

1.3.6 NO; Too Much of a Good Thing?

This certainly seems to be the case with NO production. Small amounts are beneficial in intracellular communication, vasodilation and self-defence, while unmodulated production becomes toxic. NO overproduction has been implicated in autoimmune reactions, such as DNA damage, arthritis and diabetes. Evidence of a role in septic shock indicates that overproduction of NO in response to microbial infection can also

be detrimental.[66]

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Chapter 2

Phytochemistry

2.1 Introduction

Traditional Chinese medicine has been used for thousands of years by a large population. It is currently still serving many of the health needs of the Chinese people, and still enjoying their confidence. It is practised in China in parallel with modern Western medical treatment. In addition to scientific organisations dedicated to modern Western medicine, e.g. the Chinese Academy of Medical Sciences and various medical schools, a series of parallel institutions have been established in China to promote traditional Chinese medicine, such as the Academy of Traditional Chinese Medicine and training institutions. Almost all hospitals in China have a department of traditional medicine. Furthermore, a large number of scientific journals are dedicated to traditional Chinese medicine, covering both experimental and clinical investigations.

About fifty years ago modern chemical and pharmacological methods were first used to investigate traditional medicinal materials in China. With the growth of knowledge in chemistry, biochemistry, physiology and pharmaceutics and the progress made in scientific instrumentation, there have been an increasing number of scientific reports characterising the biological activities of the chemical constituents of Chinese medicinal materials.[1]

The herb *Artemisia annua L.* (*qinghao*) has been used in Chinese traditional medicine as a treatment for many ills, including fever, haemorrhoids, consumption and malaria. In 1971 Chinese chemists isolated from the leafy portions of the plant the substance responsible for its reputed medicinal action. They named this crystalline compound qinghaosu (meaning active principle of *qinghao*), and it was also given the Western name artemisinin. This compound is a sesquiterpene lactone that bears a peroxide grouping and unlike most other antimalarials, lacks a nitrogen-containing heterocyclic ring system. Qinghaosu has been used extensively and successfully in China against both chloroquine-sensitive and chloroquine-resistant strains of the malarial parasite *Plasmodium falciparum.*[2]

Chinese researchers reported, without giving specific details, that thirty other species of *Artemisia* have been examined but that none yielded extracts with antimalarial activity.[3] Plant species of the genus *Artemisia* that are better known than *A. annua* include *A. absinthium*, used until the 1920's to prepare the narcotic and now illegal drug absinthe, and *A. drancunculus*, known as tarragon, used as a spice in cooking.

The earliest mention of *Artemisia annua L.* occurs in the Prescription *for 52 Kinds of Diseases*, so prized by the ancient Chinese that it was recorded on a piece of silk, discovered in the Mawangdui Hang dynasty tomb dating from 168 B.C. In this work the herb was recommended for treatment of haemorrhoids,[4] a translation read;

"Take 5 tou of urine and use it to boil 2 large handfuls of blue *Artemisia*, 7 gold carp the size of a hand, 6 ts'un of finely powdered cinnamon and 2 nodules of dried ginger, use to fumigate to kill worms"[5]

Li Shizhen, the famous herbalist who died 400 years ago wrote in his *Ben Cao Gang Mu* (Compendium of Materia Medica) of 1596, that chills and fever of malaria can be combated by *qinghao* preparations in the following form:

"Take a handful of *qinghao*, soak in 2 litres of water, strain the liquid and drink".

However, before 1971 the nature of the active principle was unknown and before 1976 the nature of its chemical structure remained elusive.

In the literature[6] it has been reported that the leaves and seeds of *A. annua* were prescribed, the former for children's fevers and the latter for consumption, flatulence, dyspepsia, night sweats and to destroy noxious vapours. Extracts of *A. annua* have been used for centuries in China to treat a large number of ailments including the fevers associated with malaria. Therefore *A. annua* may contain many "principles" active against these ailments. Conversely, the fever thought to be caused by various diseases may have actually been due to malaria and was therefore cured by the active principle, qinghaosu.

2.2 The Chinese Theory of Herbs

An important feature of traditional Chinese medicine is the preference for composite prescriptions (combinations of herbs) over single active ingredients. The theory behind this practice is that the multiple ingredients should produce synergistic therapeutic effects. This ancient concept is not readily compatible with modern pharmacological theory since a herbal formulation may contain between twenty and a hundred chemical compounds. In addition the amount of each compound may vary with many factors, hence quality control is a major issue. However, two recent studies support the efficacy of some herbal formulae:

In a controlled double-blind British clinical trial, a formulation of ten herbs proved to be effective in treating severe atopic eczema which was resistant to conventional Western therapies.[7] Although atopic eczema is mild in most cases there is a substantial minority in whom the disease is severe and these individuals frequently endure great suffering. Topical emollients and corticosteroids are the principal treatments and these are generally effective for mild and localised disease. However, they rarely provide adequate benefit for those in whom atopic eczema is more widespread or intense. Furthermore, the use of topical corticosteroids in children with severe eczema is associated with a risk of adverse effects as a result of systemic absorption including interference with growth.

It became known that a number of children with atopic eczema were being treated by a practitioner of traditional Chinese medicine and that 80% of such patients derived substantial benefit from this treatment. Furthermore, routine haematological and biochemical screening of these patients demonstrated no evidence of impairment of renal, hepatic or bone marrow function after 6 months continuous therapy.

In traditional Chinese medicine, atopic eczema is generally treated with medicinal plants, particularly in the form of decoctions but also as external applications. Decoctions are taken by mouth and are generally prepared daily by boiling with water

for a specified period. Prescriptions include components of several (usually 8-12) different plants selected on the basis of an assessment both of the skin and general medical state.

In this study the treatment comprised components of plants in widespread medicinal use in China. The placebo comprised a mixture of inert plant materials having a similar appearance, taste and smell, but with no known benefit in atopic eczema.

The constituents of the treatment formula were acquired from established brokers and institutes in the Republic of China, who were able to certify their identity and quality. Quality control was strictly enforced and thin-layer chromatography was used to "fingerprint" each batch of every constituent. Batches were rejected where this differed substantially from that of the reference material.

This study verified the therapeutic effect of an extract prepared from plant materials, according to traditional Chinese principles, in a group of children with severe atopic eczema. It is possible, although unproven, that several compounds act synergistically in this and other similar formulations. In this study none of the plants used were known to contain identified compounds beneficial in inflammatory skin disease.

In a randomised, controlled Japanese clinical trial it was shown that an extract of seven Chinese herbs helped prevent liver cancer in patients with cirrhosis.[8] The term "cancer chemoprevention" refers to prevention or prolongation of steps leading to carcinogenesis by intervention with drugs before the malignant (invasive) stage of carcinogenesis. Hepatocellular carcinoma (HCC) is one of the most common cancers in the world with an estimated 500,000-1 million new cases diagnosed yearly. More than 80% of patients with HCC have underlying cirrhosis.

This was the first completed, randomised and controlled trial of chemoprevention of HCC. It evaluated the preventative effect of Sho-saiko-to (Tj-9) on HCC development. Tj-9 is a Chinese herbal medicine which contains crude extracts of seven herbs.

Concentrations of the presumably active components in the Japanese preparation were regulated.

Tj-9 was administered to the trial group in a daily oral dose of 7.5 g, while conventional drugs were administered to the control group. Patients were monitored for 60 months and the cumulative incidence of HCC and the survival rate in both groups was determined.

In conclusion, Tj-9 helped prevent the development of HCC in patients with cirrhosis. In addition the higher survival rate of the cirrhotic patients in the trial group could mean that Tj-9 had beneficial effects on the clinical course of patients with cirrhosis in addition to its inhibitory effect on HCC development. The researchers also noted that it was difficult to explain the benefits of Tj-9 as the effect of a single ingredient.

2.3 Malaria Chemotherapy

How Much Qinghaosu is Present in the Herbal Decoction?

The yield of qinghaosu from *A. annua* varies between 0.01 and 0.5% depending on the origin of the herb as well as the season of harvesting. Concentrations of qinghaosu are at a maximum just prior to flowering and are generally about 0.05%, although a figure of 0.5% has been recorded for plants growing in Szechuan province in Southern China. Therefore, assuming a typical decoction contained 5 g of *A. annua*, it would contain between 0.0005 g and 0.025 g of qinghaosu.[9]

In clinical trials, significantly higher doses were prescribed; qinghaosu tablets were taken orally at a total dosage of 2.5-3.2 g, intramuscular injections of qinghaosu in oil suspension or in water suspension were administered at total doses of 0.5-0.8 g and 0.8-1.2 g respectively. The ether derivative artemether, being much more oil soluble than qinghaosu, was administered as oil solution via intramuscular injection at a total dosage of 0.08-0.22 g. Sodium artesunate, the water soluble ester derivative of qinghaosu was administered intramuscularly or by intravenous dripping at a dose of 0.15 g.[10] Thus with increasing solubility a smaller dose of the drug was required to produce a therapeutic effect.

In comparison with the dose of qinghaosu supplied by the decoction, the doses given in clinical trials were significantly higher. This provides further evidence for the benefits induced by combinations of herbs (rather than single active ingredients) employed in traditional Chinese medicine. It would appear that the multiple ingredients in the decoction produce a synergistic therapeutic effect.

It may be that other ingredients in the decoction actually solubilise qinghaosu, thus making it more active. This proposal was investigated and the solubility of qinghaosu in the presence of herbal extracts determined.

2.4 A Study of the Bioavailability of Qinghaosu in the Herbal Decoction

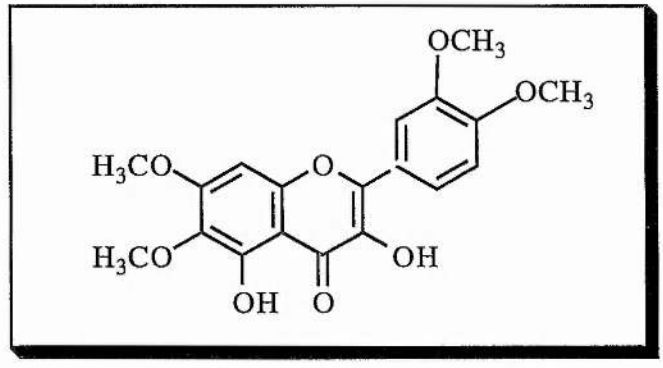
2.4.1 Introduction

According to the literature[3], the ancient Chinese remedy *qinghao* was administered in the form of an aqueous decoction. It would appear that the precise combination of herbs in the decoction affects the solubility of the active principle making it orally effective.

The solubility of qinghaosu in water was compared with the solubility of qinghaosu in water containing substances known to be present in *qinghao* leaves as well as an aqueous decoction of *qinghao*. The solubility was investigated using high performance liquid chromatography (HPLC).

2.4.2 Extracts Investigated

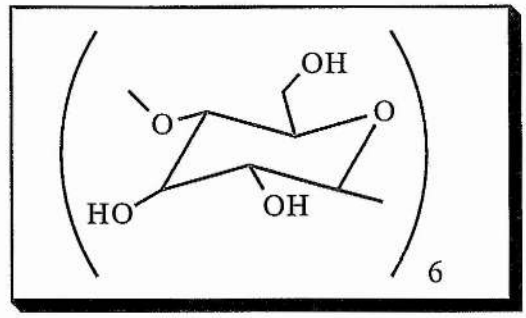
Quercetin



The actual constituent in the herb is 6,7,3',4'-tetra-O-methyl quercetagetin. This compound is not commercially available and quercetin was closest in structure.

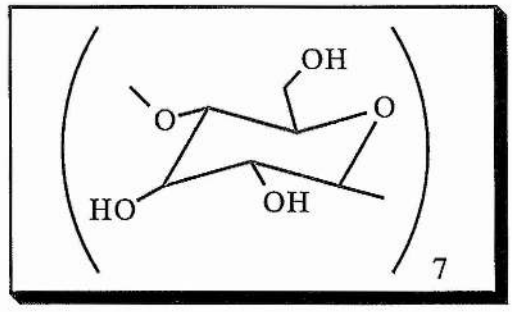
Quercetin differs from this constituent in that $-OCH_3$ groups are replaced by -OH groups.

α-cyclodextrin



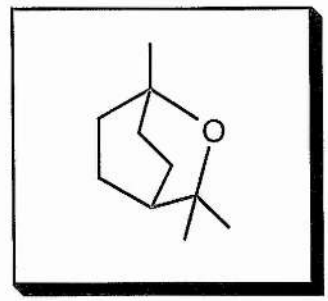
Cyclohexaamylose; a cyclic oligosaccharide composed of six D-glucose units.

β-cyclodextrin



Cycloheptaamylose; a cyclic oligosaccharide composed of seven D-glucose units.

1,8-Cineole (eucalyptol)



2.4.3 Experimental

HPLC analysis was performed using a Perkin Elmer series 410 pump, LC 235 diode array detector and GP-100 graphics printer. Column; Highchrom Lichrosorb RP-18, 10 μm (25 cm x 4.6 mm).

Chromatographic Conditions

Mobile phase: 0.01 M $NaHPO_4$ - NaH_2PO_4 buffer solution [water : methanol (3:1)] prepared with HPLC grade solvents. Flow rate; 1.3 cm^3 per minute, ambient temperature, detection wavelength; 260 nm, detection sensitivity; 0.005 aufs, injection volume; 20 μl, method for quantitation; peak mass.

2.5 mM solutions of extracts were prepared as follows; quercetin (0.085 g), α-cyclodextrin (0.24 g), β-cyclodextrin (0.28 g) and cineole (0.038 g) were prepared in water (10 cm^3). To each solution qinghaosu (0.001 g; 0.4 mmol) was added and left to stir overnight. For the control experiment the extracts were prepared as described previously but no qinghaosu was added.

A decoction of the herbal remedy was prepared by boiling the herb (250 cm^3) in water (500 cm^3) and stirring overnight at room temperature. The decoction was filtered and qinghaosu (0.001 g; 0.4 mmol) was added to the filtrate (10 cm^3). The control for this

experiment was filtrate only (10 cm^3). Each solution was left to stir overnight then filtered.

The filtrate from each solution was pre-treated before injection onto the column.[11] To each solution (0.4 cm^3), 0.2% sodium hydroxide (1.6 cm^3) was added, the mixture was heated on a water bath at 50°C for 30 minutes then cooled immediately with cold water. The solutions were extracted twice with ethyl acetate (2 cm^3). Trace amounts of ethyl acetate were removed from the aqueous layer under a gentle stream of air. 2.5 M acetic acid in ethanol (0.16 cm^3) was added and the volume brought up to 2 cm^3 with a methanol : water mixture; (1:4). All samples were filtered (0.45 μm filters) before injection.

2.4.4 Discussion

Analysis using HPLC showed that water containing the herbal decoction and that containing α-cyclodextrin increased the solubility of qinghaosu, while solutions of cineole, β-cyclodextrin and quercetin actually reduced qinghaosu solubility (appendix 1). Further studies confirmed these findings and showed that α-cyclodextrin almost doubled the amount of qinghaosu in solution. However, the herbal decoction increased qinghaosu solubility only by a factor of approximately 1.5. Therefore, an additional factor must control the solubility of the active species, making it orally effective. This may be the precise combination of herbs in the decoction which produce a synergistic effect.

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Appendices

Appendix 1

The effect of herbal extracts on the solubility of qinghaosu in water.

Sample	Peak Mass (g)
water / qhs	0.07
water / α-cyclodextrin / qhs	0.11
water / quercetin / qhs	0.03
water / cineole / qhs	0.05
water / β-cyclodextrin / qhs	0.05
herbal decoction / qhs	0.18

Control Sample	Peak Mass (g)
water only	no peak
water / α-cyclodextrin	no peak
water / quercetin	no peak
water / cineole	no peak
water / β-cyclodextrin	no peak
herbal decoction	0.09

The corrected peak mass for herbal decoction / qhs is 0.09g

Chapter 3

The Antimalarial Mechanism of Qinghaosu

3.1 Introduction

Qinghaosu is a potent antimalarial agent, but the basis for its selective toxicity is not fully understood. A number of mechanisms which differ in detail have been proposed. In this section, a range of mechanisms are reviewed and evaluated using a variety of experimental techniques in order to determine the most probable mechanism of action of qinghaosu.

It has been proposed (and is generally accepted) that the selectivity of qinghaosu for malarial parasites is due to the fact that malarial parasites are rich in haemin, derived from the digestion of host haemoglobin.[1] The human erythrocyte contains the equivalent of 20 mM haem which is almost entirely bound to haemoglobin. Malarial parasites digest approximately 25% of the host haemoglobin but do not catabolise haem. Instead, the accumulated haem is stored in food vacuoles as a polymer called haemozoin.

Meshnick *et al.*[2] claim that qinghaosu reacts within malaria infected erythrocytes to form a product with different solubility and chromatographic properties from the parent drug. In aqueous solution qinghaosu was found to react with haemin to form an adduct with identical behaviour. The product of this reaction had a molecular weight of 914 and did not appear to be directly toxic to malarial parasites. It was suggested that activated oxygen may be generated as a by-product of this reaction because, when the reaction took place in the presence of erythrocyte membranes, oxidation of protein thiols occurs. Evidence in support of the production of free radicals was provided by the observation that iron catalyses the generation of free radicals since a spin-trapped free radical signal was seen by ESR only when qinghaosu was incubated in the presence of iron. Furthermore, when *P. falciparum* infected red cells were exposed to radiolabelled qinghaosu, a haemin-qinghaosu adduct was isolated, indicating that the same reaction occurs *in situ*.[3] Direct evidence for the critical role of haemin/haemozoin in the mechanism of action of qinghaosu was obtained by Peters *et al.*,[4] who

demonstrated that qinghaosu was at least fifty times less effective against a chloroquine resistant strain of parasite called *P. berghei* which lacks haemozoin.

In the presence of iron, qinghaosu may undergo Fenton-type reactions[5] which can be summarised as follows;

Reduced metal + Oxidant ⟶ Oxidised metal + more powerful Oxidant

$$Fe(II) + H_2O_2 \longrightarrow Fe(III) + HO^{\cdot} + OH^{-}$$

In this type of reaction involving qinghaosu, hydroxyl radicals or other reactive intermediates (e.g. superoxide anion, hydrogen peroxide) may also be produced. They may serve as mediators in killing the malarial parasite which is known to be susceptible to oxidant stress.

These observations are consistent with a two step mechanism of action of qinghaosu which can be summarised as follows;

qinghaosu $\xrightarrow{Fe}$ **R·** ⟶ **free radical damage (lipid peroxidation, protein oxidation, alkylation, etc)** ⟶ **cell death**

R· = mixture of free radicals

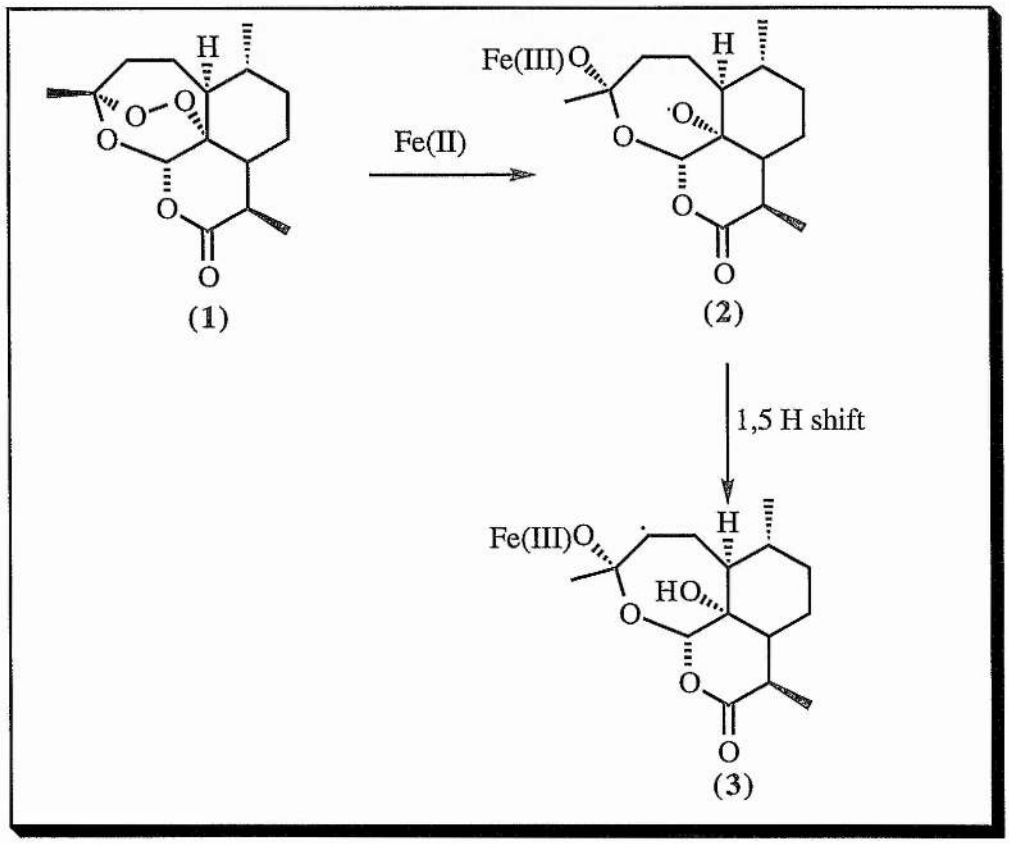
The first step is a haem catalysed cleavage of the endoperoxide bridge to form a free radical. The second step is free radical damage induced by the drug derived free radical or transient intermediate resulting in cell death. Evidence consistent with this mechanism includes observations that the endoperoxide bridge is necessary for biological activity[6] and that free radical scavengers and iron chelators antagonise the antimalarial activity of qinghaosu.[7] The necessity of the endoperoxide bridge further

enhances the hypothesis that qinghaosu exerts its antimalarial activity through the oxidant mode.

3.2 Proposed Reaction Mechanisms

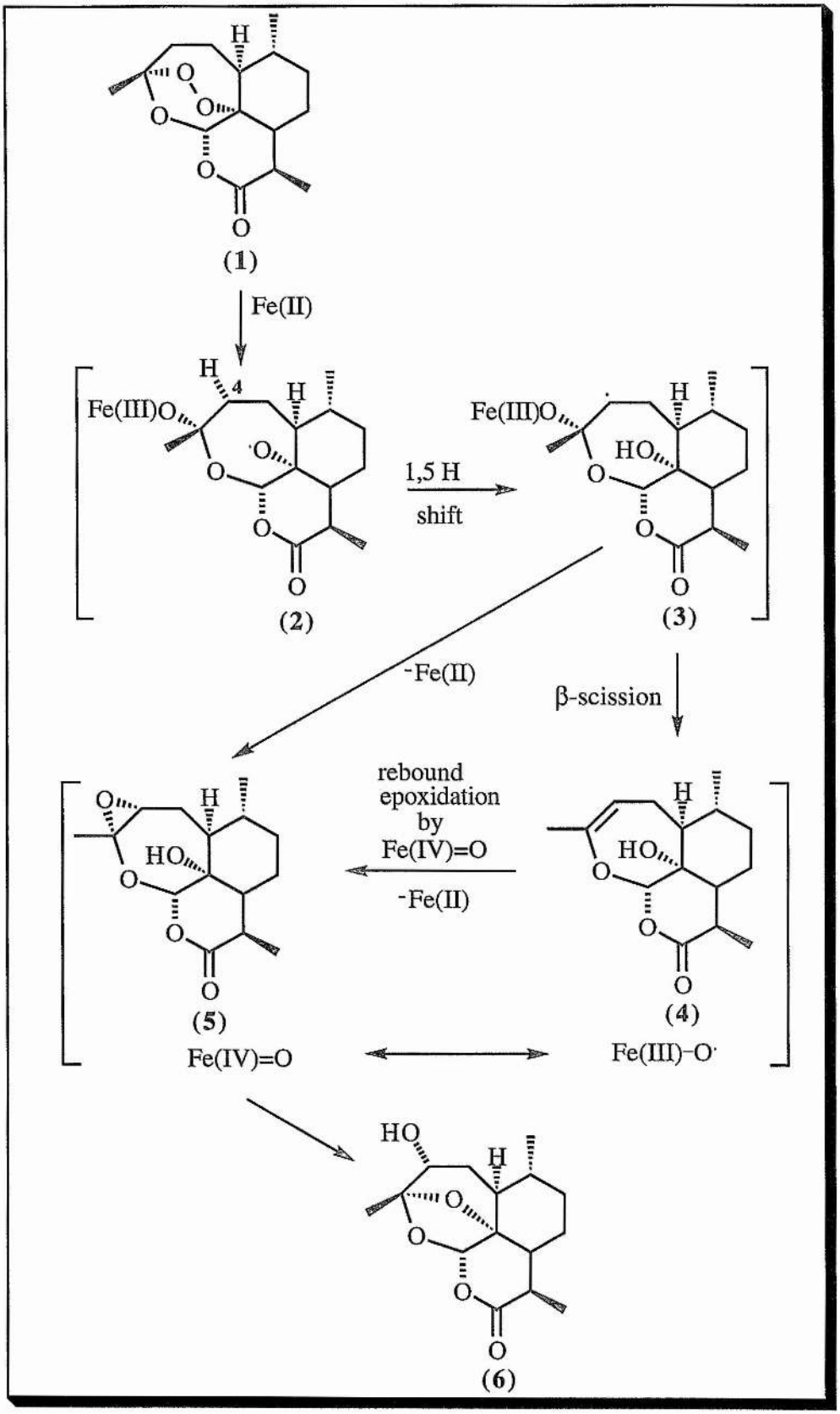
It is generally agreed that the initial step in the mechanism resulting in antimalarial activity is a one electron reduction by haem which is produced as the parasites digest haemoglobin from the host cell. What happens next is not certain. The resultant oxyl radical (**2**) (scheme 1) may be converted into a carbon radical (**3**) by a 1,5-hydrogen transfer. Carbon-centred radicals may be lethal agents, but β-scission of the C-OFe(III) bond in radical (**3**) produces an iron-oxo species and rebound epoxidation gives an epoxide, both of which may be responsible for parasite death. However no conclusive experimental evidence exists to suggest that this is the case *in vivo*

Scheme 1 1,5-hydrogen transfer



Posner *et al.*[8] have presented evidence for Fe(IV)=O production. The initial step in this mechanism is the 1,5 H shift (above), also proposed by Bloodworth *et al.*[9]

Scheme 2 Mechanism of Posner *et al.*



A molecular mechanism representing these transformations and the evidence accumulated for the existence of a high valent iron-oxo intermediate is summarised in scheme 2. By means of using an oxygen-18 labelled trioxane and some mechanism based synthetic analogues it has been claimed that a carbon centred radical formed by an oxy radical via an intramolecular 1,5 hydrogen atom shift is important for antimalarial activity.

Some experimental evidence supports the intermediacy of a high valent, non-haem, iron-oxo species resembling that characteristic of monoxygenase metalloenzymes and known to cause oxidative damage to biological macromolecules. It was proposed by Posner *et al.* that such a high valent iron-oxo species could be formed via homolytic oxygen-carbon bond scission from a β-ferryloxyethyl radical and that the highly electrophilic epoxide (**5**) (scheme 2) a potent alkylating agent was thus formed.

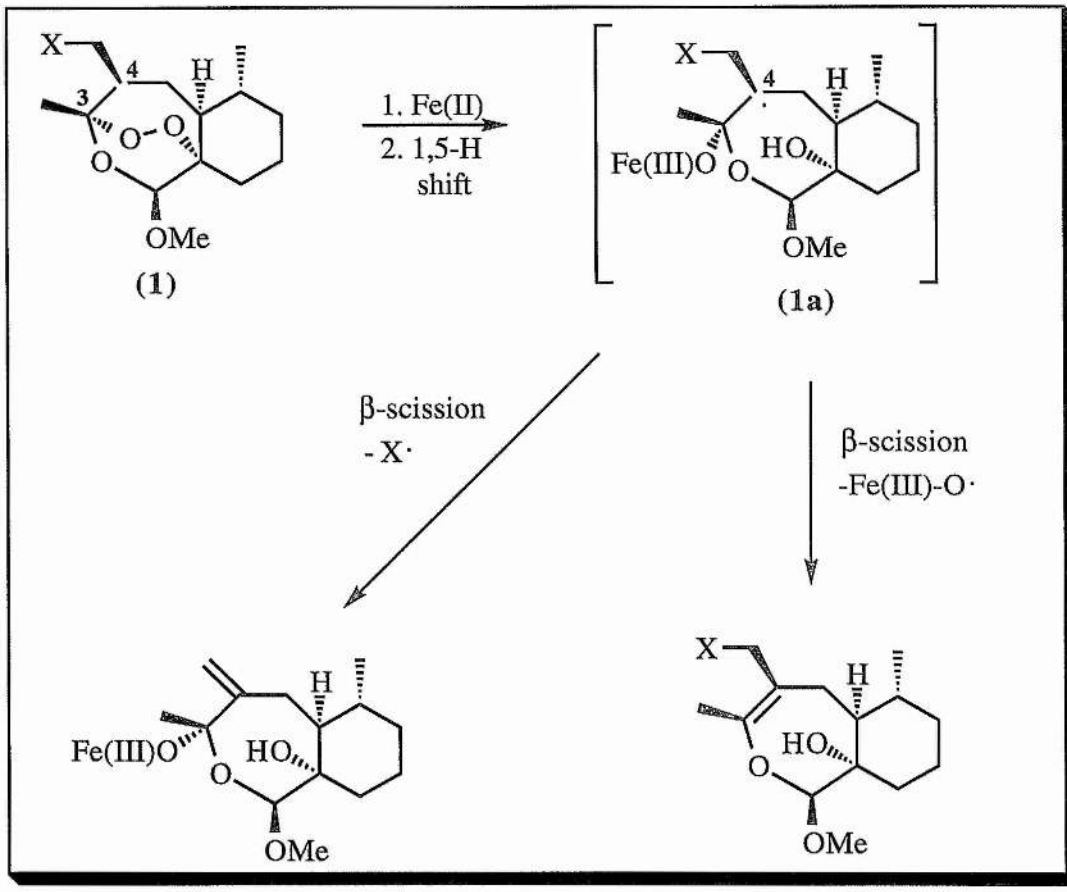
This proposed mechanism is significant in that it provides mechanistic evidence for the intermediacy of high valent iron-oxo species in the molecular mechanism of qinghaosu action. Furthermore, there is evidence that similar high valent iron-oxo species are generated from a dialkyl peroxide. It also probes the possibility that such Fe(IV)=O species are formed via homolytic oxygen-carbon bond scission from a β-ferryloxyethyl radical (**3**) (scheme 2). This is an explicit proposal of an antimalarial molecular mechanism involving highly electrophilic and cytotoxic alkylating epoxides like (**5**) (scheme 2).

However, there is a limitation to this mechanism in that C4 stabilisation is more than that of a tertiary radical and seems to shunt the iron(II) reduction of that analogue towards a different pathway, thereby resulting in low antimalarial activity.

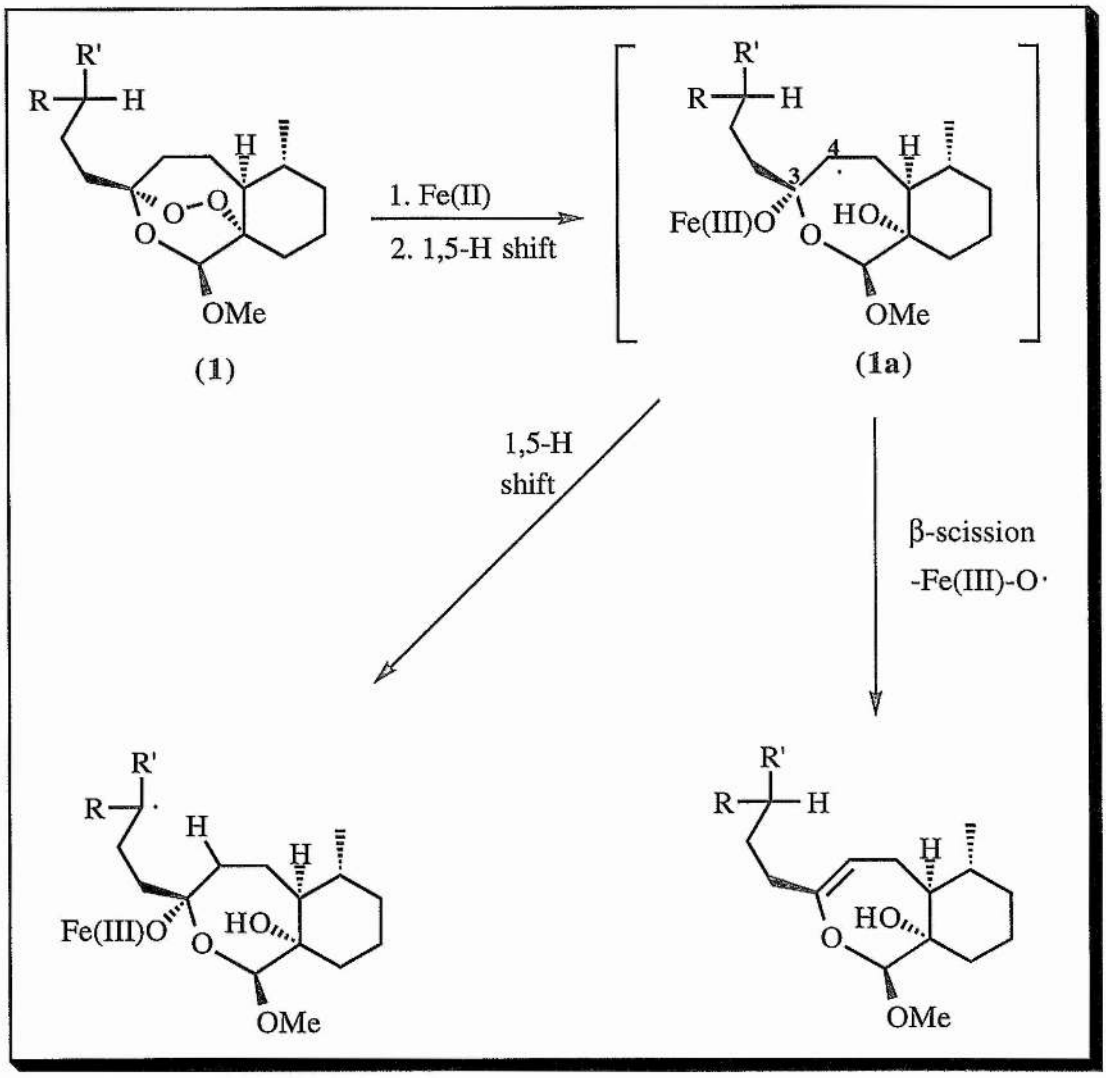
Furthermore, Posner *et al.*[10] have designed and synthesised some structurally simplified 1,2,4-trioxanes in order to determine whether they are reduced by ferrous iron to form the same kinds of radical intermediates as formed upon qinghaosu reduction. The simplified trioxanes were formulated such that an intermediate carbon-

centred radical could be intercepted before β-scission of Fe(III)-O· [Fe(IV)=O] either by a competing β-scission of a better radical leaving group e.g. X· from a radical intermediate (**1a**, scheme 3) or by a competing subsequent 1,5 hydrogen atom shift in a radical intermediate (**1a**, scheme 4).

Scheme 3

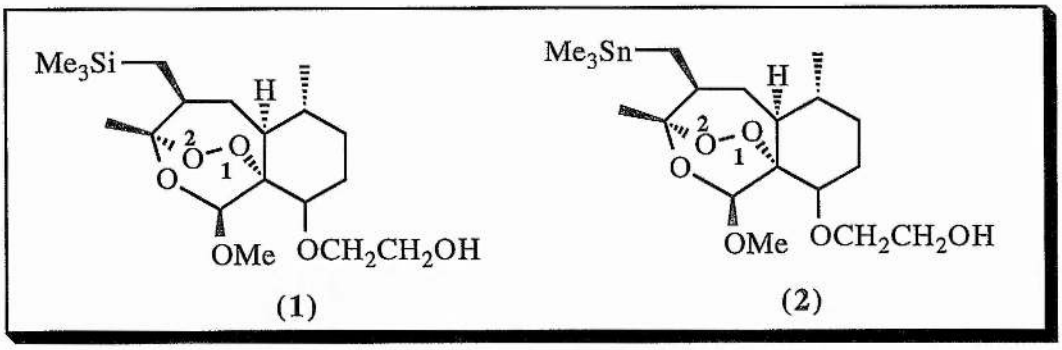


Scheme 4



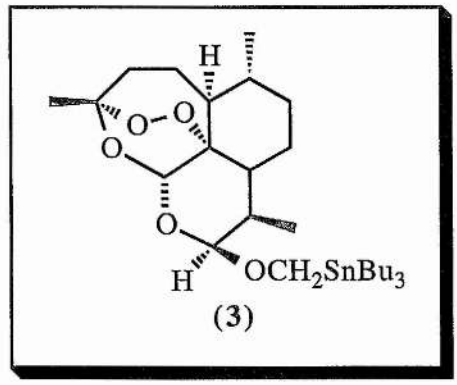
Such mechanistically competing reactions were expected to interrupt formation of a high valent iron-oxo species and thus diminish or undermine the antimalarial activity of analogues. The β-scission pathway is supported by *in vitro* antimalarial studies.

Figure 1



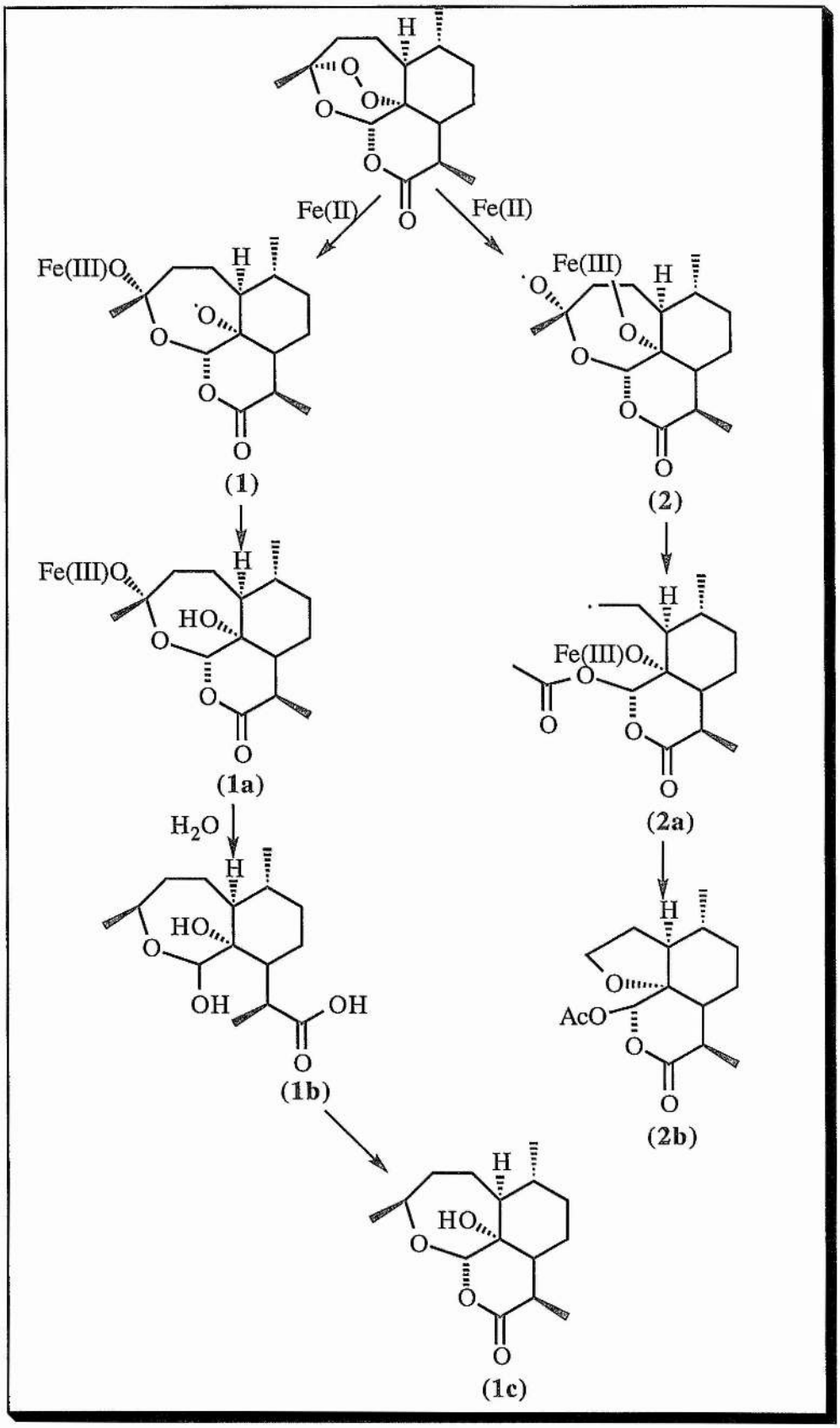
The trioxanes (**1**) and (**2**) (figure 1) differ by only one atom: silicon vs. tin. Despite this small structural difference within a complex organic endoperoxide, C_4 β–silyl analogue (**1**) had significant antimalarial activity *in vitro* whereas tin analogue (**2**) was virtually inactive. These results are consistent with selective β-scission of Me_3Sn (rather than retention of Fe(III)-O·). In order to show that the presence of a tin atom does not destroy a trioxane's antimalarial activity, a tin containing dihydroartemisinin analogue (**3**) (figure 2) was synthesised and examined.

Figure 2



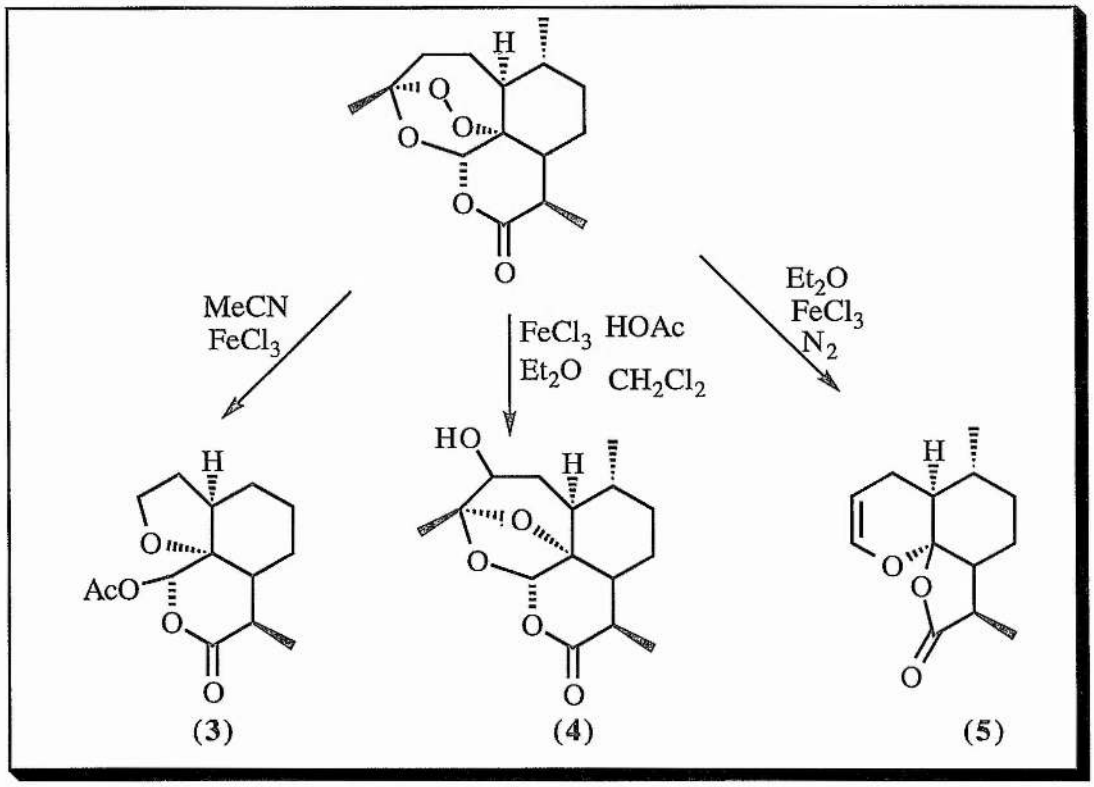
It has been proposed that it is haem iron(II) which reduces qinghaosu, with the reduction of haemin iron(III) to haem iron(II) being induced by an exogenous, possibly thiol-based, electron source.[11] Recent work with either qinghaosu or synthetic trioxanes has focused on Fe(II) and commenced with the premise that reductive cleavage of the peroxide bridge leads to carbon-centred radicals which may be the biologically active species. However, haemin iron in oxidation state (III), either in haemin itself or in haemozoin may also be a target for qinghaosu interaction. Haynes and Vonwiller[12] evaluated the behaviour of both iron(II) and iron(III) in aqueous media which mimicked physiological conditions. Initially a Fenton-type cleavage of the peroxide bridge gave radicals (**1**) and (**2**) (scheme 5) with haemin [chloroprotoporphyrin IX iron(III)] in aqueous acetonitrile, oxygen loss from the peroxide bridge of qinghaosu takes place to give desoxoqinghaosu (**1c**), a known malaria-inactive metabolite. In addition, radical (**2**) may undergo β-scission to the primary carbon-centred radical (**2a**) which then forms tetrahydrofuran (**2b**).

Scheme 5 Mechanism of Haynes and Vonwiller (haem iron)



Studies of the behaviour of qinghaosu with non-haem iron(II) and (III) in non-aqueous media caused consideration of an alternative viewpoint.[13] In aprotic solvents with $FeCl_3$/N-acetylcysteine or $FeCl_2$, qinghaosu undergoes rearrangement to give the tetrahydrofuran acetate (**3**), 4-hydroxydesoxoqinghaosu (**4**) or the enol lactone (**5**) (scheme 6) depending on the catalyst used and the polarity of the reaction medium.

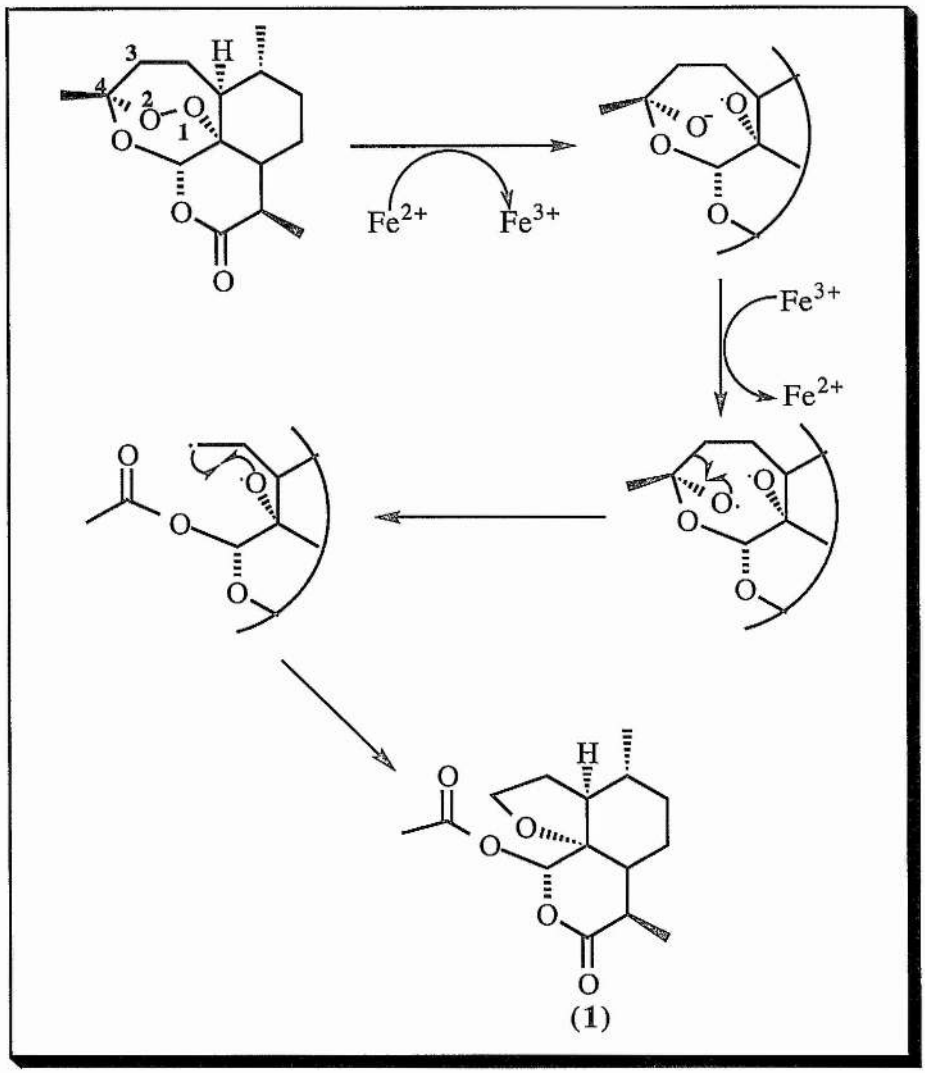
Scheme 6 Mechanism of Haynes and Vonwiller (non-haem iron)



Wu *et al.*[14] have proposed the following mechanism involving the cleavage of the peroxy bond in qinghaosu with ferrous iron.

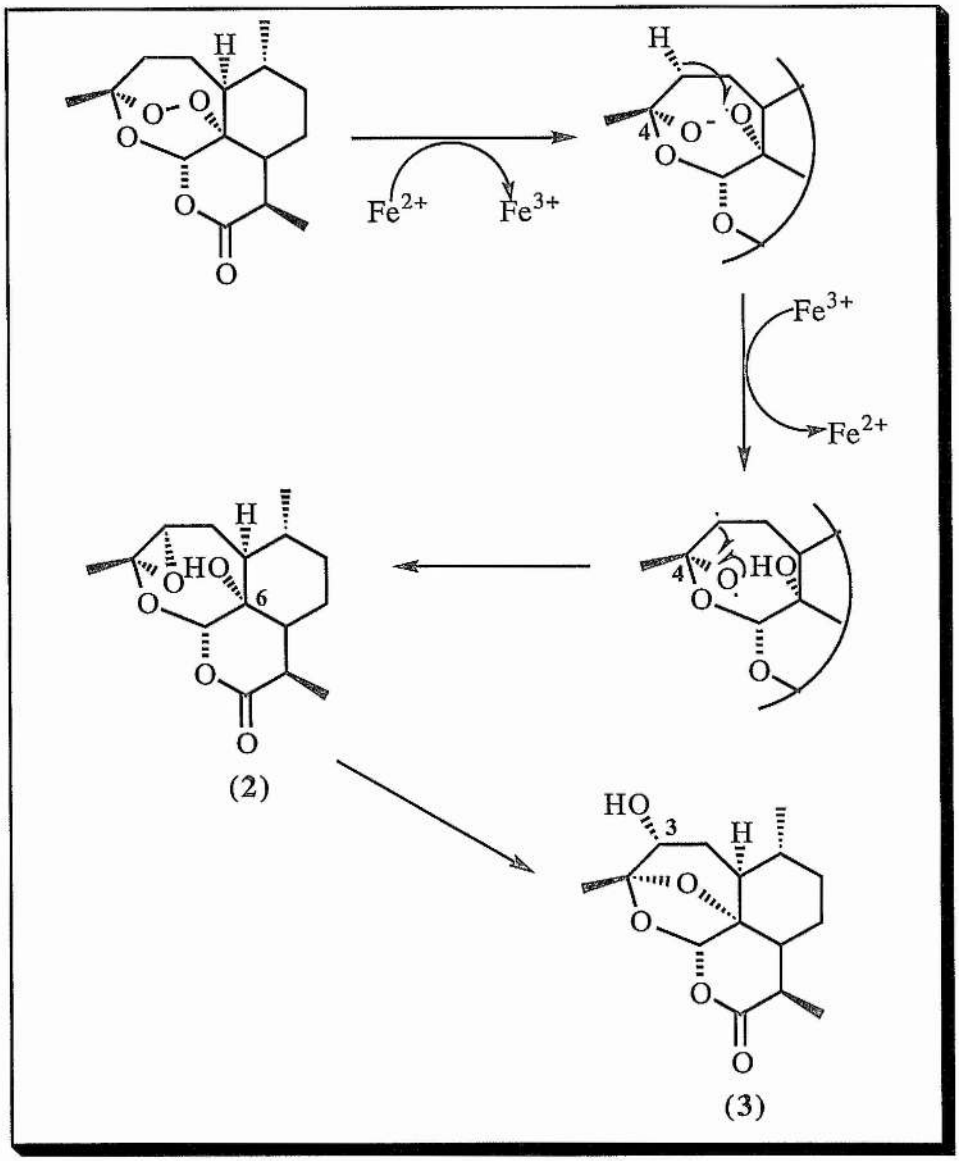
Initially the peroxy bond was cleaved by receiving one electron from Fe(II) to give a free radical at O1 and an anion at O2 which further reacted with the resulting Fe(III) in two different ways;

Scheme 7 Mechanism 1 of Wu *et al*



Starting with transfer of an electron from O2 to Fe(III), β-scission followed to give a carbonyl at C4, leaving a radical at C3 which coupled simultaneously with the O1 radical to afford compound (**1**).

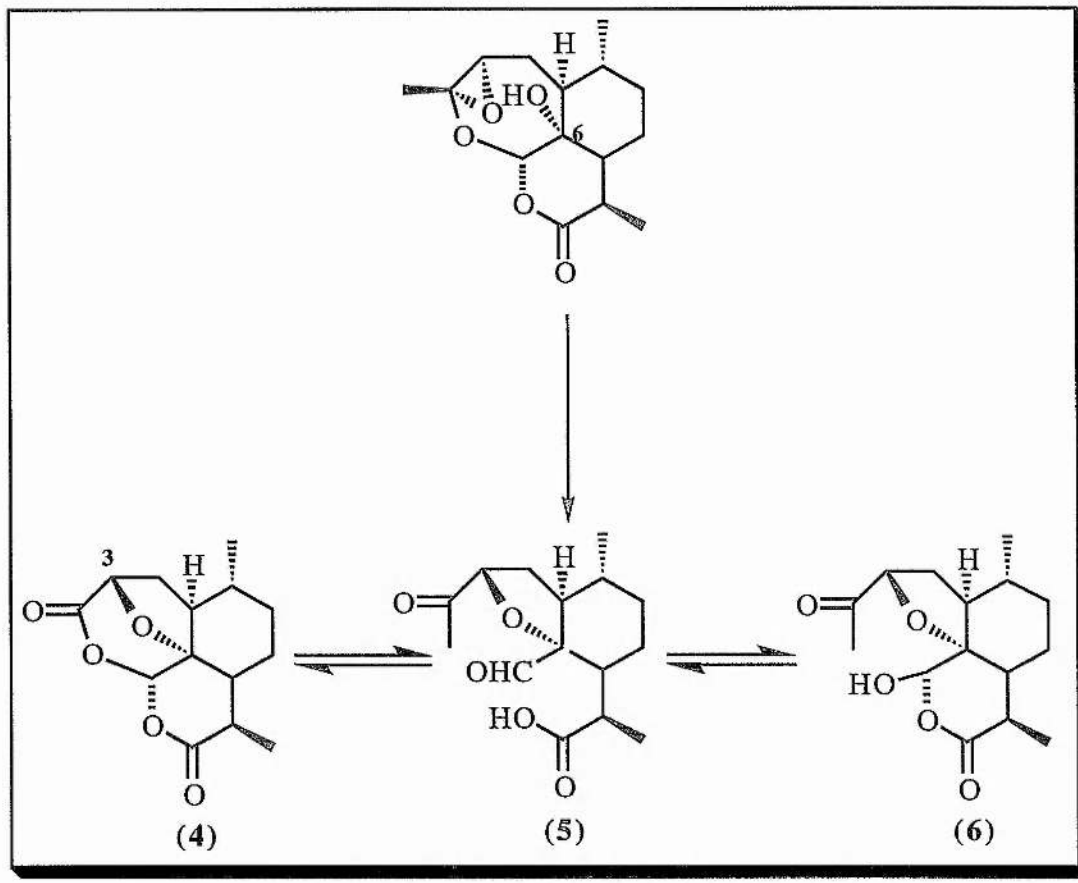
Scheme 8 Mechanism 2 of Wu *et al.*



An alternative mechanism is intramolecular hydrogen abstraction, initiating with the radical at O1 abstracting the α -hydrogen from C3. The newly formed C3 radical then coupled with O2, after donating an electron from oxygen back to Fe(III), forming the epoxide (**2**) (scheme 8). This epoxide could be easily opened by the attack of 6-OH; the attack at C4 resulted in the formation of major product (**3**) (scheme 8). This is consistent with the product isolated by Posner *et al.* [8] Formation of compound (**3**) was confirmed by preparative studies (page 65). However compound (**1**) could not be isolated.

Alternatively 6-OH attack at C3 gave an equilibrating mixture of compounds (**4**), (**5**) and (**6**); scheme 9)

Scheme 9 Mechanism 2a of Wu *et al.*



This mechanism was postulated in order to rationalise experimental outcomes. Serious damage to DNA chains by this cleavage has also been observed. These results provide a rational explanation for why qinghaosu affects only the red cell infected by the parasite, not the normal red cell [both are rich in Fe(II)]-the former has a cell nucleus whereas the latter does not. The site of DNA damage (the carbohydrate or the base moiety) is still under investigation. In all the proposed mechanisms it must be noted that the reactions were carried out *in vitro*, and therefore may not accurately mimic what occurs *in vivo*.

3.3 Experimental

3.3.1 Instrumentation and General Techniques

Routine melting points were determined with open glass capillaries using an Electrothermal IA 9000 series digital melting point apparatus. Infra-red spectra were recorded on a Perkin-Elmer 1330 infra red spectrophotometer. NMR spectra were recorded on a Bruker AM 300 spectrometer operating at 300 MHz for ^{1}H and 75.4 MHz for ^{13}C. Chemical shifts (δ) for ^{1}H and ^{13}C are reported in ppm. Mass spectra were recorded on an A.E.I MS-50 spectrometer and fragment ions indicated as m/z units.

3.3.2 Preparation of Compound (3)

A mixture of qinghaosu (0.56 g; 2 mmol) and ferrous sulphate (0.56 g; 2 mmol) in aqueous acetonitrile (1:1, pH 4) was stirred under nitrogen while temperature was maintained at 37°C. The reactants dissolved to form a yellow/orange solution and the reaction was continued until no starting material was detectable by TLC. The reaction mixture was extracted with ethyl acetate (3 x 20 cm^3) and the combined organic layers were washed with brine, dried over anhydrous sodium sulphate and evaporated *in vacuo*. The residue was separated by flash chromatography [silica gel, ethyl acetate-petroleum ether (1:1) as eluent]. White needles of compound (**3**), (scheme 8) were obtained upon recrystallisation from ethanol and diagnostic assignments made; yield 0.21 g (38%); m.p 149-151°C (lit[14] 150-152°C); $\upsilon_{max.}$ (nujol)/cm^{-1} 1745 (C=O), 1270 (OH); δ_H (300 MHz; $CDCl_3$); 5.60 (1H, s, OH), 4.33, 4.56 (1H, dd, C<u>H</u>-OH); δ_C (75.4 MHz; $CDCl_3$); 171.23 (C=O), 82.84 (C-OH), 68.98 (<u>C</u>H_2-C-OH); *m/z* 265 (M^{+}-OH).

3.4 Studies of the Reaction between Iron and Qinghaosu

3.4.1 Electron Spin Resonance (ESR) Studies

As most proposed mechanisms feature free radicals, ESR was seen as the most suitable technique to investigate further the antimalarial activity of qinghaosu. Spin traps (diamagnetic molecules) were used to scavenge reactive free radicals and hence form a more stable radical, readily detectable by ESR spectroscopy. The mechanism proposed by Wu *et al.*[14] was used as a model for the experiments performed.

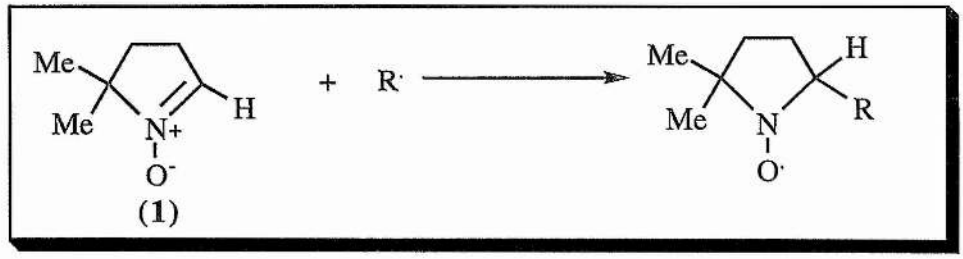
Experimental

ESR spectra were recorded on a Bruker ESP 300 spectrometer equipped with an X-band klystron and 100 kHz modulation. The hyperfine splittings and g-values were determined from the spectrometer field scan, this having been calibrated with the signal from Fremy's salt (a_N 1.309 mT, g 2.0055).

ESR experiments involved mixing aqueous acetonitrile (1:1) solutions of Fe(II) sulphate (2 mM), qinghaosu (2 mM), and the spin trap [typically 200 mM for DMPO (5,5-dimethyl-1-pyrroline N-oxide) and 25 mM for DBNBS (3,5-dibromo-4-nitrosobenzene sulphonate)]. All spin traps were purified (recrystallised and frozen) prior to use. The resultant solution was placed in a standard flat-cell for use with aqueous solutions and acquisition of the first spectrum started *ca.* 1 minute after mixing.

Results

Spin-trapping with DMPO (**1**)

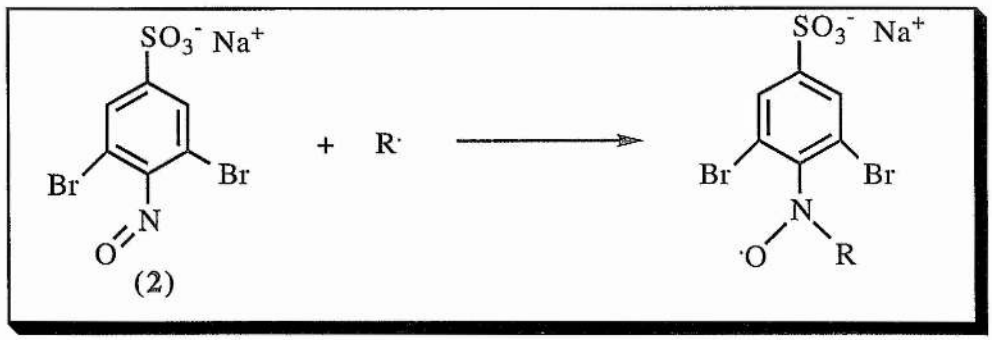


Reaction of a radical with DMPO gives a nitroxide which usually has a β-hydrogen splitting characteristic of the initial radical. For example, typically, a carbon-centred radical gives $a_{(\beta\text{-H})}$ 20-22 Gauss whilst an alkoxyl radical gives $a_{(\beta\text{-H})}$ 15-17 Gauss. A mixture of Fe(II), qinghaosu and DMPO (50 mM) gave very weak signals which could not be unambiguously interpreted (spectra dmpo_1 and dmpo_2). Use of a higher concentration of DMPO (200 mM) gave stronger signals (dmpo_3, dmpo_4 and dmpo_5) which were interpreted as follows:

a_N Gauss	**a_H Gauss**	**Assignment**	**Symbol (on spectra)**
15.7	22.25	carbon-centred	*
14.72	-	Spin trap degradation product	Δ
15.04	18.24	$CO_2^{-\cdot}$	x

(for spectra see appendix 1)

Spin-trapping with DBNBS (2)



DBNBS usually only forms long-lived adducts with carbon-centred radicals, but has the advantage that more information on the structure of the initial radical can be obtained. In particular, the number of large proton splittings observed gives the number of protons at the α-carbon. A mixture of Fe(II) sulphate, qinghaosu and DBNBS (2.5 mM) gave very weak signals which could not be unambiguously interpreted (dbnbs_1 and dbnbs_2). Use of a higher concentration of DBNBS (25 mM) gave stronger signals (dbnbs_3, dbnbs_4 and dbnbs_5). It is interesting to note that the signal intensity grows with time which is indicative of a slow rate of reaction between Fe(II) and the peroxide. The signals were interpreted as follows:

a_N Gauss	a_H Gauss	Assignment	Symbol (on spectra)
~13.35	~13.35 (3H)	$\cdot CH_3$	*
13.45	11.80 (2H)	$\cdot CH_2R$	Δ
13.40	6.80 (1H)	·CHRR'	x

(for spectra see appendix 1a)

Discussion

The detection of spin adducts of carbon-centred radicals is consistent with degradation of qinghaosu by Fe(II) occurring via a radical mechanism. The proposed mechanism for oxidation as shown in scheme 10 leads to the formation of three radicals; these are the methyl radical, a primary radical (**3**) and a secondary radical (**4**). Species of this type were trapped in the studies using DBNBS as the spin trap.

Reaction in the presence of DMPO lead to the trapping of carbon-centred radicals which is again consistent with the proposed mechanism. The nitroxide radical detected, which

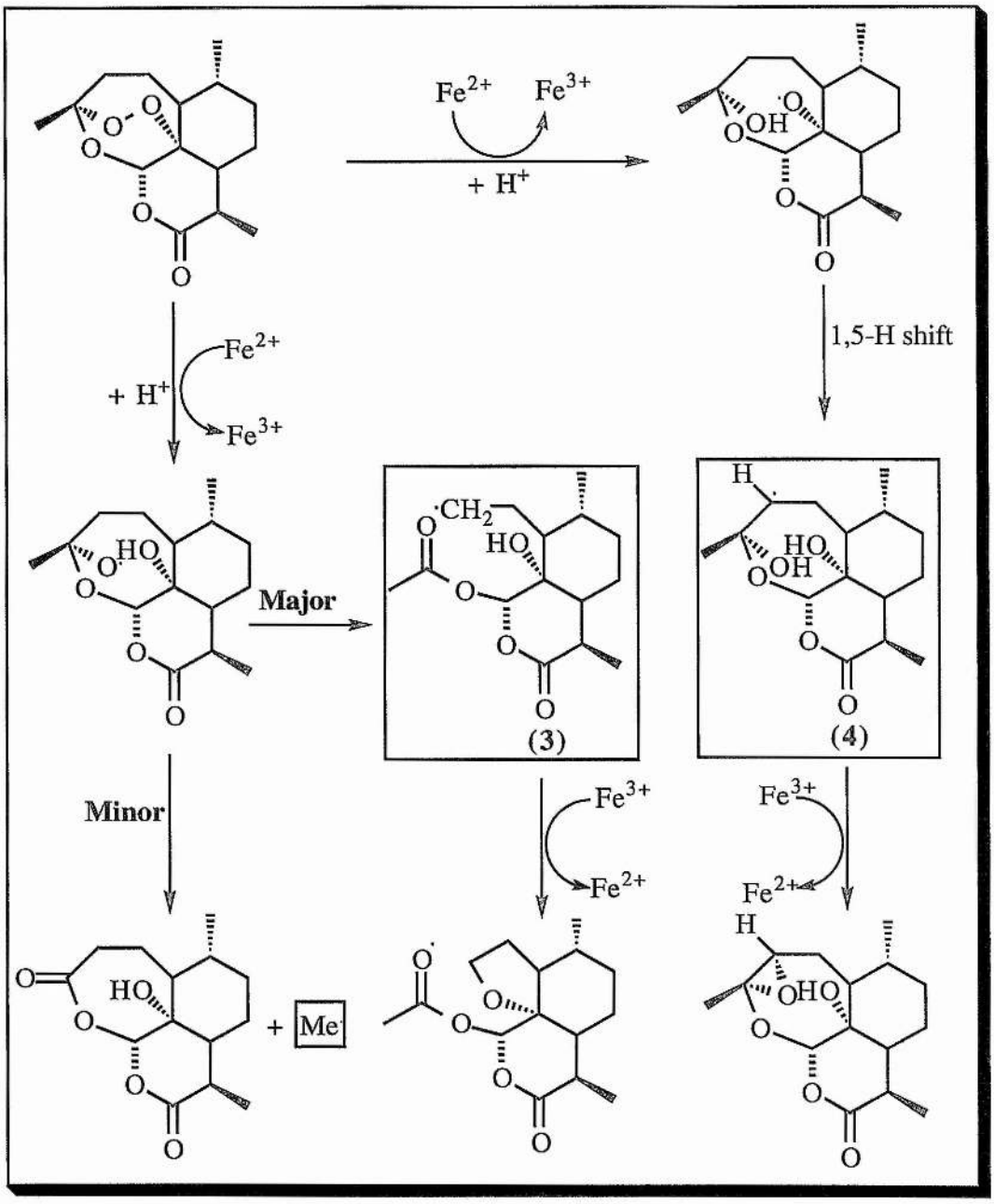
lacked a β-splitting, is probably formed as a decay product of some of the initial adducts, consistent with an increase in intensity with time. From where the adduct which is assigned $CO_2^{-\cdot}$ arises is uncertain. The lack of observation of an alkoxyl radical is not surprising in view of the rapid intramolecular rearrangement expected for this species.

It should be noted that if the radicals formed are free to diffuse away from the Fe(II) formed with them (*i.e.* out of the solvent cage) then protonation of the oxygen anion will occur. This diffusion is necessary if the radical is to react with the spin trap. This protonation is likely to occur faster than the rearrangements. Oxidation of the rearranged radicals to cations by the Fe(III) [and regenerating the Fe(II)] and subsequent rearrangement will still give the expected products.

The conclusions of these preliminary ESR experiments are entirely consistent with the mechanism proposed by Wu *et al.*[14] which avoids postulation of a high valence iron-oxo intermediate. Evidence was presented for the trapping of primary radical (**3**) and secondary radical (**4**) (scheme 10). These radicals are intermediates in the formation of compound (**1**) (scheme 7) and compounds (**2**) and (**3**) (scheme 8) respectively. From the findings of this study there is no evidence whatsoever to suggest the formation of a high valent iron-oxo species such as that proposed in the mechanism of Posner *et al.*[8] (scheme 2).

ESR has proven to be a valuable tool in further elucidating the antimalarial mechanism of qinghaosu. It will therefore be employed in further studies with qinghaosu and its ether derivatives artemether and arteether, in order to probe further the mode of action of these compounds.

Scheme 10 Radicals Detected from Reaction of Qinghaosu and Iron (II)



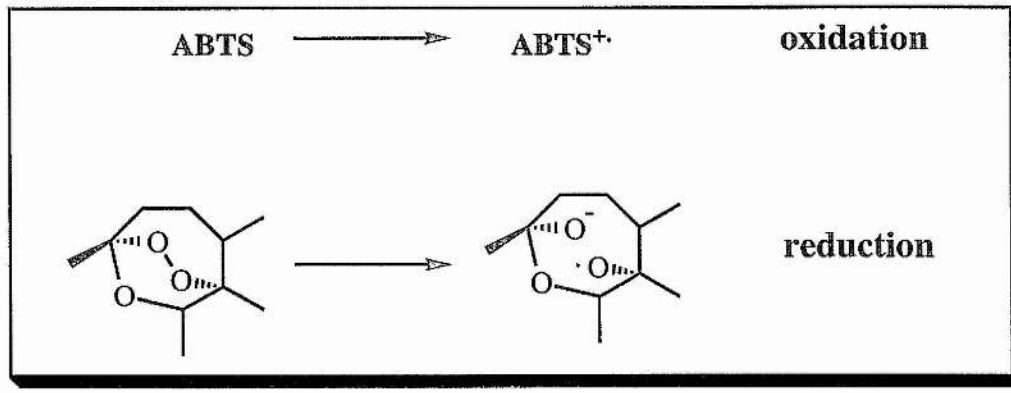
The spin adducts of carbon centred radicals $\cdot CH_3$, $\cdot CH_2R$, $\cdot CHRR'$ were detected, and are highlighted in the above scheme.

3.4.2 A Mechanistic Study Involving ABTS

Much evidence has been gathered to support the hypothesis that qinghaosu exerts its antimalarial activity through the oxidant mode. The activity of qinghaosu was studied in the following experiments.

The reaction of qinghaosu with FeTSPP [tetra (4-sulphonato-phenyl) porphyrin Fe(III)] was investigated using an excess of ABTS [2,2'-azinobis (3-ethyl-2,3-dihydrobenzothiazole-6-sulphonate)] as a one electron oxidant trap. If, in the presence of Fe(III) qinghaosu acts as oxidising agent, ABTS will be oxidised to the relatively stable blue-green cation $ABTS^{+\cdot}$ ($\lambda_{max.}$ 660 nm, ε_{660} 1200 m^2 mol^{-1})[15] which is detected by spectrophotometry.

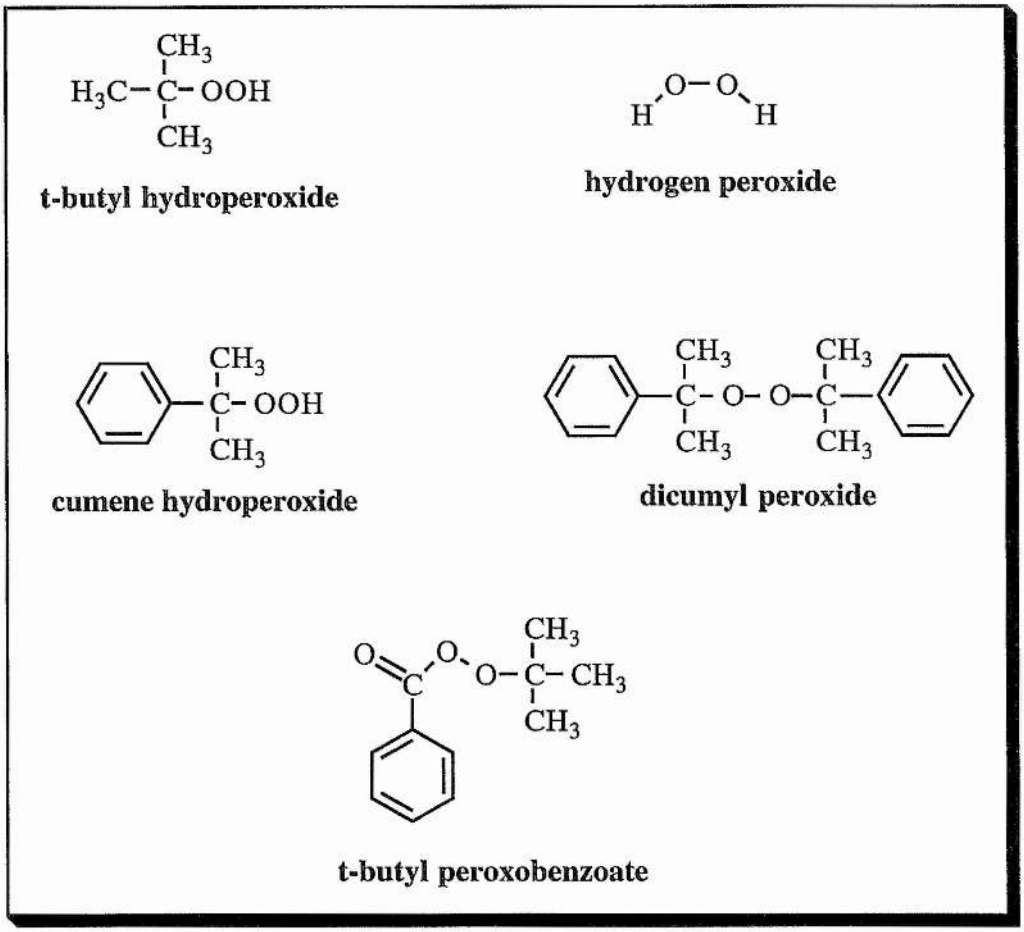
In the presence of FeTSPP the following reactions might be expected;



The radical species formed by reduction of qinghaosu could be responsible for the destruction of membranes of *Plasmodium*.[16]

Test Compounds - Peroxides and Hydroperoxides

Various peroxides and hydroperoxides which exhibit antimalarial activity were used to probe the mode of action of qinghaosu.



The Antimalarial Activity of Test Compounds

The following compounds have already been tested *in vitro* on *P. falciparum* [17]

Test Compound	D-6 clone IC_{50} (μM)	W-2 clone IC_{50} (μM)
qinghaosu	0.005	0.002 (most effective)
cumene hydroperoxide	62.2	51.0
dicumyl peroxide	62.2	60.0
t-butyl peroxobenzoate	391	202 (least effective)

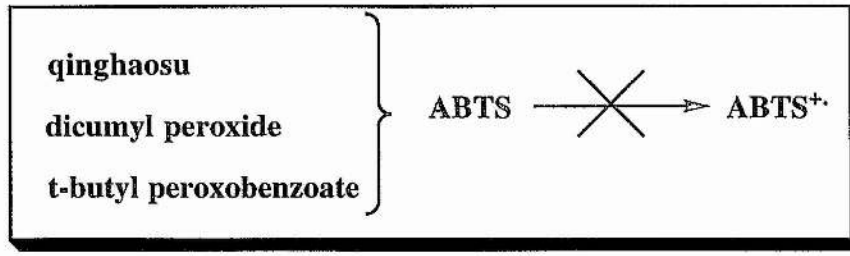
Experimental

Spectrophotometry was carried out using a Phillips PU 8730 UV/Vis Scanning spectrophotometer and a Pye Unicam temperature control unit. Temperature was maintained at 30°C.

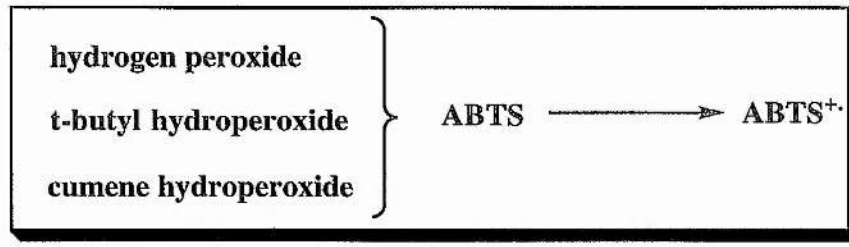
Test compounds were prepared in acetic acid-acetate buffer (0.1 M) pH 5.2. No reaction occurred between $(NH_4)_2$ABTS and the test compound in the absence of FeTSPP. Experiments involved FeTSPP (0.009 mmol), $(NH_4)_2$ABTS (0.009 mmol) and test compound (0.06 mmol), and were repeated using EDTA (0.01 mmol) as an iron chelator in all control samples.[18]

Results

Peroxides



Hydroperoxides



Discussion

This study provides evidence for the oxidation of ABTS to $ABTS^{+\cdot}$ by hydroperoxides in the presence of Fe(III). Under these conditions hydrogen peroxide, cumene hydroperoxide and t-butyl hydroperoxide instantaneously oxidised ABTS to $ABTS^{+\cdot}$ ($\lambda_{max.}$ 660 nm). However, other peroxides studied (qinghaosu, t-butyl peroxobenzoate and dicumyl peroxide) did not oxidise ABTS in the presence of Fe(III). From the results obtained there is no evidence to suggest a correlation between the antimalarial activity of the test compounds and their ability to act as oxidising agents. Qinghaosu, the most effective antimalarial test compound, was not a good oxidising agent in the presence of Fe(III). Although both hydroperoxide and other peroxides are antimalarial agents only the former oxidise ABTS to $ABTS^{+\cdot}$. Therefore, we cannot use the ABTS reaction as an indicator of possible antimalarial activity.

Due to solubility problems this experiment could not be repeated using a Fe(II) salt. However, the reaction of qinghaosu with iron(II)/(III) salts in a variety of solvents was followed using spectrophotometry. In all experiments there was little evidence of reaction, even after many hours no significant spectral changes occurred.

The results obtained are consistent with the mechanism proposed by Wu *et al.*[14] which involves Fe(II). It explains why no evidence for the production of $ABTS^{+\cdot}$ in the presence of Fe(III) was obtained, and is consistent with no reaction between Fe(III) and qinghaosu. Also, in the proposed mechanism, Fe(II) is regenerated and may explain the absence of spectral changes.

3.5 Evidence for the Binding of Qinghaosu to Serum Proteins

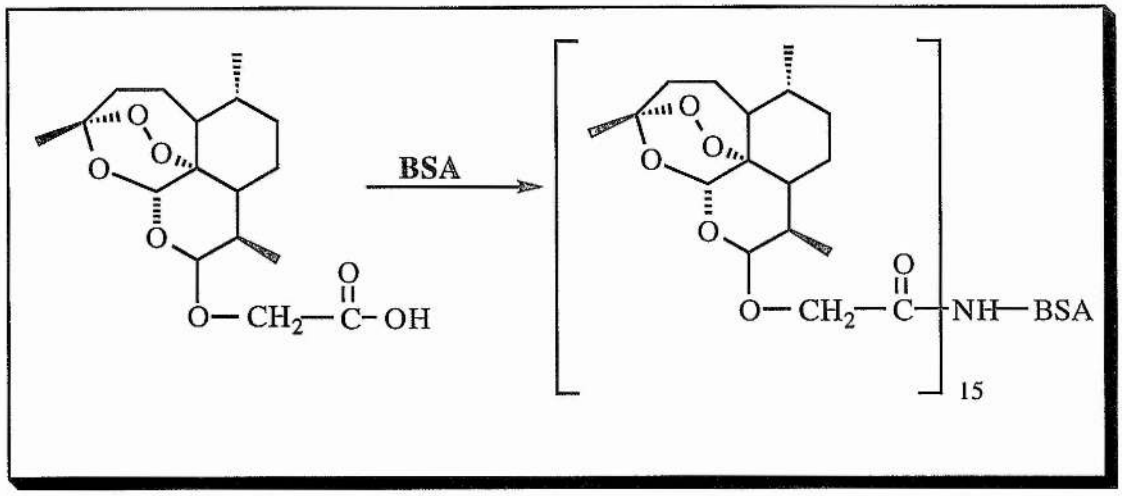
Introduction

Iron catalyses the activation of qinghaosu to produce free radicals. Evidence for this was presented by the ESR study (3.4.1). Free radical reactions appear to be critical in the mechanism of action of the drug.

There is an account in the literature[19] which describes the binding of qinghaosu and its derivatives to human serum proteins, it is also suggested that this plays a role in the antimalarial activity exhibited by these compounds. Qinghaosu, dihydroqinghaosu, artemether and artesunate bind to human proteins to different degrees, the percentage of compound bound to protein has been determined as 64, 43, 76 and 59 respectively.

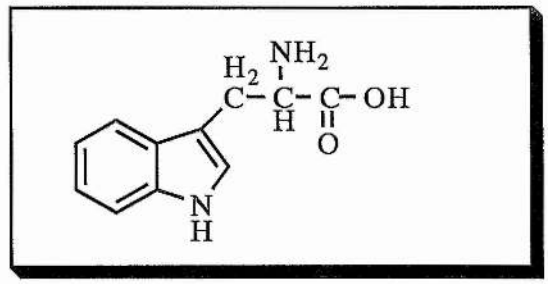
Yang *et al.*[20] claim that human serum albumin was alkylated by qinghaosu, but provided no conclusive evidence to this effect. The occurrence of reactions between the drug and host proteins may have important implications in toxicity and therefore in the clinical use of qinghaosu. In order to further investigate this claim, the reaction between qinghaosu and bovine serum albumin was studied.

It is claimed that dihydroqinghaosu-12-O-acetic acid binds to bovine serum albumin to form the following product;



The reaction of qinghaosu with BSA was studied using fluorescence emission spectroscopy. BSA has two tryptophan residues. Each residue is a chromophore which exhibits an intrinsic fluorescence. This fluorescence can be used as a probe to study the binding of qinghaosu.[21] Excitation of the tryptophan residues in the high affinity site occurs at 290 nm. The main pathway for the dissipation of absorbed energy is fluorescence at 350 nm.

Tryptophan



Experimental

Fluorescence measurements were performed using a Perkin Elmer Luminescence spectrophotometer, model L3 50B. Data manipulation was carried out using an Elonex PC 45011 and graphs printed on a Hewlett Packard Desk Jet 560C. All solutions were prepared in isotonic buffer pH 7.2 and scanned in polymethacrylate cells. Excitation wavelength was 290 nm and emission spectra were recorded between 300 and 450 nm while temperature was maintained at 25°C.

Experiment 1: The Effect of Qinghaosu on the Emission Spectrum of Albumin

Albumin (1.5×10^{-7} M) and qinghaosu (0.1 g) were combined. Control solutions; albumin (1.5×10^{-7} M) in buffer, qinghaosu (0.1 g) in buffer and buffer only.

Experiment 2: Further Studies of the Binding of Qinghaosu to BSA

In order to prevent a dilution effect the following solutions were prepared;

Albumin (1.5×10^{-7} M) - solution B

Solution B (50 cm^3) containing qinghaosu (0.1 g) - solution A

Control solutions; qinghaosu (0.1 g) in buffer (50 cm^3) and buffer only. Test solutions were prepared as follows; 8 cm^3 A + 2 cm^3 B, 6 cm^3 A + 4 cm^3 B, 5 cm^3 A + 5 cm^3 B, 4 cm^3 A + 6 cm^3 B, 2 cm^3 A + 8 cm^3 B.

Experiment 3: Is the Quantity of Qinghaosu Important for its Binding Ability?

Solutions were prepared as in **Experiment 2**, except solution A which was prepared using solution B + qinghaosu (0.01g).

In all experiments the test solutions and controls were stirred overnight and filtered. The emission spectra of all solutions were recorded.

Discussion

In combination with qinghaosu the intensity of the emission spectrum of albumin decreased significantly (appendix 2; experiment 1). Upon mixing solutions A and B the solution containing most albumin had the most intense spectrum while the solution containing most qinghaosu (least albumin) had the least intense spectrum (appendix 2; experiment 2). The difference in intensity observed between albumin and albumin/qinghaosu was not dependent on the amount of qinghaosu used i.e. 0.1 or 0.01 g (appendix 2; experiment 3). However, qinghaosu is not readily soluble in aqueous medium and the findings of experiment 3 indicate that a certain amount of qinghaosu (less than or equal to 0.01 g) has gone into solution.

The reduction in intensity of the emission spectrum of albumin observed upon addition of qinghaosu is consistent with it binding to bovine serum albumin. These results indicate only this and provide no evidence of alkylation as proposed by Yang *et al.* [20] However, the binding of qinghaosu to proteins has implications for our understanding of the drug's pharmacology. Qinghaosu is known to be selective for malarial parasites containing iron. It is proposed that the binding of qinghaosu to the parasite protein may have a role in the selective toxicity observed *i.e.* qinghaosu binds strongly to malarial parasites containing iron and much less strongly to parasites which lack iron. In effect the selective toxicity of qinghaosu may be due to the presence of iron and we find no evidence that the situation is complicated by the ability of qinghaosu to effect alkylation of human serum albumin.

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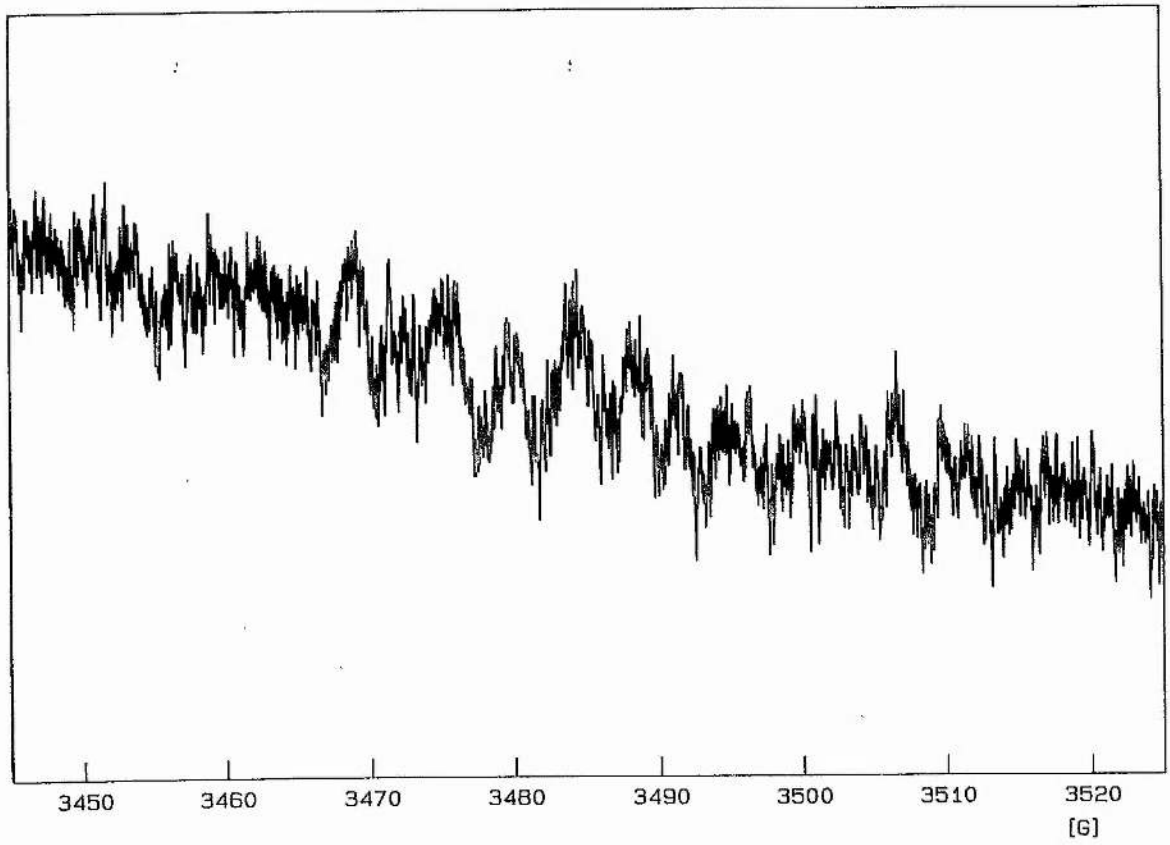
Appendices

Appendix 1

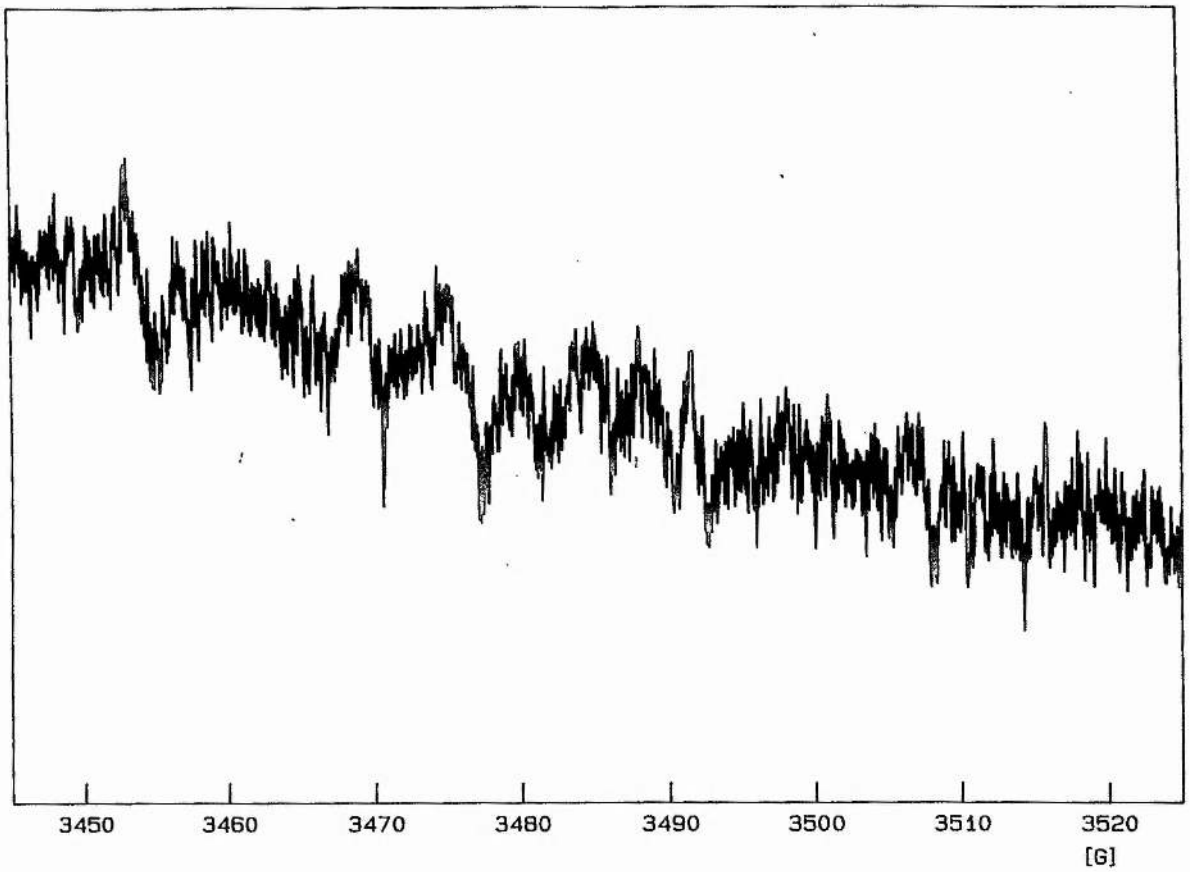
spin-trapping with DMPO

2 mM Fe(II), 2 mM qinghaosu, 50mM DMPO, MeCN:H_2O (1:1) after 1 min

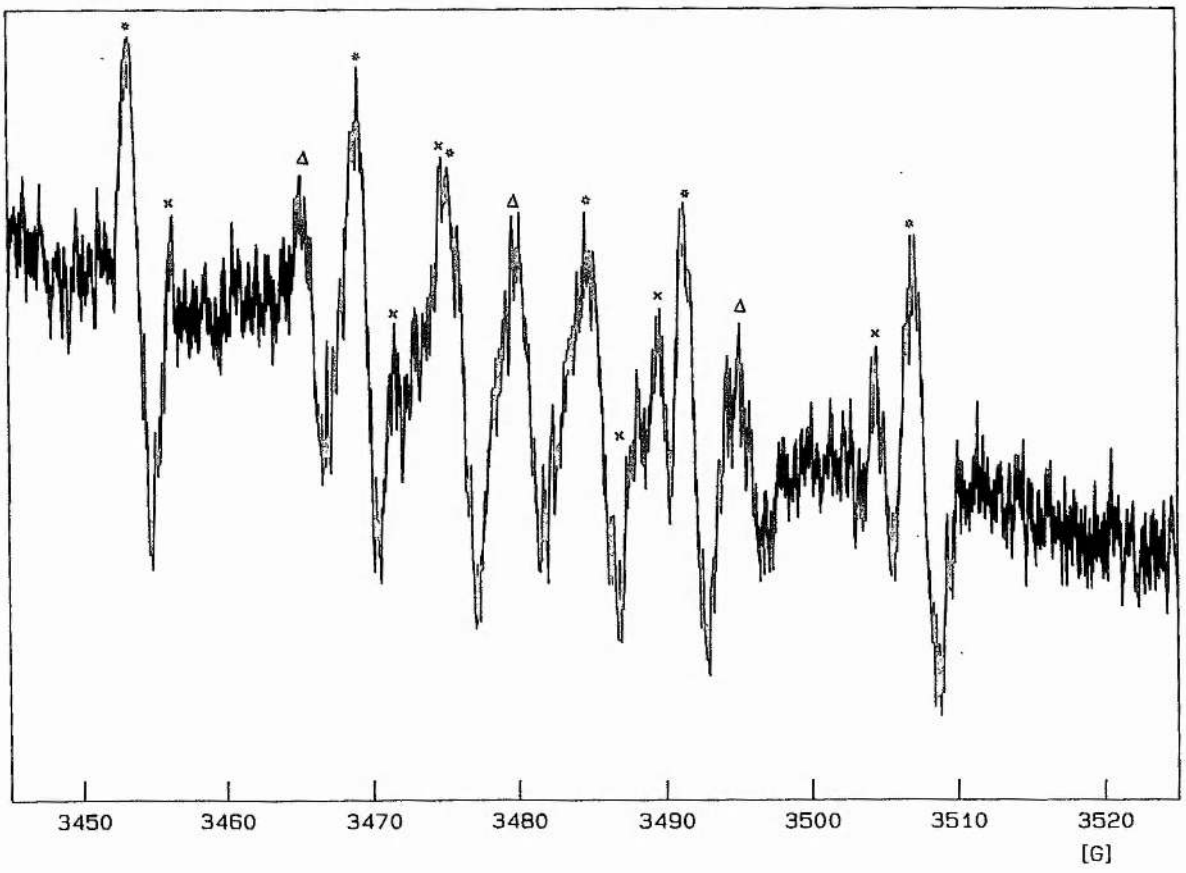


dmpo_1

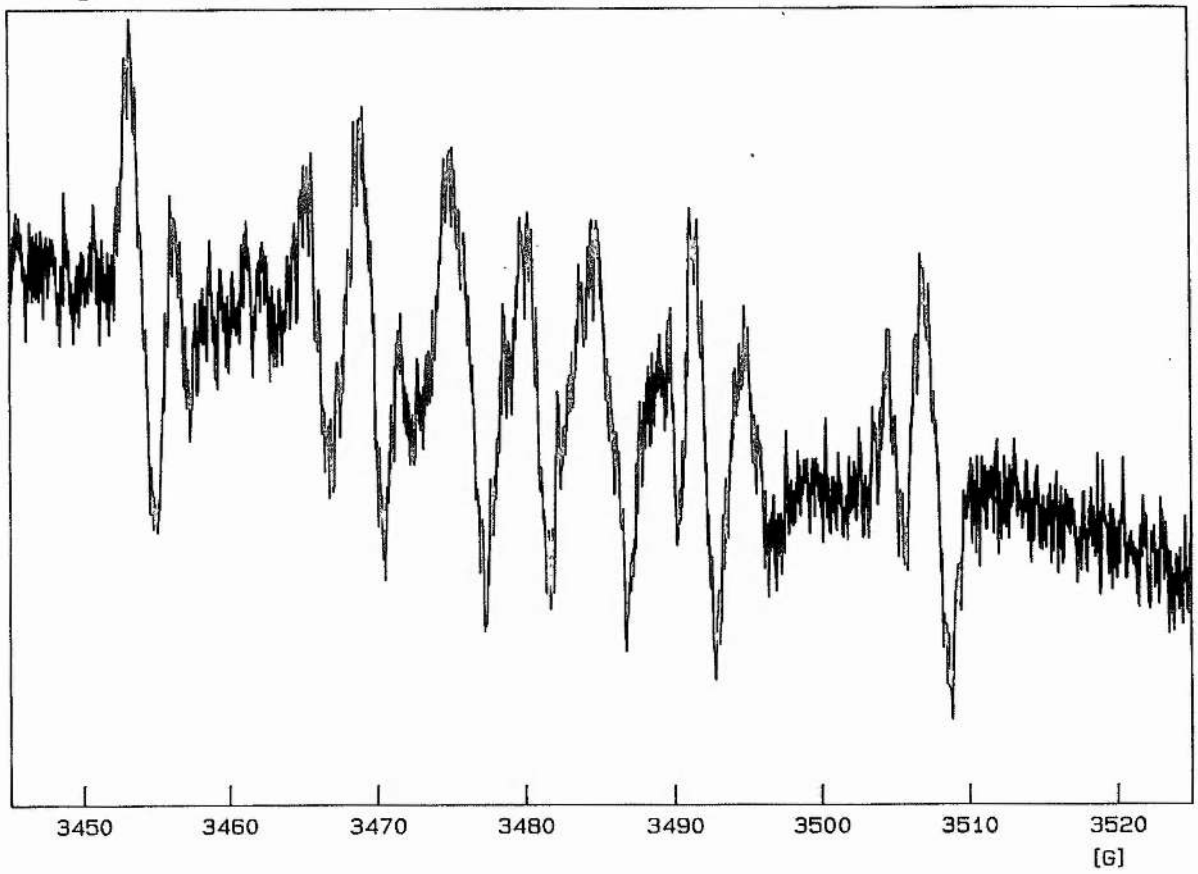
dmpo_2 2 mM Fe(II), 2 mM qinghaosu, 50 mM DMPO, MeCN:H_2O (1:1) after 15 mins



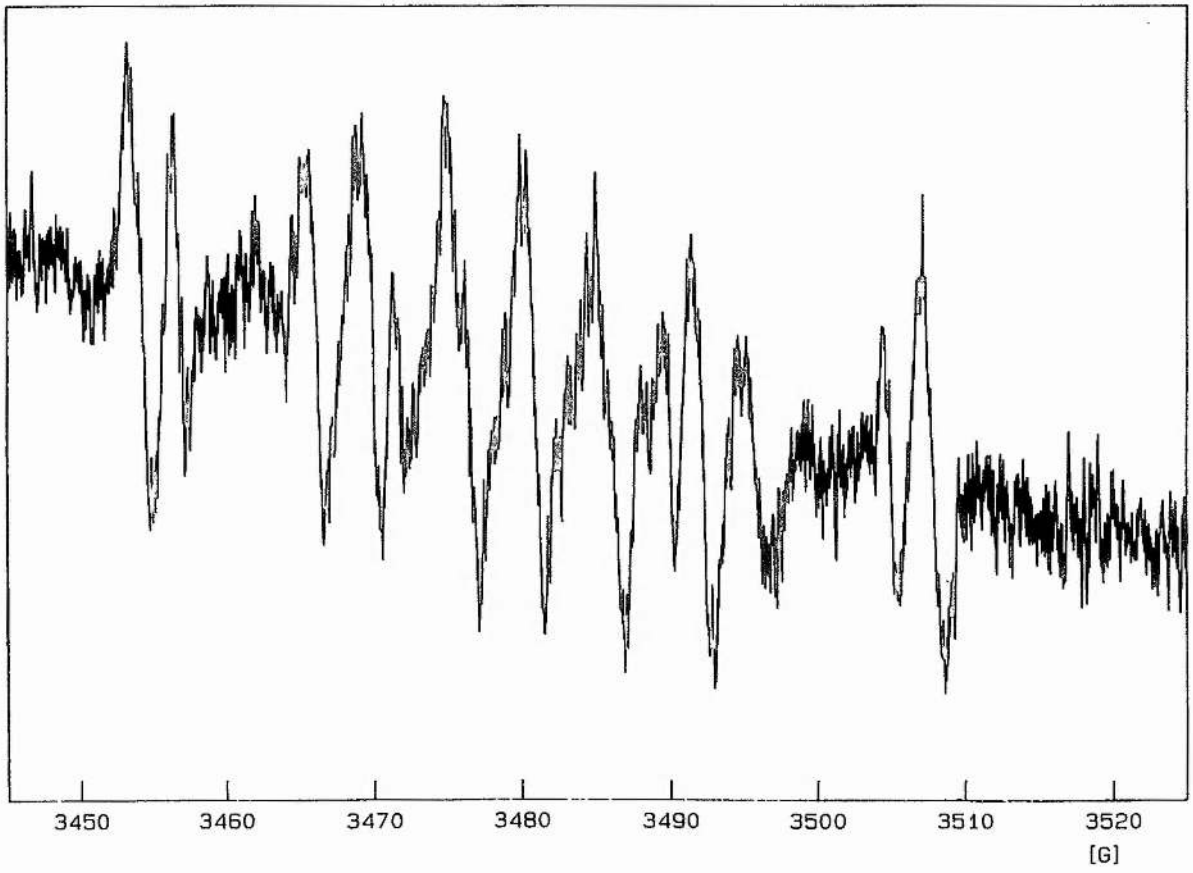
dmpo_3 2 mM Fe(II), 2 mM qinghaosu, 200 mM DMPO, MeCN:H_2O (1:1) after 2 mins



dmpo_4 2 mM Fe(II), 2 mM qinghaosu, 200 mM DMPO, MeCN:H_2O (1:1) after 20 mins



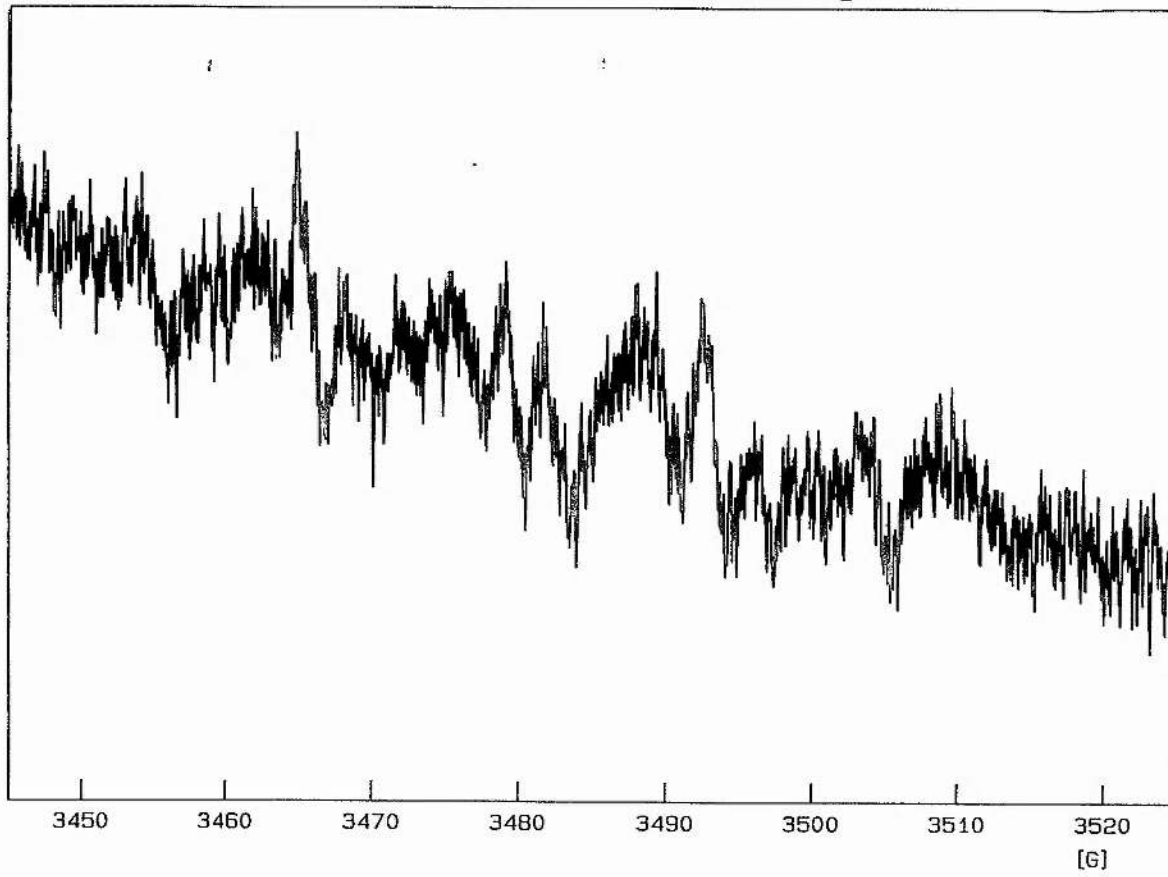
dmpo_5 2 mM Fe(II), 2 mM qinghaosu, 200 mM DMPO, MeCN:H_2O (1:1) after 40 mins



Appendix 1a

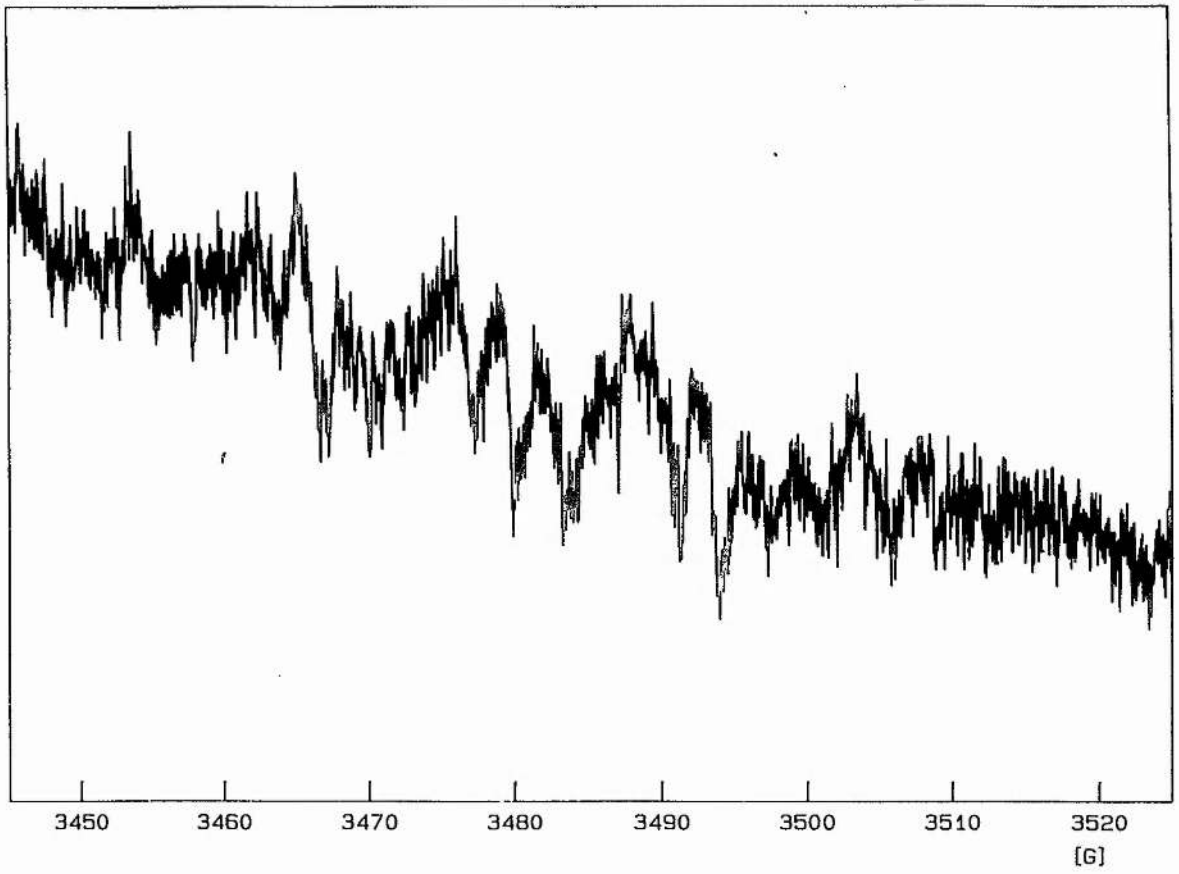
Spin-trapping with DBNBS

2 mM Fe(II), 2 mM qinghaosu, 2.5 mM DBNBS, MeCN:H_2O (1:1) after 1 min

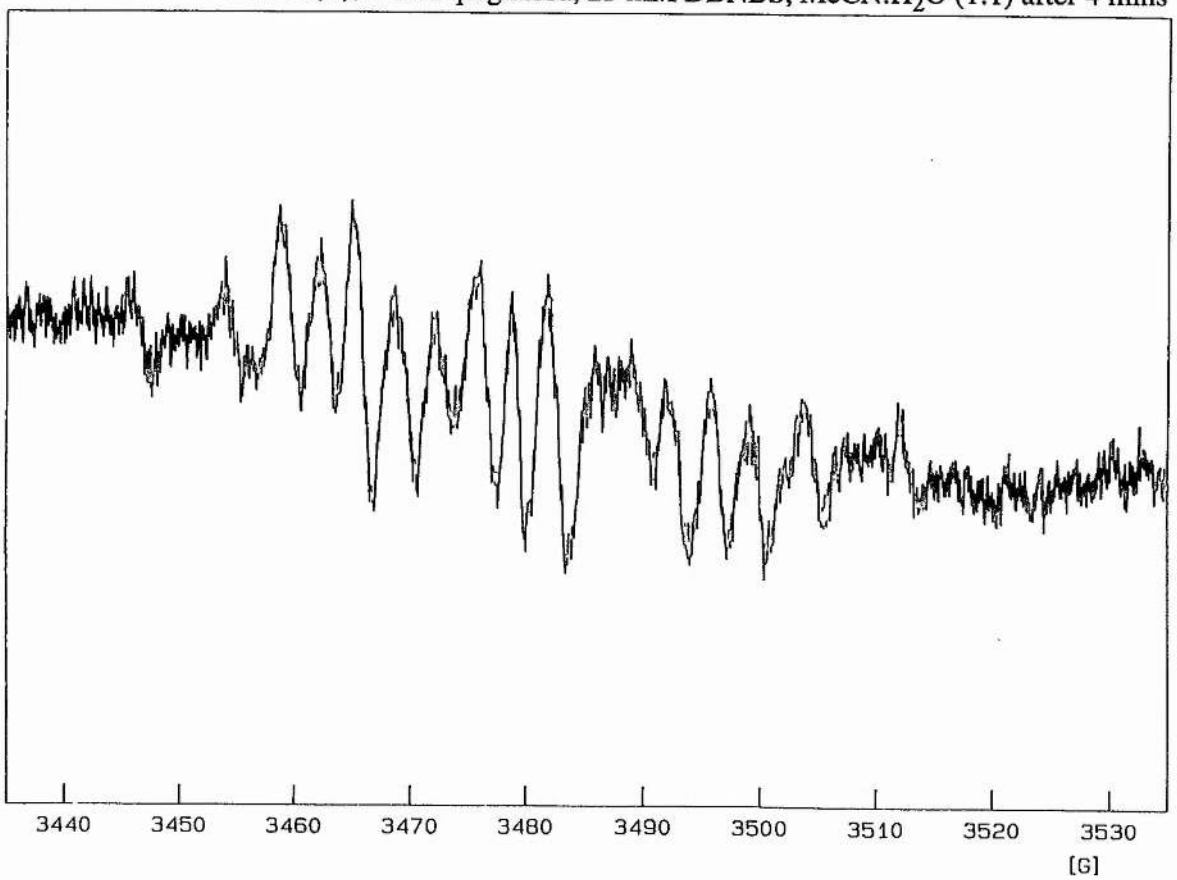


dbnbs_1

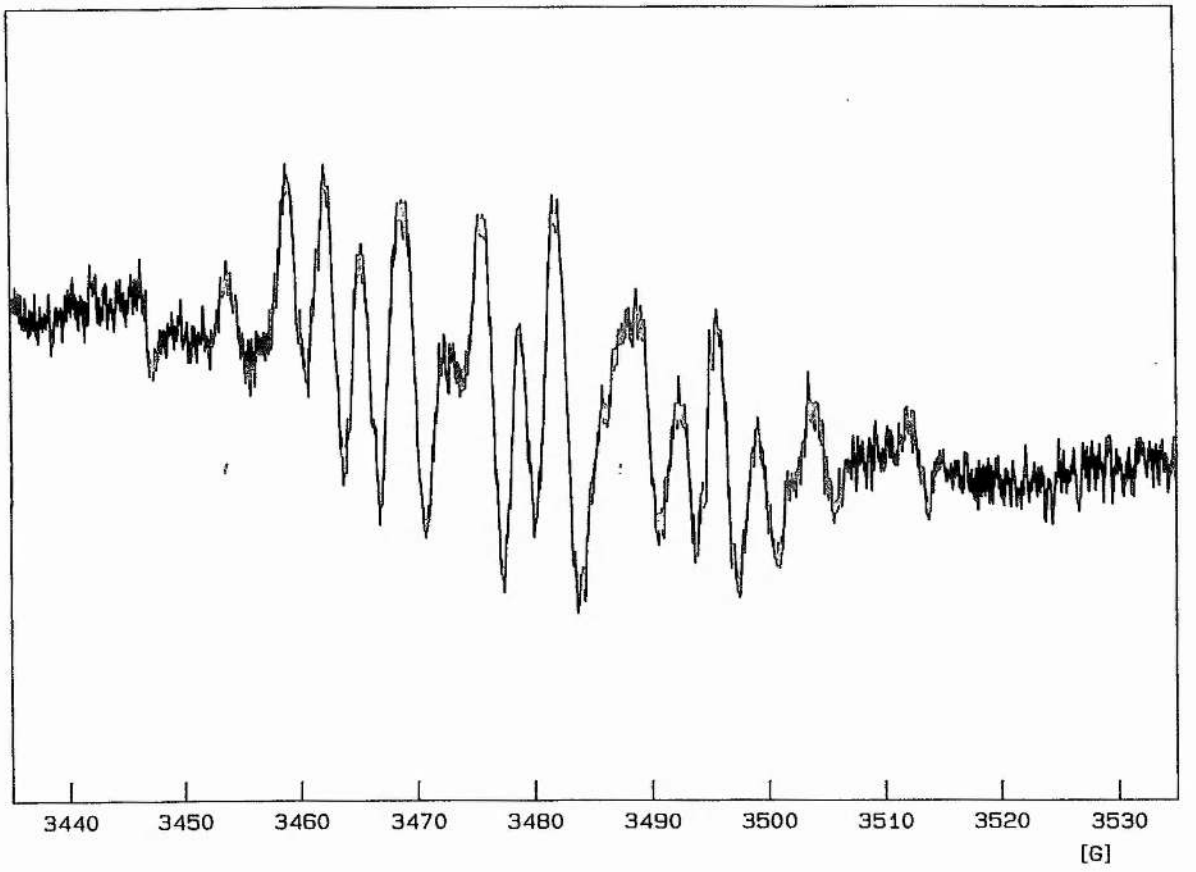
dbnbs_2 2 mM Fe(II), 2 mM qinghaosu, 25 mM DBNBS, MeCN:H_2O (1:1) after 1 min



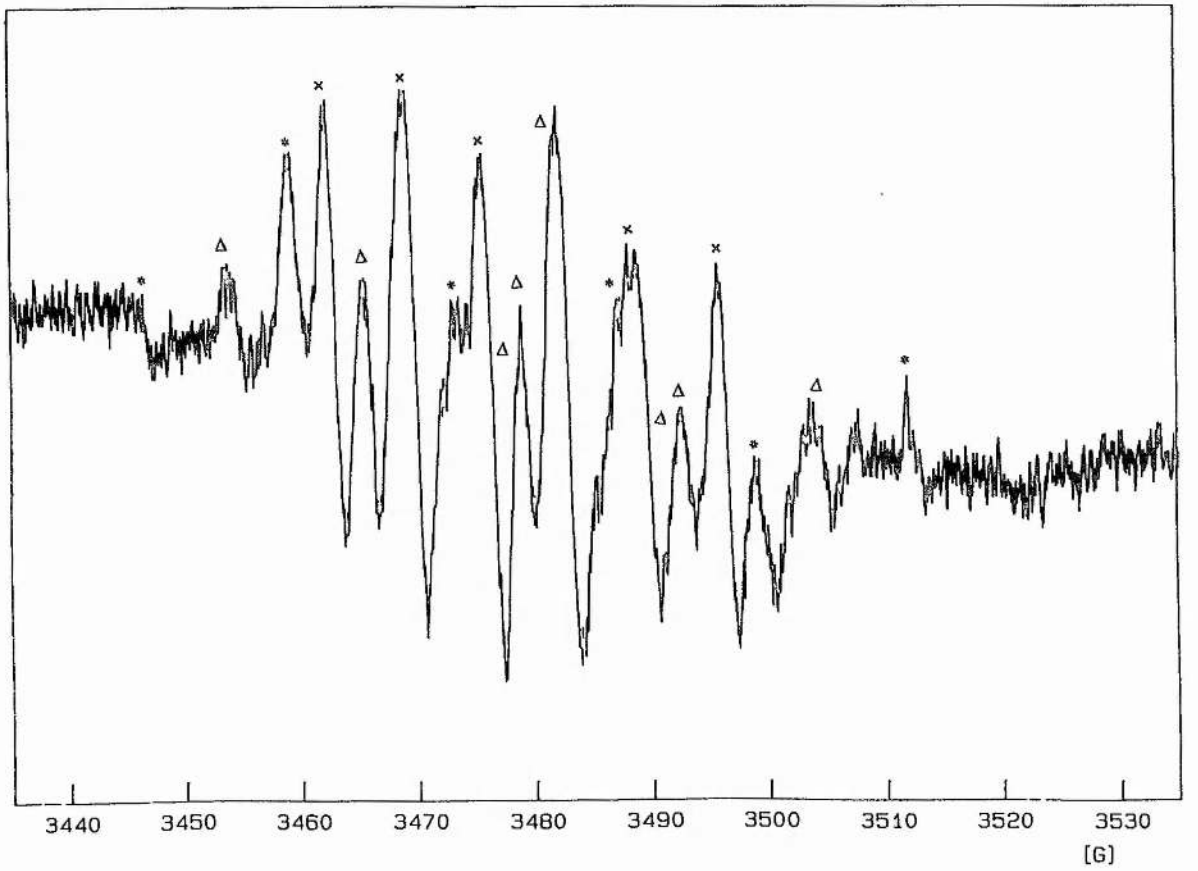
dbnbs_3 2 mM Fe(II), 2 mM qinghaosu, 25 mM DBNBS, MeCN:H_2O (1:1) after 4 mins



dbnbs_4 2 mM Fe(II), 2 mM qinghaosu, 25 mM DBNBS, MeCN:H_2O (1:1) after 20 mins



dbnbs_5 2 mM Fe(II), 2 mM qinghaosu, 25 mM DBNBS, MeCN:H_2O (1:1) after 35 mins



Appendix 2

Fluorescence Studies

Experiment 1

(ex. slit: 10, em. slit: 10)

	Intensity (I) at 350 nm
albumin (1.5×10^{-7} M)	110
albumin (1.5×10^{-7} M) + qinghaosu (0.1 g)	70
qinghaosu (0.1 g) + buffer	15
buffer only	20

Experiment 2

(ex. slit: 5, em. slit: 5)

		Intensity (I) at 350 nm
albumin (1.5×10^{-7} M) - solution B		590
albumin (1.5×10^{-7} M) + qhs (0.1 g) - solution A		350
qinghaosu (0.1 g) + buffer		100
buffer only		100
Solutions	8 cm^3 A : 2 cm^3 B	350
	2 cm^3 A : 8 cm^3 B	500

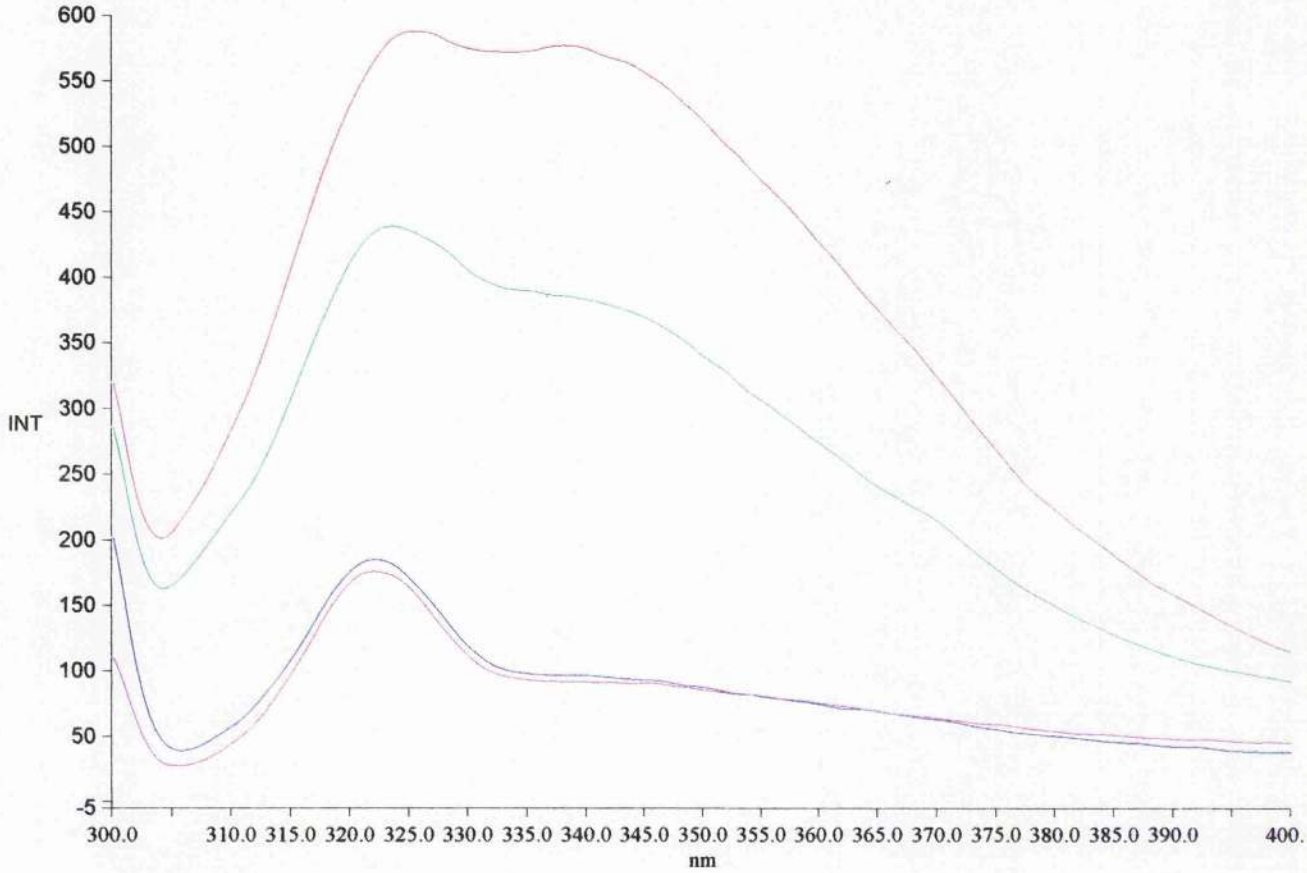
The solutions prepared between these extremes had very similar intensities.

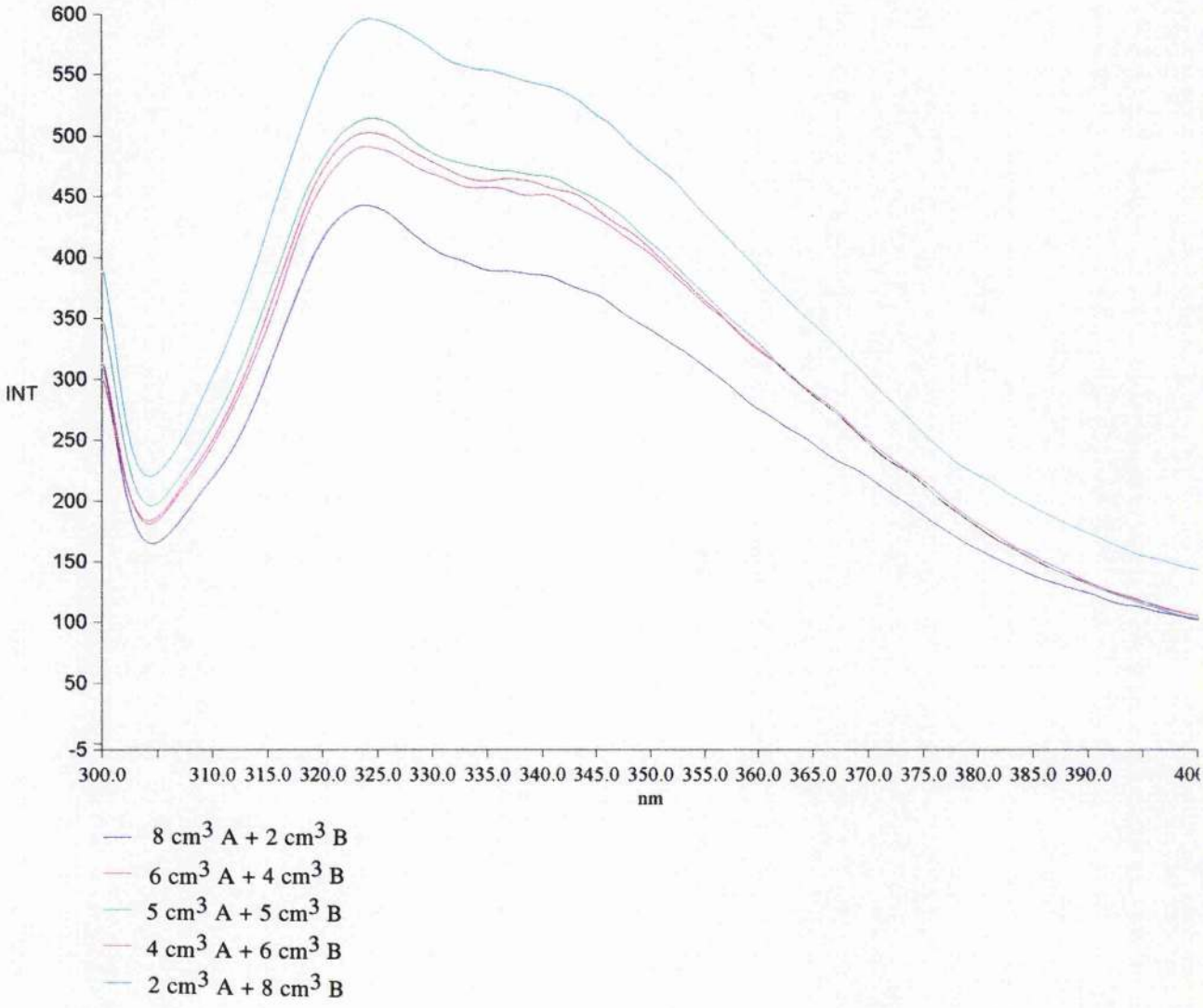
Experiment 3

(ex. slit: 10, em. slit: 10)

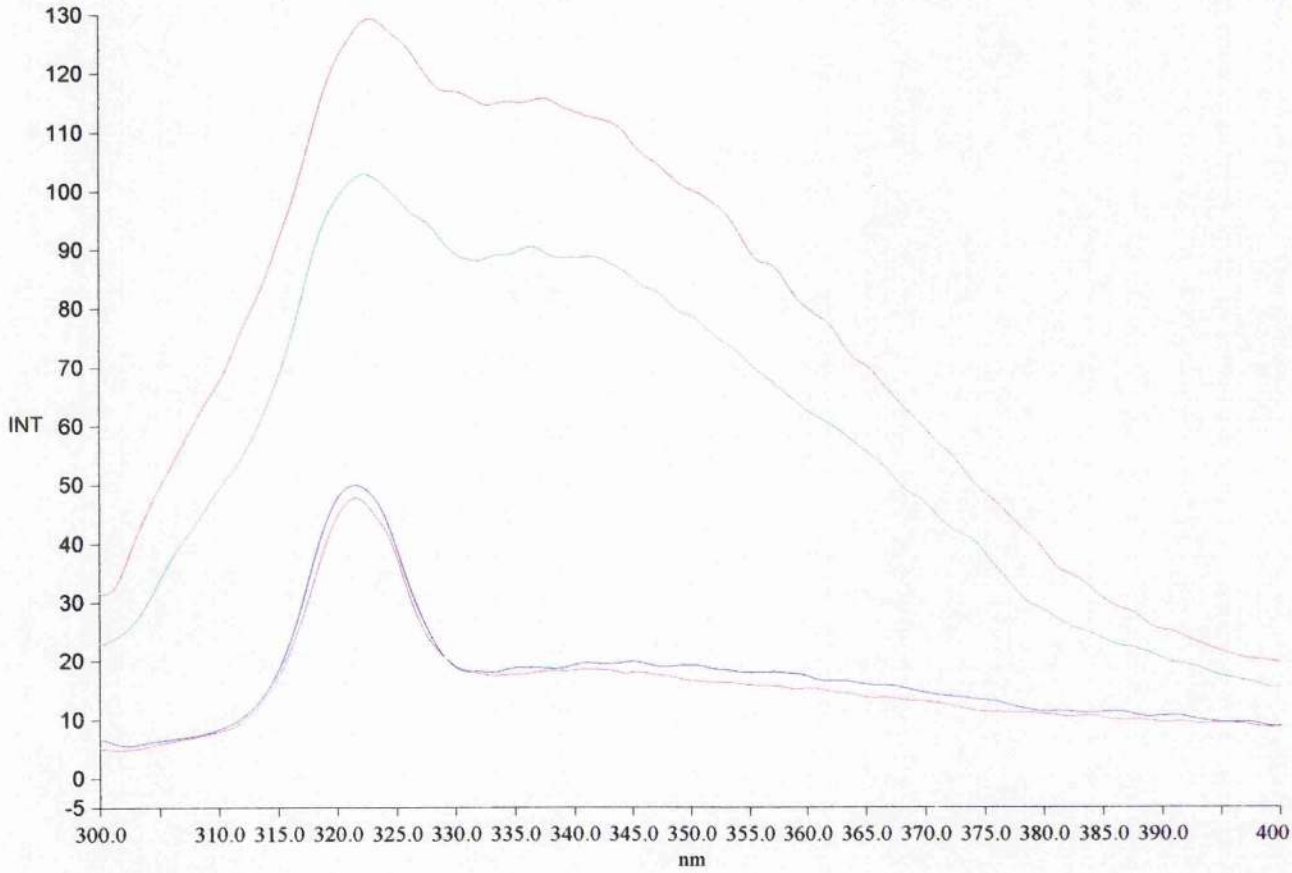
	Intensity (I) at 350 nm
albumin (1.5×10^{-7} M)	105
albumin (1.5×10^{-7} M) + qhs (0.1 g)	70 (**see experiment 1**)
albumin (1.5×10^{-7} M) + qhs (0.01 g)	75
buffer only	15
qinghaosu (0.01 g) + buffer	12

Experiment 2




600
550
500
450
400
350
300
250
200
150
100
50
-5
INT
300.0
310.0 315.0 320.0 325.0 330.0 335.0 340.0 345.0 350.0 355.0 360.0 365.0 370.0 375.0 380.0 385.0 390.0
nm
8 cm3 A + 2 cm3 B
6 cm3 A + 4 cm3 B
5 cm3 A + 5 cm3 B
4 cm3 A + 6 cm3 B
2 cm3 A + 8 cm3 B

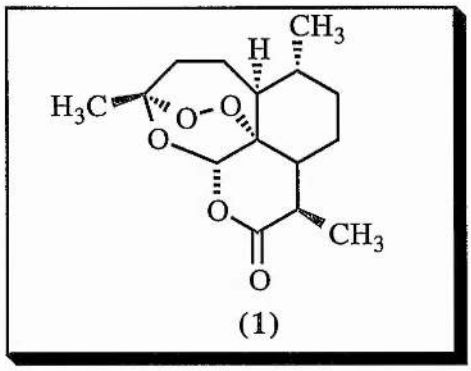
Experiment 3



Chapter 4

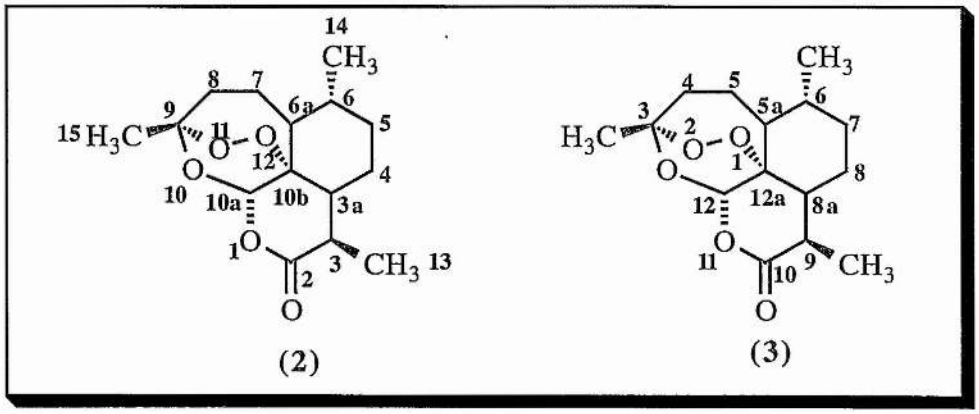
^{1}H and ^{13}C NMR Assignments of Arteether and a Comparison with those of Qinghaosu

4.1 Introduction



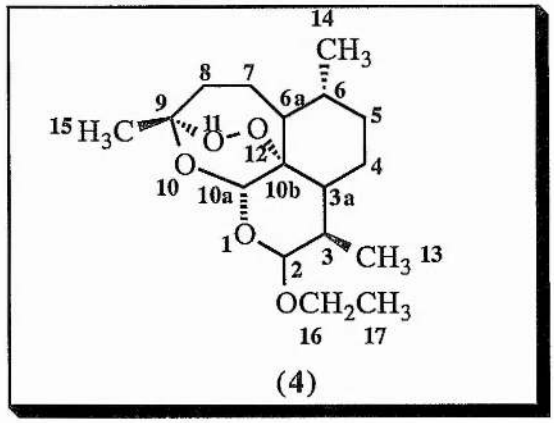
(1)

The structure of qinghaosu (**1**) was deduced using regular spectroscopic techniques and confirmed by X-ray crystallographic studies.[1] The correct naming of this compound and therefore the correct numbering, is a matter of some complexity. In a recent review,[2] the name 3,6,9-trimethyl-9,10b-epidioxyperhydropyrano[4,3,2-*jk*]benzoxepin-2-one was used. This conforms to IUPAC conventions and gives the numbering system indicated in (**2**). Several other numbering systems have been used and in most cases they are incorrect. However, in a paper by Cordell *et al.*[3] an alternative numbering system was used (**3**). This is also consistent with IUPAC conventions.



(2) (3)

Most previous NMR studies have used qinghaosu, therefore the ether derivative arteether (**4**) was selected for this study. The aim of which was to provide an unambiguous assignment of the NMR of (**4**). The numbering system shown in (**4**) is used throughout this chapter.



(4)

Qinghaosu has been the subject of several NMR spectroscopic studies. Three early studies[4] produced conflicting assignments for the ^{13}C NMR spectrum, which were made even more confusing by different numbering. A later study[5] used selective heteronuclear decoupling experiments for assigning the carbon signals, but the proton data were recorded without spectroscopic assignments of non-equivalent protons. A substantial study on qinghaosu by Cordell *et al.*,[6] used homonuclear COSY and 2 dimensional nOe (NOESY) measurements to assign the proton signals.

An interesting aspect of the current study is the comparison of the spectra of qinghaosu and arteether and highlighting the differences in chemical shift which arise. In addition, the effect of the ether group on the conformation of the ring and consequently the whole molecule was investigated. All experiments were performed at 500 MHz rather than the 300 MHz used by Cordell *et al.*, with the expectation that some overlapping multiplets may be more fully resolved.

4.2 Experimental

Spectra were obtained using a Varian Unity + 500 spectrometer, operating at 500.2 MHz (^{1}H) and 125.7 MHz (^{13}C). Standard pulse programs were used to generate the COSY and DEPT spectra.

4.3 Results and Discussion

The ^{1}H NMR spectrum (appendix; figure 1) showed, as expected, a number of multiplets of intensity 1H, most of which were sufficiently different in chemical shift to permit location. However, in the region δ1.44-1.60 there were a number of overlapping multiplets and assignments in this group were obviously impossible. Elsewhere in the spectrum there were a number of easily recognised signals where assignment was immediate and unambiguous.

In the homonuclear COSY spectrum (appendix; figure 2), some cross peaks were missing and justification of the assignments came only after examination of the heteronuclear COSY spectrum (appendix; figure 3). Coupling constants were obtained from the J-resolved spectrum (appendix; figure 4).

The only triplet of intensity 3H in the ^{1}H spectrum is at δ1.20, and this was assigned to C-17. In the 2D homonuclear spectrum this correlated with two signals at δ3.50 and δ3.90, which we assign to the protons at C-16. Both are quartets of doublets, indicating that the protons on the methylene group are non-equivalent and each quartet is further split by the proton at C-2.

One of the few simple doublets corresponding to one proton is at δ4.90 and to this chemical shift the proton at C-2 was assigned. It correlates with a multiplet at δ2.60 (proton at C-3) which in turn correlates with a doublet of 3 protons at δ0.95 (C-13) and a multiplet at δ1.24 (C-3a). By the same process the following multiplets were

assigned; δ1.75 and δ1.84 to the α- and β-protons at C-4 and the signals at δ1.50 and 1.64 to the α- and β-protons at C-5. The single proton at C-6 was assigned to a multiplet at δ1.40, while the methyl group at C-14 was assigned to a doublet at δ0.89, which was nicely separated from the methyl group at C-13 in an expanded version of the low field spectrum.

The multiplet of intensity 1H at δ1.36 was assigned to the proton at C-6a; unfortunately there are no cross peaks for this signal. However, it was assumed that the signals for α- and β-protons at C-7 are two overlapping multiplets around δ1.50. From this signal there are strong cross peaks at δ2.05 and δ2.38 to which the α- and β-protons at C-8 were assigned. An increased chemical shift is consistent for protons which are closer to the oxygen of the ring.

The J-resolved 2D spectrum showed an interesting splitting pattern for these two signals. One is a triplet of doublets and the other a doublet of triplets. If it is assumed that geminal coupling makes each a doublet, then trans coupling to one of the protons at C-6 will create two double doublets. With overlapping of the two inner lines these could appear as two triplets and coupling to the other proton at C-6 would split each line of the triplet into a doublet.

A relayed-COSY experiment was performed, however no additional information was obtained from this.

Table 1

^{1}H NMR Spectral Assignments for Arteether

Proton	Chemical Shift (δ)	Multiplicity	Coupling Constant (Hz)
2	4.90	d	3
3	2.60	m	3.5
3a	1.24	m	-
4	1.75	m	-
	1.84	m	-
5	1.50	m	-
	1.64	m	-
6	1.40	m	-
6a	1.36	m	-
7	1.50	m	-
	1.54	m	-
8	2.05	td	15, 4.5
	2.38	dt	14, 4
10a	5.40	s	-
13	0.95	d	6.2
14	0.89	d	7.5
15	1.44	s	-
16	3.50	qd	7.5, 10.5
	3.90	qd	7.5, 10.5
17	1.20	t	7.5

The ^{13}C NMR spectrum is altogether simpler and the 2D heteronuclear spectrum was used to confirm the assignments made in table 1. The signals are listed in table 2. The distinction between quaternary, CH, CH_2 and CH_3 was made by means of the DEPT pulse sequence.

Table 2

^{13}C NMR Assignments for Arteether

Carbon	Chemical Shift (δ)	Assignment in DEPT
2	101	CH
3	31.5	CH
3a	53	CH
4	24.5	CH_2
5	35	CH_2
6	45	CH
6a	37.1	CH
7	25	CH_2
8	36.4	CH_2
9	104	C
10a	88	CH
10b	87	C
13	20	CH_3
14	13	CH_3
15	26	CH_3
16	64	CH_2
17	16	CH_3

The signal at δ24.5 has a cross peak with a signal at δ1.75-1.84 and was therefore assigned to C-4, while that at δ25.0 correlates with a signal at δ1.55 and was assigned to C-7. From the cross peak the signal at δ31.5 is clearly C-3. Likewise the signal at δ35 is C-5. The signal at δ36.4 is correlates with two signals in the ^{1}H NMR spectrum at δ2.05 and δ2.38 and was assigned to C-8. The signal at δ37.1 was assigned to C-6a while that at δ45 to C-6 and δ53 to C-3a. The signals at δ64 correlate with two signals in the ^{1}H NMR spectrum at δ3.50 and δ3.90 and is therefore C-16. The remaining signals at δ88 and δ101 were assigned to C-10a and C-2 respectively.

The chemical shifts for all the protons and carbon atoms in arteether have now been unambiguously assigned, although little progress has been made in disentangling the α- and β-protons at positions 4,5,7 and 8. There is an expected large difference in the chemical shifts of the α- and β-protons at C-8. This is probably due to the proximity of the ring oxygen.

Inspection of a molecular model showed that in both methylene groups (C4/5 and C7/8) the protons are staggered but the proximity of the ring oxygen does set C-8 apart from the others.

The assignments made were compared with those of Cordell *et al.* for qinghaosu (table 3). There are a number of differences within the ^{13}C NMR spectrum but nothing of great significance. However, within the ^{1}H NMR spectrum our assignments show that for C-4, C-5 and C-7 the α- and β-protons have very similar chemical shifts but Cordell *et al.* report differences ranging from δ0.75 to δ0.53. A difference of δ0.33 was found for C-8 and this was explained previously.

For C-8 (C-4 in their numbering) Cordell *et al.* find the smallest difference between the α- and β-protons. It is likely that the differences between the sets of data is due to the fact that in arteether there is an ether group at the same position as a carbonyl group in qinghaosu. Therefore there will be changes in the conformation of the ring to which these groups are attached and a smaller effect on the conformation of the other rings.

Evidence for this argument was obtained when potential changes in conformation were determined by comparison of the X-ray structures of qinghaosu[7] and artemether.[8] Artemether was used as a model for arteether as its coordinates were readily available in the literature. It contains the $-OCH_3$ group as opposed to $-OCH_2CH_3$ in arteether.

Artemether and qinghaosu have different crystal structures. Artemether has a monoclinic crystal structure with space group $P2_1$. Its unit cell parameters are a = 9.868, b = 18.324 and c = 10.172. In comparison, qinghaosu has an orthorhombic crystal structure with space group $P2_1P2_1P2_1$ and unit cell parameters a = 24.077, b = 9.443 and c = 6.356.

X-ray structures (appendix; figure 5) show that the six membered ring containing the carbonyl group in qinghaosu and that containing the ether group in artemether are different. This was confirmed by comparison of torsion or conformation angles (table 4). Most of the measured conformation angles in this ring differ significantly for qinghaosu and artemether, while only slight differences were observed for the remainder of the molecule. In addition, computer generated molecular models of qinghaosu were produced and observed at various angles (appendix; figure 5).

Table 3

A Comparison of our Data with those of Cordell *et al.*

Cordell's Numbering	Chemical Shift (δ)		Our Numbering	Chemical Shift (δ)	
	H	C		H	C
9	3.40	32.8	3	2.60	31.5
8a	1.75	44.8	3a	1.24	53.0
8	1.87	23.3	4	1.75	24.5
	1.12			1.84	
7	1.08	33.4	5	1.50	35.0
	1.79			1.64	
6	1.42	37.4	6	1.40	26.0
5a	1.37	49.9	6a	1.36	37.5
5	1.47	24.8	7	1.50	25.0
4	2.05	35.8	8	2.05	36.5
	2.43			2.38	
3	-	105.2	9	-	104.0
12	5.9	93.6	10a	5.4	88.0

Table 4

Comparison of Torsion (Conformation) Angles of Qinghaosu and Artemether

				qinghaosu	artemether
(1)	(2)	(3)	(4)	(angle)	(angle)
O(1)	C(2)	C(3)	C(3a)	-30	51
O(1)	C(2)	C(3)	C(13)	-158	-177
O(1)	C(10a)	C(10b)	C(3a)	44	-55
O(5)	C(2)	O(1)	C(10a)	-170	69
O(5)	C(2)	C(3)	C(3a)	156	-74
O(5)	C(2)	C(3)	C(13)	28	57
C(2)	C(1)	C(10a)	C(10b)	-23	55
C(10b)	C(3a)	C(3)	C(13)	178	177
C(10b)	C(10a)	O(1)	C(2)	-25	55
C(10a)	O(1)	C(2)	C(3)	16	-54
C(10a)	C(10b)	C(3a)	C(3)	-58	51

At the conclusion of this study there was brought to our attention a paper by Prakash *et al.*,[9] which investigated the 2D spectral assignments of arteether at 400 MHz. The majority of assignments made were in agreement with those for qinghaosu, published by Cordell *et al.* [6] Criticism of this paper by Misra *et al.* [10] altered assignments to further coincide with those for qinghaosu.

However, from the evidence presented in this study the spectral assignments of arteether differ from those of qinghaosu. Evidence from spectroscopic studies was strengthened by the determination of differences in conformation angles when qinghaosu containing C=O (sp^2) and artemether containing C-OCH_3 (sp^3) were compared. It has been shown that qinghaosu and artemether have different crystal structures, space groups, cell parameters and conformation angles. Therefore differences in chemical shift values for qinghaosu and arteether would not be unexpected and were in fact observed.

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Appendices

Figure 1

^{1}H NMR of Arteether

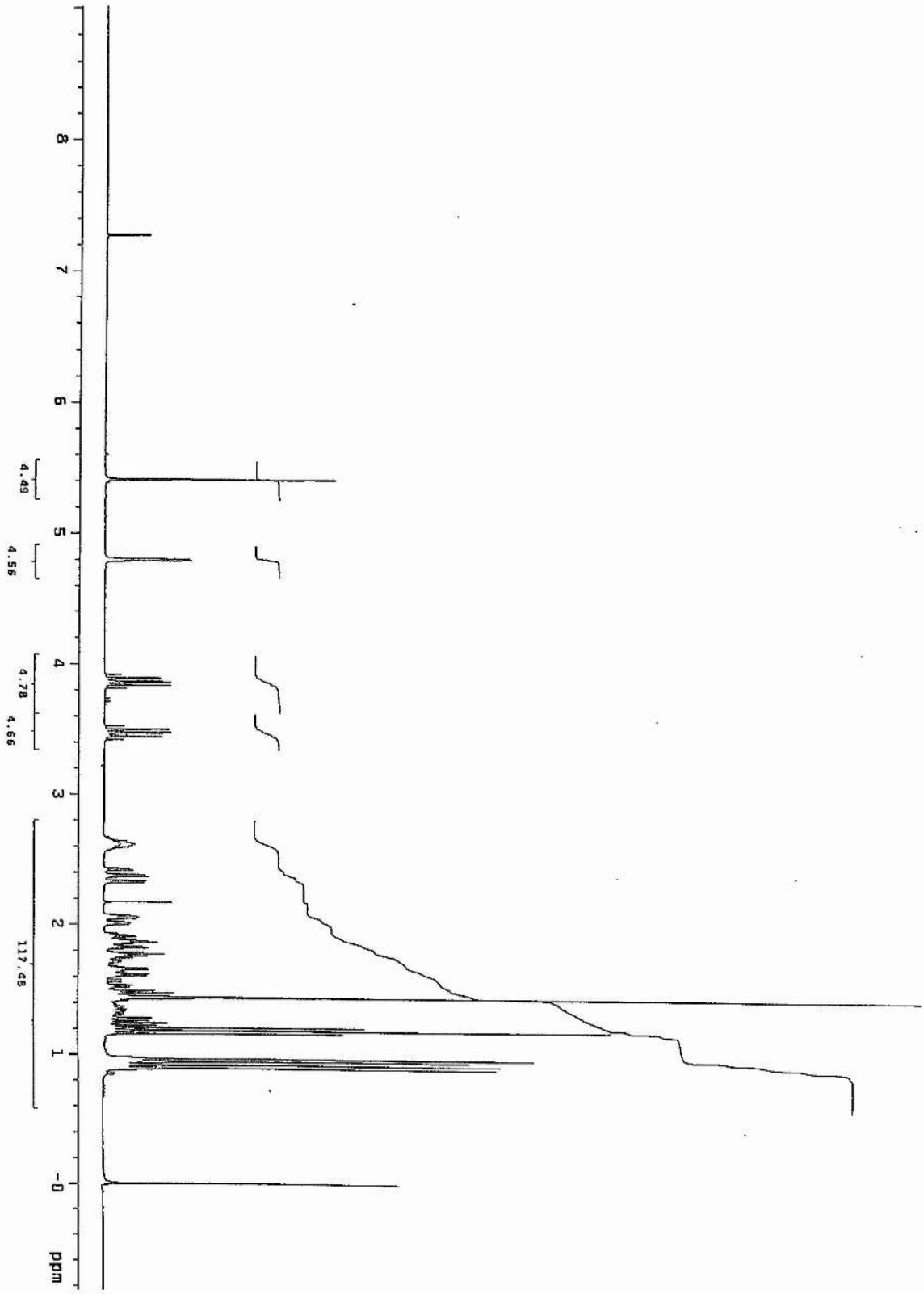


Figure 2

Homonuclear COSY Spectrum of Arteether

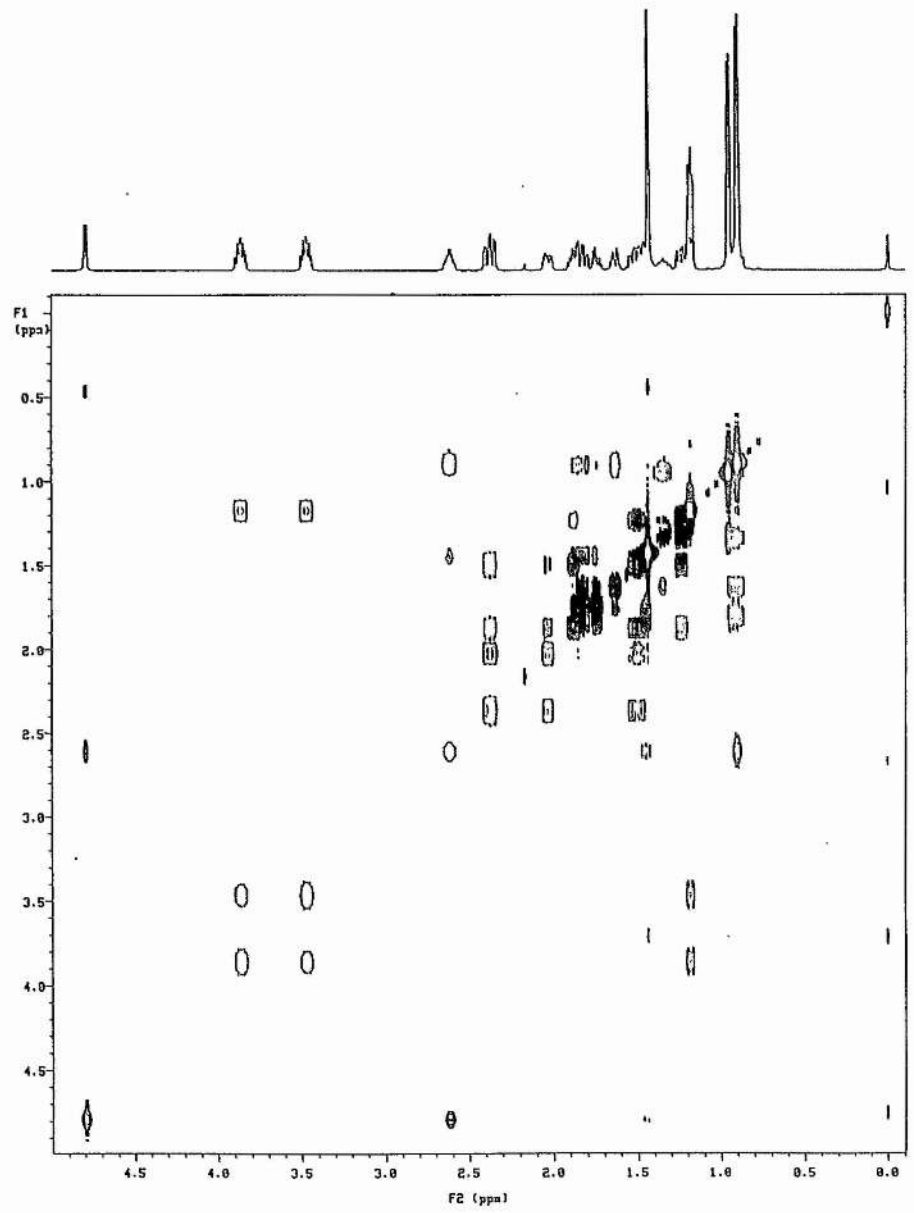


Figure 3

Heteronuclear COSY Spectrum of Arteether

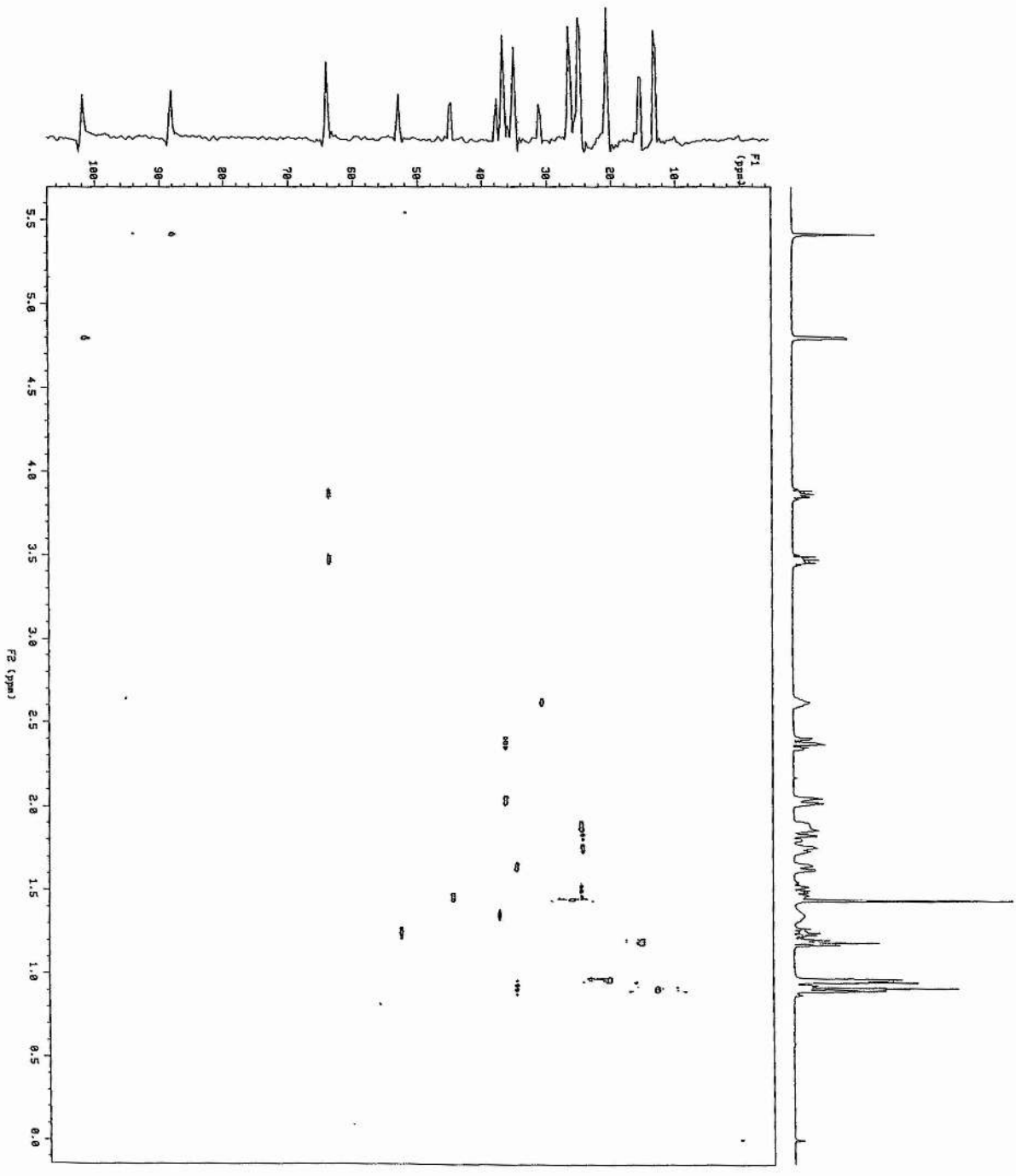


Figure 3

(Expansion)

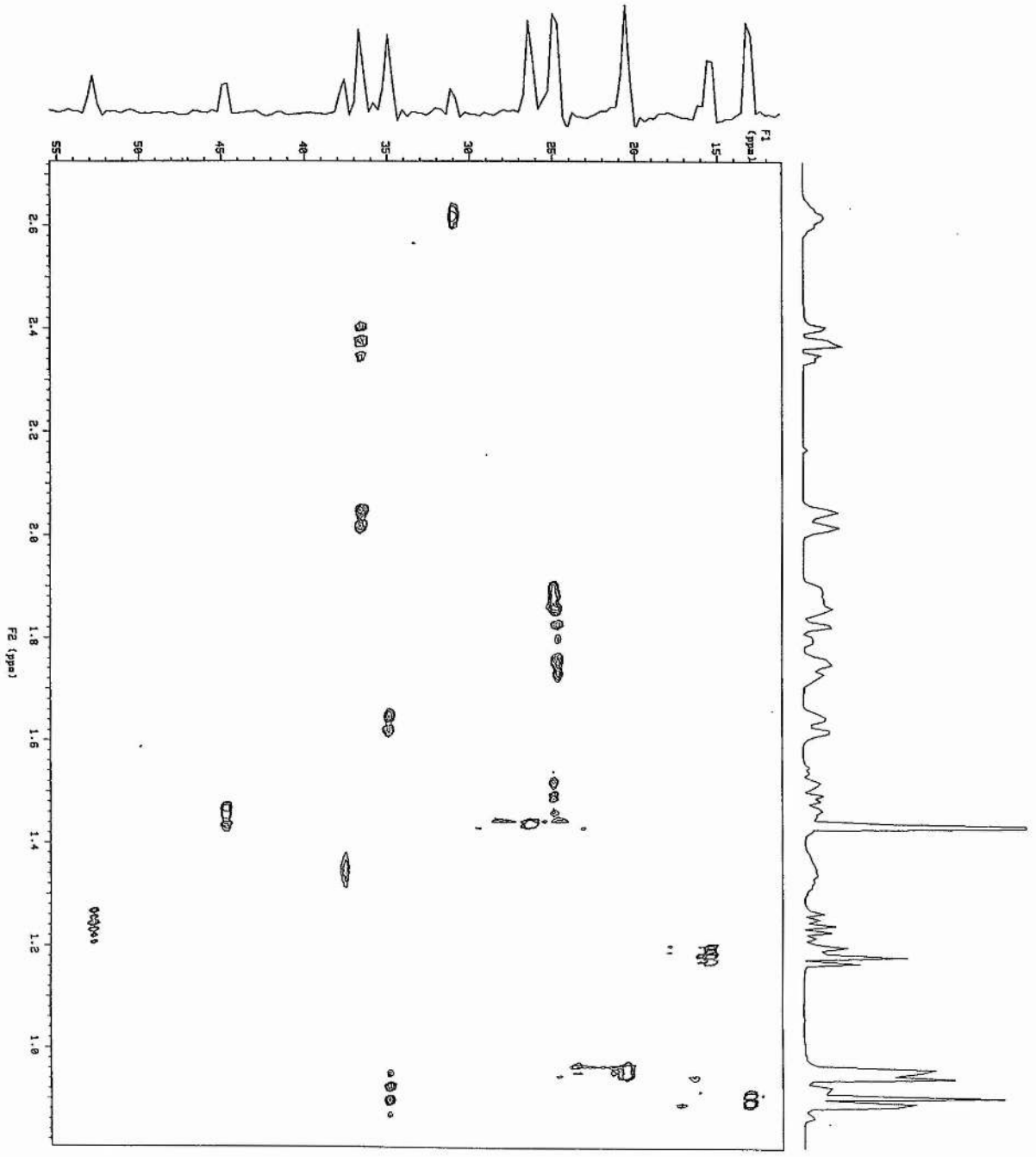


Figure 4

J-resolved Spectrum of Arteether

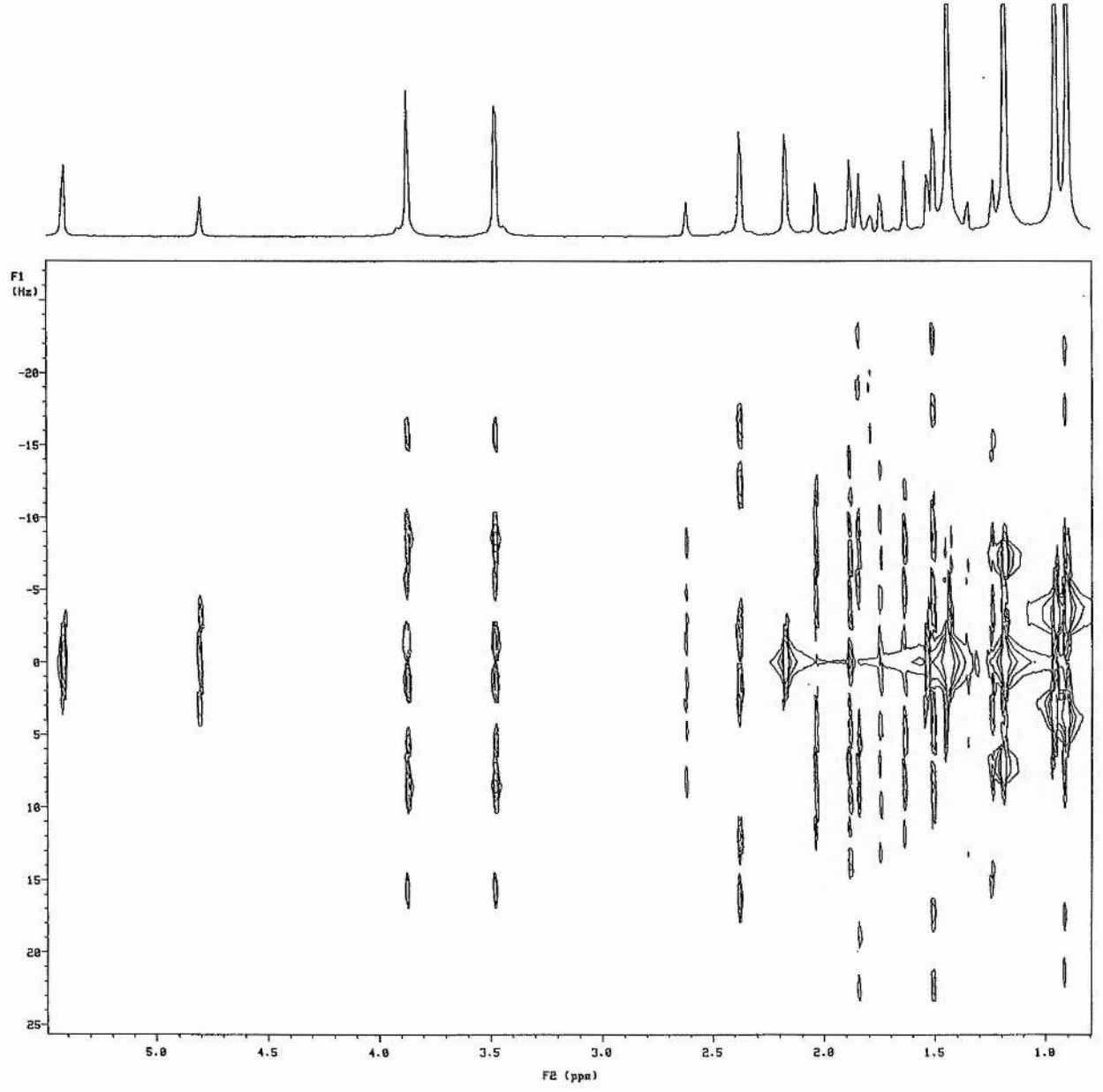
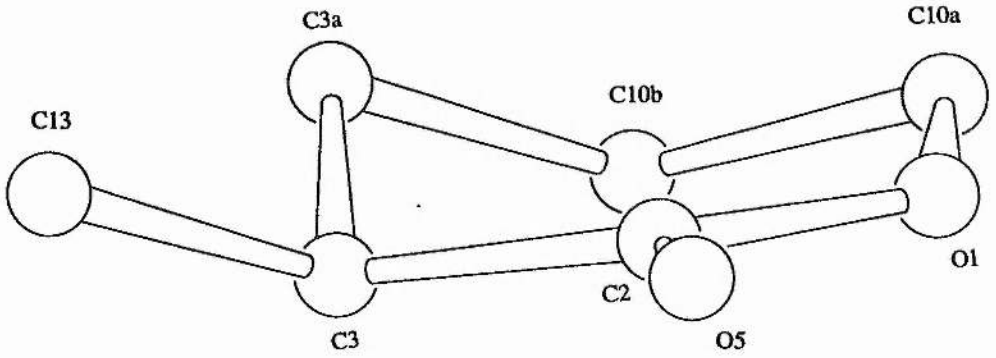


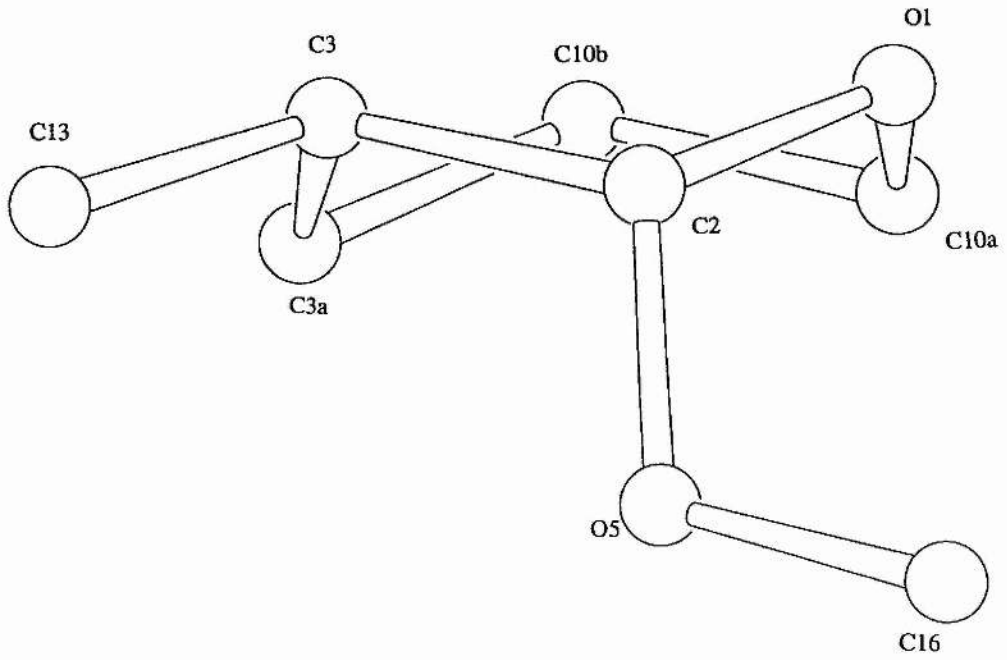
Figure 5

X-Ray Structures of Qinghaosu and Artemether

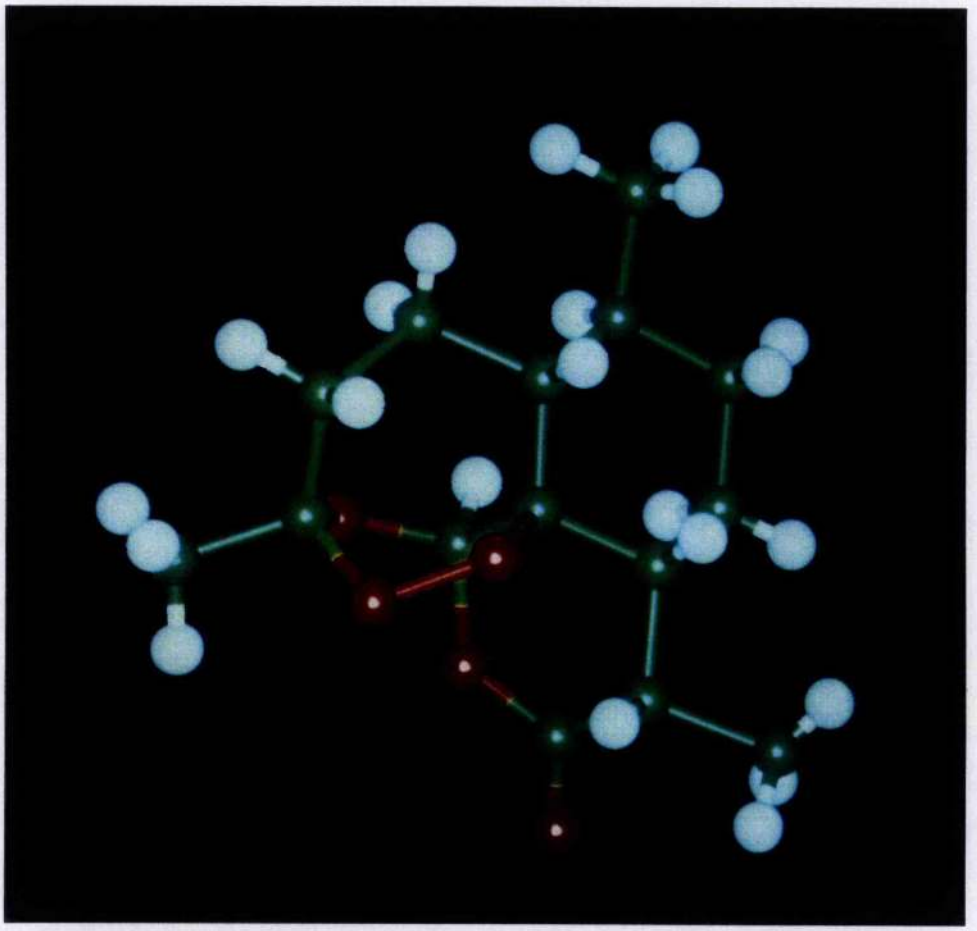
Qinghaosu

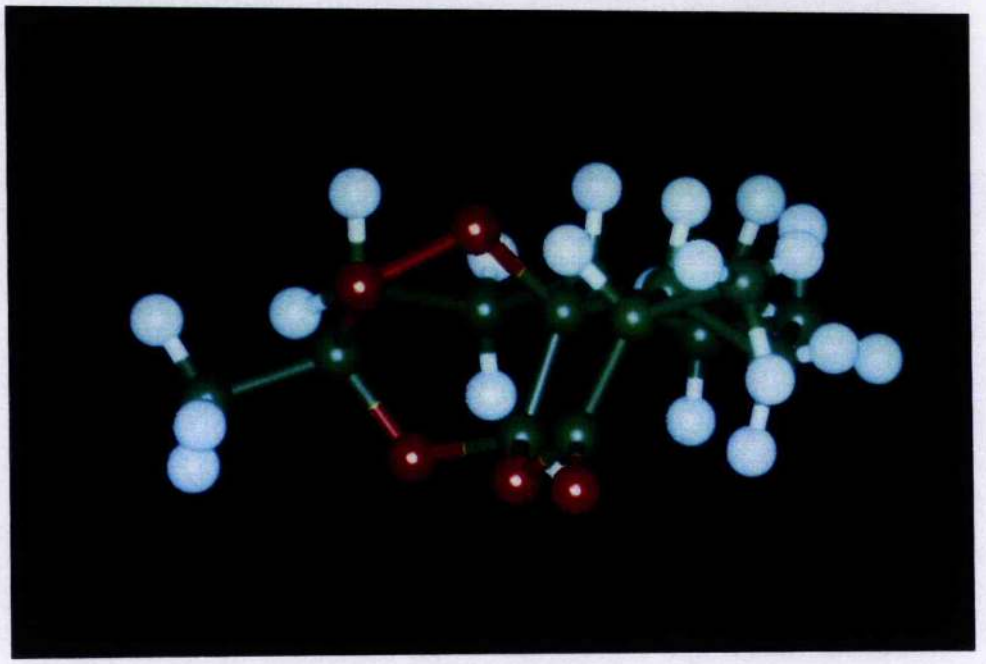


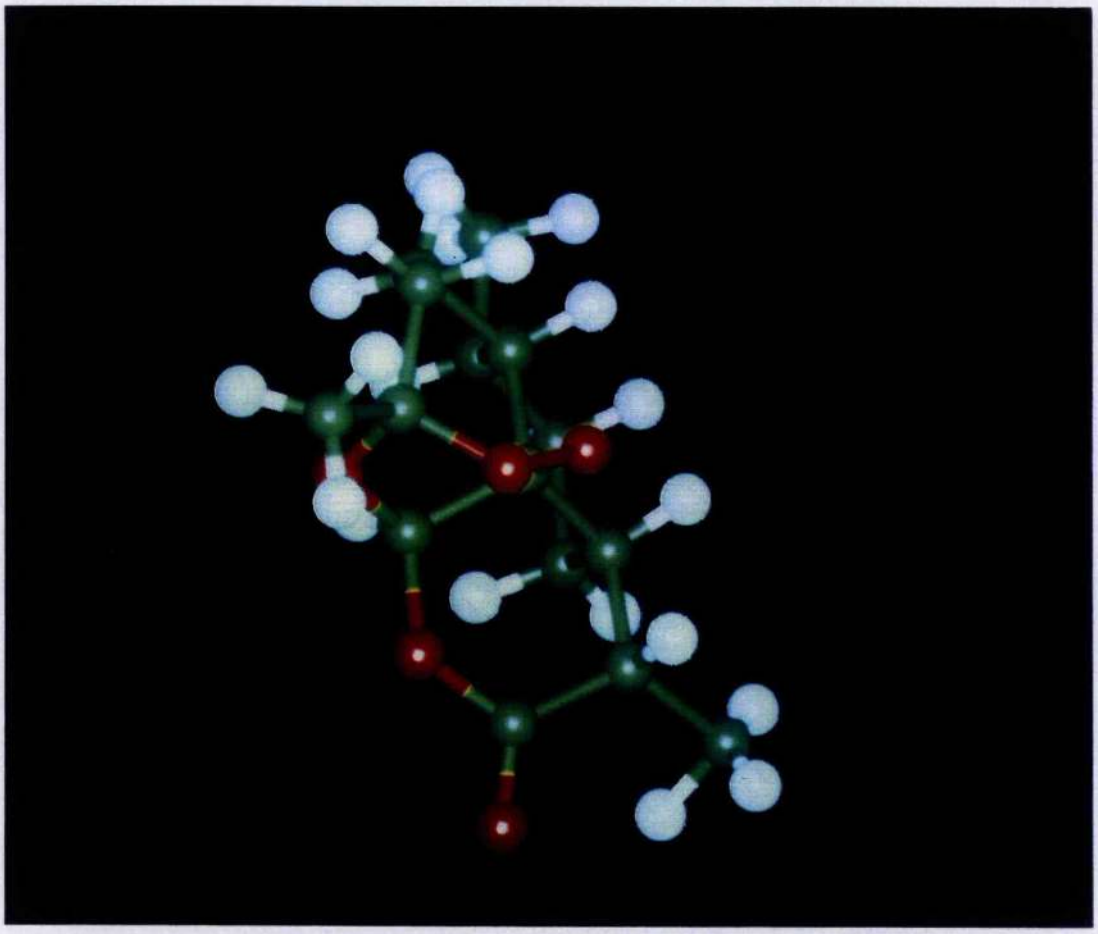
Artemether

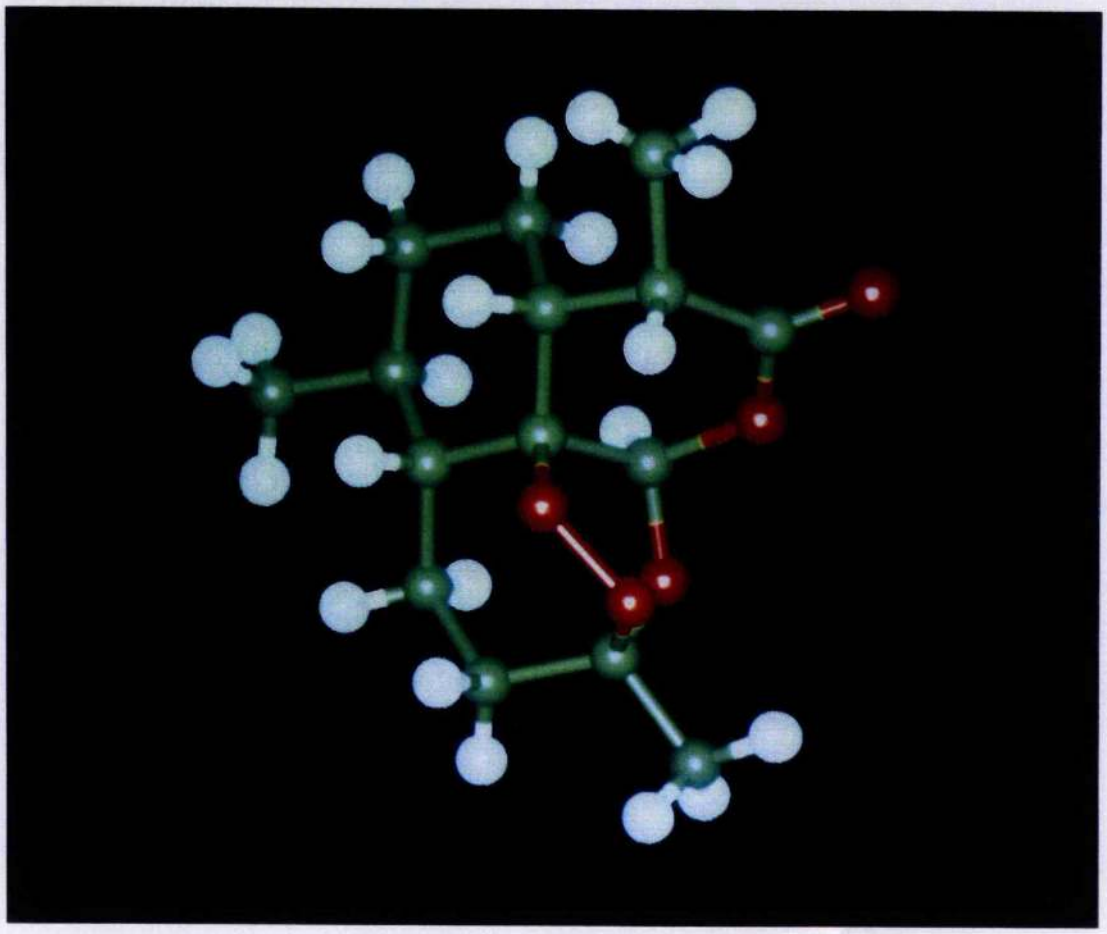


Molecular Models of Qinghaosu









Chapter 5

Nitric Oxide and the Immune System

5.1 Introduction

Nitric oxide (NO) and/or NO related compounds are involved in ridding the body of unwanted microbes. Therefore it is important to understand the chemistry of these compounds. Nitric oxide (NO, nitrogen monoxide) is a colourless, paramagnetic and relatively stable gas. The electronic structure of nitric oxide may be considered advantageously in the molecular orbital (MO) theory. NO contains one more electron than dinitrogen and one less electron than dioxygen and, according to simple MO theory, this extra single electron occupies an antibonding π MO. Removal of this electron is not too difficult, and indeed the ionisation potential (9.5 eV) is somewhat lower than in other diatomic molecules such as CO and N_2 (14.5 eV). However, NO is not very reactive chemically. As an oxidant the electrode potential of NO is not very high (E = +1.18V for NO/N_2O) and as a reducing agent the electrode potential is not very low (E = +0.35V for NO_2^-/NO).

NO has a short physiological half-life (between 6 and 30 seconds). However, this cannot be due to oxidation by molecular oxygen as reaction with oxygen in aqueous solution is much slower than the loss of NO over this time period. However, there is strong evidence that the only product obtained in these circumstances is nitrite. It appears that NO reacts rapidly with NO_2 as it is formed to give N_2O_3, the anhydride of nitrous acid as shown in the following reactions

$$NO + {}^1/_2O_2 \longrightarrow NO_2$$

$$NO_2 + NO \longrightarrow N_2O_3$$

$$N_2O_3 + H_2O \longrightarrow 2HNO_2$$

Whereas in the gas phase the following reactions occur;

$$2NO + O_2 \longrightarrow 2NO_2 \rightleftharpoons N_2O_4$$

$$N_2O_4 + H_2O \longrightarrow HNO_2 + HNO_3$$

The chemistry of the formation and reactivity of nitrosothiols, various nitrosyl complexes and its redox related species NO^+ (the nitrosonium cation) and NO^- (the nitroxyl anion) is central to an understanding of the biology of NO.[1]

NO contains N(II), the nitroxyl ion N(I) and the nitrosonium ion N(III) and each of them has a distinctive chemistry unique to itself. NO tends to react rapidly with other atoms or molecules that also contain unpaired electrons.

Table 1

Characteristics of NO and its redox forms

species	oxidation number	bond order	bond length(Å)	N-O stretching freq (cm^{-1})	unpaired electrons
NO	2+	2.5	1.15	1840	1
NO^+	3+	3	0.95	2300	0 (diamagnetic)
NO^-	1+	2	1.26	1290	2 (triplet) or 0 (singlet)

The formation of nitrosyl complexes from the reaction of NO with haem proteins, such as guanylate cyclase or non-haem iron-sulphur centres is very important in the biological chemistry of NO. NO is reactive towards metal complexes forming a wide range of nitrosyl complexes. The reaction of NO with a metal-bearing protein is intriguing because it can have either of two opposing effects. In some cases NO can activate the protein, for example by triggering the catalytic effects of an enzyme. In other instances, however, NO binds to the metal at the protein's active site, preventing it from carrying out its normal function. It is not surprising therefore, that the biological chemistry of NO is intimately associated with iron proteins.[2]

5.1.1 Antibacterial Activity

Viruses, mycobacteria, protozoans, helminths, bacteria and fungi are among a growing list of microbes that are susceptible to nitric oxide. In order to establish the antimicrobial activity of nitric oxide donor compounds the bacterium *Escherichia coli* was selected, one reason being that parasites are difficult to culture. An attraction of working with *E. coli* is that it represents a simplified system. The possibility of harvesting a large number of cells in a short time and the advantage offered by a haploid organism containing only one chromosome and which can double every 20 minutes have prompted many investigators to use *E. coli*.

Growing bacteria, dividing by binary fission, exhibit exponential or logarithmic growth kinetics until a point of saturation of the culture is reached. During this time the increase in the number of bacteria (N) per unit time (t) is proportional to the number of bacteria present in the culture.

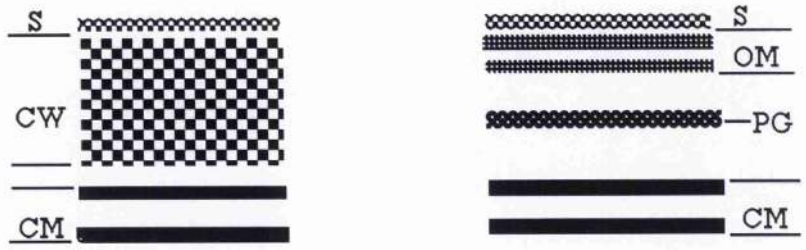
$$\mathbf{dN/dt = kN} \quad \text{where k is a growth constant}$$

E. coli can grow on a simple chemical medium in which glucose is the carbon and energy source, provided the medium is buffered at a pH near 7 and contains magnesium, phosphate and a nitrogen source (ammonium chloride or ammonium sulphate). However, strains of *E. coli* grow more rapidly in a rich broth because it supplies many of the compounds which the cell would otherwise have to synthesise. *E. coli* is capable of growing either in the presence of air (aerobically) or in its absence (anaerobically). However the growth rate in the absence of air is significantly poorer than that achieved with good aeration.[3]

Cell Walls and the Associated Surface Layers

E. coli is defined as a gram-negative bacterium. The cell wall is responsible for the gram staining reaction. The property of being coloured dark violet or not, by the staining procedure developed by Gram in 1884 is an important taxonomic feature which correlates with many other properties of bacteria. The cell wall may be a barrier to the delivery of toxic species to the cell. It is a unique structure which contains murein, also known as peptidoglycan. This is composed of a backbone of alternately repeating units of the amino sugars N-acetylglucosamine and N-acetylmuramic acid. The cell wall is very important because it protects the cell against osmotic shock. Without a cell wall cells would burst from the osmotic pressure exerted on their cytoplasm membranes as these organisms normally exist in dilute aqueous environments.

The cell wall of gram-positive bacteria such as *Clostridium sporogenes* has a peptidoglycan layer that is relatively thick and comprises approximately 90% of the cell wall. This layer acts to fulfil the primary protective function of the wall. The gram-negative cell wall also has an outer membrane which is composed of additional lipopolysaccharides and proteins not present in gram-positive cell walls. Figure 1 shows the cell envelopes of gram-positive and gram-negative bacterial cells.



(a) (b)

Figure 1

Figure 1 is a schematic diagram of gram-positive (a) and gram-negative (b) cell envelopes containing crystalline surface layers. The cytoplasmic membrane (CM) consists of phospholipid and membrane-bound proteins; the cell wall (CW) and peptidoglycan (PG) layers are composed of peptidoglycan and/or other polymers. The outer membrane (OM) is composed of phospholipid membrane proteins, lipoproteins and lipopolysaccharides; the crystalline S-layer (S) contains proteins of the outer cell wall.

When chemicals attack bacterial cell walls a reaction on the cell wall or membranes can alter the permeability of the cell. This can impair the passage of nutrients into the cell, as well as allow leakage of cellular constituents from the cell. Damage to the cell wall alone does not usually kill the microbial cell. Once inside, the chemical may cause the lysis of the cell membrane or precipitate in the cytoplasm of the cell.[2]

The mechanism by which nitric oxide exerts antimicrobial activity is not clear. Attack by nitric oxide and reactive nitrogen intermediates on vital iron-dependent enzymes has been thought to contribute to metabolic dysfunction.[4] A decrease in cellular respiration and replication has been shown to be associated with high levels of nitric oxide produced by cytokine-activated macrophage cells.[5]

5.2 Research Programme

The toxicity of a number of NO-containing or producing compounds towards *E. coli* was examined in order to understand better the nature of the non-specific immune system. The particular topics under scrutiny were:

- the ease of NO release

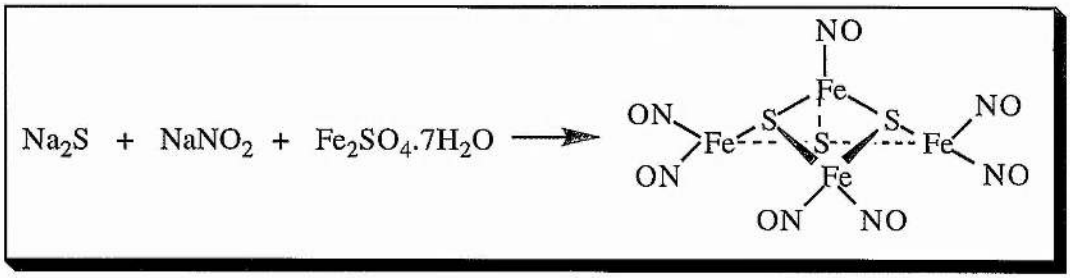
- the innocence of the NO ligand (is it NO, NO^+, NO^- ?)

- the barrier provided by the bacterial cell wall

5.2.1 Test Compounds

(a) Roussin's Black Salt (RBS) $Na[Fe_4S_3(NO)_7]$

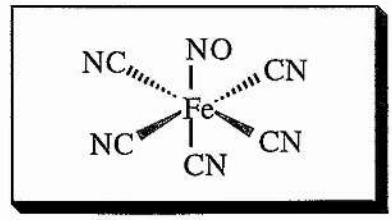
Roussin's salts (black and red) are the best known examples of iron-sulphur-nitrosyl complexes first described by Roussin in 1858.[6] Roussin's black salt [(heptanitrosyl-tri-μ_3-thioxotetraferrate (1-)], was prepared by reacting iron(II) sulphate heptahydrate, sodium nitrite and sodium sulphide in hot aqueous solution under nitrogen gas. This reaction is an example of self assembly.



RBS has essentially C_3V molecular symmetry and contains a flattened Fe_4 tetrahedron, of which three faces are triply bridged by sulphur atoms.

(b) Sodium nitroprusside (SNP) $Na_2[Fe(CN)_5NO]$

Sodium nitroprusside (SNP) has been known for more than a hundred years. It was synthesised by Playfair in 1848 and is one of the oldest metal nitrosyl complexes. It is stable in solution in the absence of light. This complex was of interest because it has a very high N-O stretching frequency and is used clinically to lower blood pressure.



Schematic structure of the nitroprusside anion

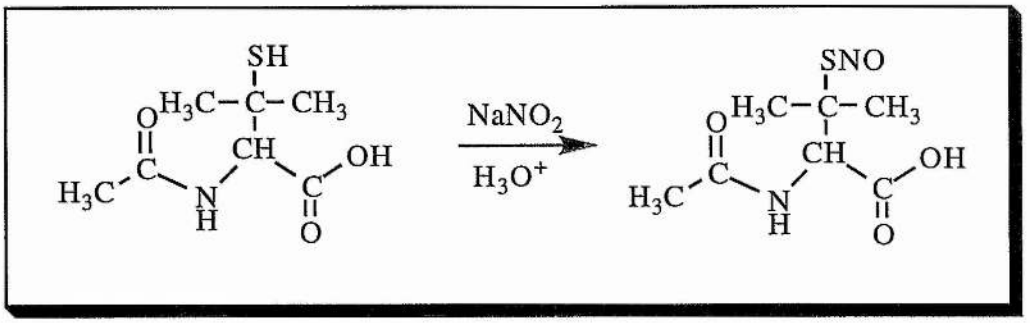
It has six ligands co-ordinated to a centre iron atom, the iron is present as Fe(II). SNP is a nitrosating agent, it reacts with a range of nucleophilic compounds such as azides, hydroxylamines, amines, carbanions and thiols.[7]

S-nitrosothiols

The nitrosation of thiols readily forms S-nitrosothiols.[8] These compounds decompose thermally and photochemically to form the disulphide and NO. The release of NO from these compounds is well documented and several have been used clinically to prevent platelet aggregation and adhesion to the vessel wall.[9]

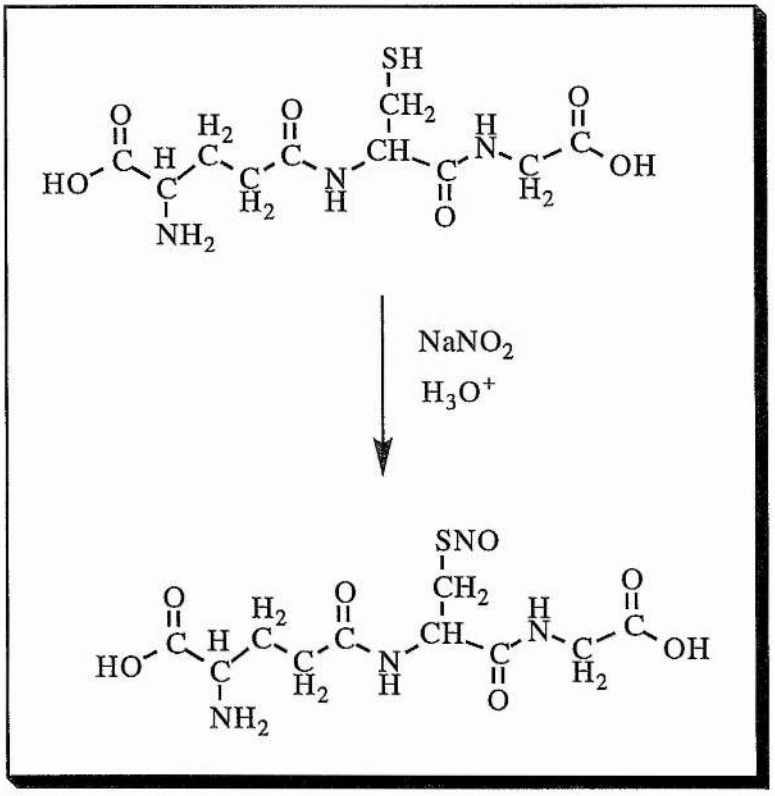
(c) S-nitroso-N-acetylpenicillamine (SNAP)

This thionitrite from N-acetyl-D,L-penicillamine is unusually stable and was synthesised by the method of Field *et al.*[10]



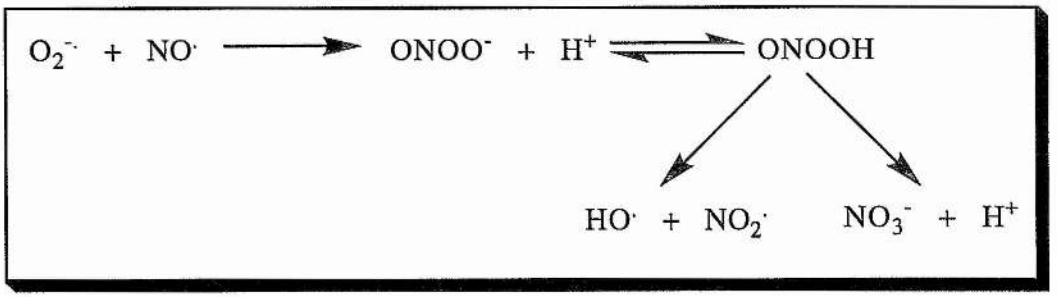
(d) S-nitrosoglutathione (GSNO)

This nitrosothiol was synthesised by the method of Hart.[11]



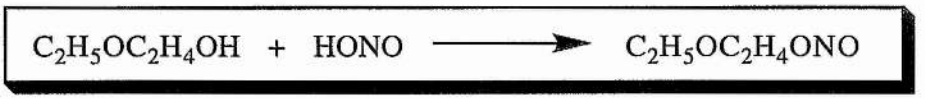
(e) Peroxynitrite

It has been proposed that NO reacts with superoxide ($O_2^{-\cdot}$) in many pathological states to yield a secondary cytotoxic species; the peroxynitrite anion ($ONOO^-$).[12] Formation of peroxynitrite from activated phagocytic cells could mediate NO-dependent microbial killing. Peroxynitrite is a potent bactericidal agent.[13]



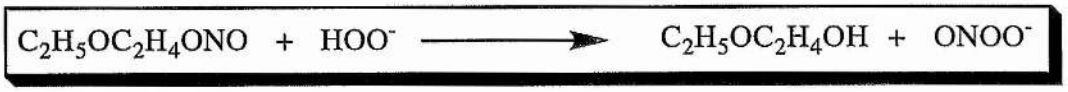
(i) Preparation of 2-ethoxyethyl nitrite

This first step in peroxynitrite synthesis involves the reaction of 2-ethoxyethanol with sodium nitrite.[14]



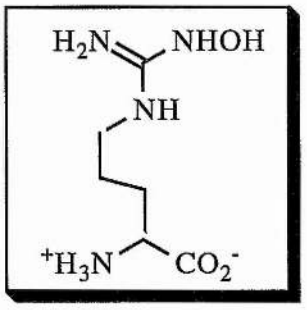
(ii) Peroxynitrite formation

This stage involves the reaction of 2-ethoxyethyl nitrite with equimolar hydrogen peroxide in basic medium, which ensures that no excess hydrogen peroxide is present in the final solution. The yellow colour of this solution is due to the presence of the peroxynitrite anion and can be detected by spectrophotometry.[15]



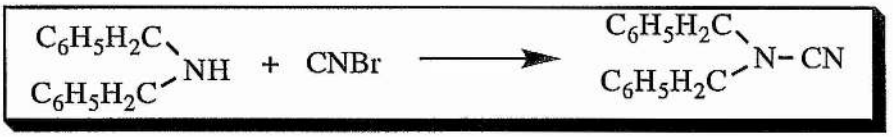
(f) Hydroxyguanidines

These compounds are an interesting target for synthesis since the discovery that the biosynthesis of nitric oxide from L-arginine occurs via hydroxyarginine.[16]

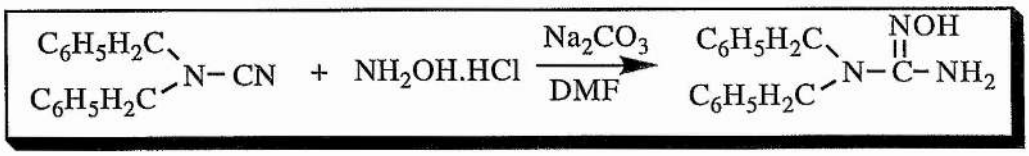


Success was achieved in the formation of dibenzylhydroxyguanidine via dibenzylcyanamide by the method of Bailey and De Grazia.[17]

(i) Formation of dibenzylcyanamide



(ii) Formation of dibenzylhydroxyguanidine



5.2.2 Antibacterial Studies

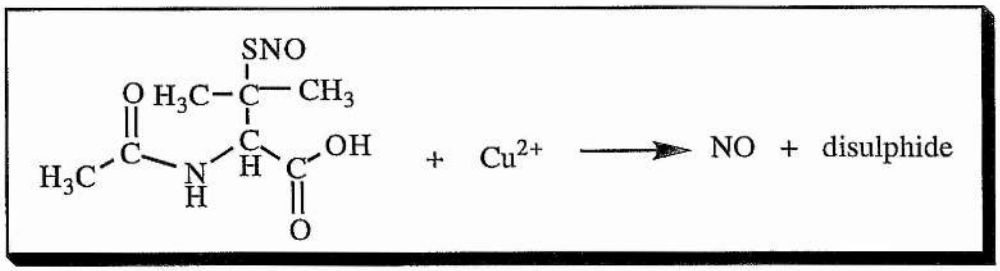
i) The toxic effects of a range of NO donor compounds on *E. coli* were investigated.

ii) Disruption of the bacterial cell wall: experiments were performed whereby the cell wall of the gram-negative bacterium *E. coli* was disrupted using ampicillin and lysozyme, thus reducing the barrier preventing access of the toxic species.

iii) It has been proposed that peroxynitrite is a potential bactericidal agent.[13] Once protonated peroxynitrite decomposes to form a species with reactivity similar to that of a hydroxyl radical plus nitrogen dioxide. The toxic nature of peroxynitrite was investigated using L-ascorbic acid (a scavenger of HO·) and its effect on the ability of peroxynitrite to kill *E. coli* was observed.

iv) Electron spin resonance (ESR) spectroscopy was used to detect radical formation from peroxynitrite. According to the literature preparation of peroxynitrite[15] all of the hydrogen peroxide used is consumed in the reaction. Therefore, any hydroxyl radicals trapped must arise from the decomposition of peroxynitrite.

v) SNAP decomposition: the decomposition of this S-nitrosothiol by Cu(II) acetate was investigated and the effect on antibacterial activity assessed.



5.2.3 The Nature of the Toxic Species

The antibacterial effects of a number of NO^+ containing complexes have been studied using the bacterium *Clostridium sporogenes*. It was discovered that RBS was several thousand times more toxic towards *C. sporogenes* than nitrite.[18] These compounds contain the NO^+ moiety and are good nitrosating agents towards a variety of substrates including amines, carbanions and thiols.[19] Their inhibitory effect may be due to the reaction of the coordinated NO^+ with cellular targets rather than to the release of NO. This is shown by the fact that NO is less toxic to *C. sporogenes* than iron-nitrosyl complexes.[18] The site of action of the toxic species may be the cell membrane. Payne showed by electron microscopy that cells treated with RBS had distorted shapes.[20]

In recent literature[21] it has been reported that RBS generated NO/NO^+ and caused cell clumping, while SNP generated NO/NO^+ and caused cell lysis. It was suggested that SNP released NO when illuminated and NO^+ in the dark. However NO^+ may interact with thiols to give nitrosothiols which then release NO. The relative toxicity of SNP and RBS under controlled conditions was investigated in order to determine the nature of the toxic species.

5.2.4 Distribution Studies

The theory that the greater toxicity of RBS is due to increased membrane solubility was investigated. The organic solvent octanol was used to mimic the cell membrane, and solubility was determined by spectrophotometry.

5.2.5 Reactions Monitored Using a NO Sensor

A sensor was used to measure the production of NO in the following circumstances

i) the decomposition of SNAP catalysed by copper (II) ions.

$$\text{SNAP} + Cu^{2+} \longrightarrow \text{NO} + \text{disulphide}$$

ii) the decomposition of SNAP by copper (II) in the presence of hydrogen peroxide.

iii) reaction between cysteine and SNP.

iv) reaction between cysteine and RBS.

5.3 Experimental

Instrumentation and General Techniques

Routine melting points were determined with open glass capillaries using an Electrothermal IA 9000 series digital melting point apparatus and were uncorrected. Infra-red spectra were recorded on a Perkin-Elmer 1330 Infrared Spectrophotometer. NMR spectra were obtained on a Varian Gemini spectrometer operating at 200 MHz for ^{1}H and 50.3 MHz for ^{13}C or as specified on a Bruker AM 300 operating at 300 MHz for ^{1}H and 75.4 MHz for ^{13}C. Chemical shifts (δ) for ^{1}H and ^{13}C are reported in ppm. Mass spectra were recorded on an A.E.I MS-50 spectrometer and fragment ions indicated as m/z units. Spectrophotometry was carried out using a Phillips PU 8730 UV/Visible scanning spectrophotometer. Quartz cells of 1 cm path length were used for measurements. The measurement of nitric oxide was carried out using a World Instruments Inc. isolated nitric oxide meter and sensor, model ISO-NO-B. ESR studies were performed using a Bruker ER 200D instrument.

Preparation of Roussin's Black Salt (RBS) (a)

Sodium nitrite (8 g; 0.02 mol) and sodium sulphide (11.3 g; 0.05 mol) were dissolved in water (160 cm^3) and the mixture heated to boiling under nitrogen. The reaction mixture was removed from the heat source and ferrous sulphate (2 g; 3 mmol) in water (80 cm^3) was added carefully followed by addition of 20% ammonia solution (20 cm^3). The reaction mixture was allowed to heat for a further 5 minutes before being filtered through a hot hyflo (treated with silica/water). The filtrate was cooled in ice water and filtered. The crude product was recrystallised from water to form shiny black crystals, yield 1.5 g (90.4%); $\nu_{max.}$ (THF)/cm^{-1} 1795, 1742, 1726 (NO).

Preparation of S-nitroso-N-acetylpenicillamine (SNAP) (c)

N-acetyl-D,L-penicillamine (1.91 g; 10 mmol) was dissolved in methanol and 1 M hydrochloric acid (40 cm^3; 1:1), conc. sulphuric acid (2 cm^3) was added dropwise. Sodium nitrite (1.38 g; 20 mmol) was dissolved in water (2 cm^3) and added very slowly with vigorous magnetic stirring. After 30 minutes the precipitate was washed with water and air dried, forming deep green crystals with red reflections. The reaction mixture and product were protected from light at all stages of the reaction; yield 1.56 g (71%); m.p. 152°C (lit[8] 152-154°C); $\nu_{max.}$ THF/cm^{-1} 1720 (NO).

Preparation of S-nitrosoglutathione (GSNO) (d)

To a stirred cold aqueous solution of glutathione (1.53 g; 5 mmol) containing 2 M hydrochloric acid (2.5 cm^3) was added in one portion sodium nitrite (0.35 g; 5 mmol). After 50 minutes at 5°C the resulting pale red precipitate was filtered and washed successively with ice-cold water (3 x 2 cm^3) and absolute ethanol (2 x 4 cm^3). The reaction mixture and product were protected from light at all stages of the reaction; yield 0.88 g (52%); m.p. 174°C (lit[9] 175-176°C); $\nu_{max.}$ (THF)/cm^{-1} 1720 (NO).

Preparation of 2-ethoxyethyl nitrite (ei)

Sodium nitrite (95 g; 1.4 mol) and water (375 cm^3) were stirred mechanically and cooled below 0°C in an ice/salt bath. A mixture of water (25 cm^3), conc. sulphuric acid (34 cm^3) and 2-ethoxyethanol (112.8 g; 1.25 mol) were cooled to 0°C and introduced slowly beneath the surface of the nitrite with stirring. The solution was added at such a rate that practically no gas evolved and the temperature remained at approx. 1°C. The resulting mixture was allowed to stand in an ice/salt bath until it separated into layers. The 2-ethoxyethyl nitrite was dried over magnesium sulphate, then purified by distillation; b.p. 112°C (15 mm Hg).

Preparation of peroxynitrite (eii)

In order to prepare a solution of 0.016 M peroxynitrite, 0.016 M hydrogen peroxide (15 cm^3), 2 M sodium hydroxide (15 cm^3) and water (70 cm^3) were combined. 2-Ethoxyethyl nitrite (0.2 cm^3) was added and after a few minutes the solution turned yellow due to the presence of the peroxynitrite anion ($ONOO^-$) which was determined by spectrophotometry, $\lambda = 302$ nm, $\varepsilon = 1670$ dm^3 mol^{-1} cm^{-1}.

Preparation of dibenzylcyanamide (fi)

Cyanogen bromide (4 g; 0.04 mol) in methanol (7 cm^3) was added dropwise with stirring to a mixture of dibenzylamine (6.78 g; 0.03 mol), anhydrous sodium acetate (2.83 g; 0.03 mol) and methanol (3 cm^3). This was stirred for 2.5 h at 5°C then at ambient temperature for 2 h. The reaction mixture was concentrated under vacuum and treated with water (8 cm^3) then extracted with dichloromethane (3 x 10 cm^3) washed with 2 M hydrochloric acid (10 cm^3) and brine (10 cm^3) then dried over magnesium sulphate. The product was recrystallised from methanol, yielding cream coloured needles; 5.4 g (71%); m.p. 42-44°C; $\nu_{max.}$ (Nujol)/cm^{-1} 3420, 3380 (NH), 3060, 3020, 1562, 1493, 735, 695 (CH), 2196 (CN); δ_H (300 MHz: $CDCl_3$); 4.62 (4H, s, CH_2), 7.30 (10H, m, Ar); δ_C (75.4 MHz: $CDCl_3$); 54.73 (benzyl carbon), 134.61, 129.23, 129.45, 129.10 (Ar carbons), 139.5 (CN); *m/z* 222 ($M^{+\cdot}$); (Found: C, 81.2; H, 6.65; N, 12.6, $C_{15}H_{14}N_2$ requires C, 81.1; H, 6.35; N, 12.6%).

Preparation of dibenzylhydroxyguanidine (fii)

To a solution of hydroxylamine hydrochloride (0.21 g; 3 mmol) and dibenzylcyanamide (0.44 g; 2 mmol) in purified dimethylformamide (5 cm^3) was added sodium carbonate (0.59 g; 5.6 mmol) over 10 minutes. The resultant solution was heated over a steam bath for 1 h, cooled to 50°C and filtered. The salts were washed with warm dimethylformamide and the filtrate and washings combined and reduced under vacuum. The residue was added dropwise to ice-water with much scratching and stirring. The product was purified by recrystallisation from water yielding white needles; 0.42 g (82%); m.p. 120-121°C; $\nu_{max.}$ (nujol)/cm^{-1} 3448 (OH), 3315, 1630 (NH), 1575,1455, 755, 696 (CH); δ_H (200 MHz; $CDCl_3$); 7.35 (10H, m, Ar); δ_C (50.3 MHz; $CDCl_3$); 51.42 (benzyl carbon), 138.3, 127.9, 129.1, 127.7 (Ar carbons), 157.3 (guanidino); *m/z* 255 ($M^{+\cdot}$); (Found: C, 70.54; H, 6.50; N, 16.36, $C_{15}H_{17}N_3O$ requires C, 70.56; H, 6.71; N, 16.46%).

5.3.1 Antibacterial Studies

i) Toxicity Studies

In order to investigate the toxicity of test compounds towards bacteria, the bacterium *Escherichia coli* DH5 strain was used. To obtain a standard stock culture a loopful of *E. coli* from a slope of pure agar was transferred to a minimal defined media (10 cm^3), and the prepared culture was incubated at 37°C.

The test compounds: SNAP, GSNO and RBS were prepared as saturated solutions in phosphate buffered saline (PBS) and their concentrations determined by spectrophotometry. The stock solutions were further diluted with PBS. DBHG was prepared as a stock solution in dimethyl sulphoxide (DMSO) and diluted with PBS. Peroxynitrite was prepared immediately prior to each experiment. Cysteine and SNP were sufficiently soluble and were diluted to the required concentrations.

Experimental controls: PBS only as controls for SNAP, SNP, GSNO and RBS test solutions, PBS/DMSO for DBHG while for the peroxynitrite solution; PBS, sodium hydroxide, hydrogen peroxide and water was used as the control. All test solutions and controls were filtered (0.45 μm sterile filters) before use. The solutions were exposed to light (200 watt bulb at a distance of 30 cm) or kept in darkness (wrapped in aluminium foil).

A growing DH5 culture (10 μl) was added to each of the prepared solutions (1 cm^3) in sterile bottles. Each bottle was flamed upon addition of culture in order to prevent contamination. An aliquot (100 μl) from each solution was transferred to a sterile agar plate and spread over the surface. The plates were incubated at 37°C for 24 h, after this time the colonies were counted.

ii) Disruption of the Bacterial Cell Wall

Ampicillin or lysozyme (1 mg) was added to the bacterial culture (10 cm^3) and shaken at 37°C for 1 h. The culture was added to the test solutions as described in toxicity studies (i). Control experiments were run in parallel.

iii) Peroxynitrite Toxicity

L-ascorbic acid (1 mg/100 cm^3) was added to the peroxynitrite solution in order to scavenge hydroxyl radicals. The culture was added to the test solutions as previously described (i).

iv) ESR Studies

Peroxynitrite (1 cm^3) and trapping compound 5,5-dimethyl-1-pyrroline-N-oxide [(DMPO), 0.05 cm^3] were combined in a quartz tube and degassed with nitrogen. This procedure was carried out at ambient temperature.

v) SNAP Decomposition

Cu(II) acetate (1-2 mg) was added to SNAP (1 cm^3) or the control solution - PBS (1 cm^3) - and shaken before the addition of *E. coli*.

5.3.2 Media preparation

Nutrient Agar (L-Agar)

Oxoid: 28 g of powder per litre of distilled water. Dissolved by boiling and sterilised by autoclaving at 121°C for 15 minutes. The solution was allowed to cool to 45°C then dispensed into sterile petri dishes.

Minimal defined media (M9)

per litre:

disodium hydrogen phosphate (Na_2HPO_4)	6 g
potassium dihydrogen phosphate (KH_2PO_4)	3 g
sodium chloride (NaCl)	0.5 g
ammonium chloride	1 g
magnesium sulphate ($MgSO_4.7H_2O$)	(1 cm^3)
20% glucose solution	(10 cm^3)
0.01 mol dm^{-3} calcium chloride ($CaCl_2$)	(10 cm^3)

The prepared solutions were sterilised by autoclaving at 121°C for 15 minutes, allowed to cool then combined. To the prepared media (10 cm^3) vitamin B_1 (1 mg) was added and the solution filtered before the addition of *E. coli*.

5.3.3 Distribution Studies[22]

Saturated solutions of octanol in water were prepared by shaking octanol (100 cm^3) with water (25 cm^3) or by shaking water (100 cm^3) with octanol (25 cm^3). SNAP, RBS and SNP were dissolved in 5 cm^3 of each solution to a final concentration of 1 x 10^{-3} mol dm^{-3} (SNAP) and 1 x 10^{-5} mol dm^{-3} (RBS and SNP). Each solution was shaken and the spectrum of the aqueous and organic layers recorded.

5.3.4 NO Sensor Experiments

(The electrode was calibrated for temperature before use)

The test compounds were prepared in phosphate buffer pH 7.4 and purged with argon.[23] The vessel was sealed thoroughly to prevent leakage of NO and measurements were recorded.

i) SNAP decomposition with Cu(II)

SNAP 9.1 x 10^{-6} mol dm^{-3} + Cu(II) 4.76 x 10^{-6} mol dm^{-3}

ii) SNAP decomposition with Cu(II): the effect of H_2O_2

SNAP 9.1 x 10^{-6} mol dm^{-3} + Cu(II) 4.76 x 10^{-6} + H_2O_2 9.3 x 10^{-6} mol dm^{-3}

iii) Reaction between cysteine and SNP

cysteine 1 x 10^{-3} mol dm^{-3} + SNP 1.25 x 10^{-3} mol dm^{-3}

iv) Reaction between cysteine and RBS

cysteine 1 x 10^{-3} mol dm^{-3} + RBS 1.25 x 10^{-3} mol dm^{-3}

5.4 Discussion

Roussin's Black Salt (RBS) was a potent bactericidal agent towards *E. coli* killing 50% of colonies at 4 x 10^{-5} mol dm^{-3}. This toxicity increased with contact time and varied with concentration when tested in both light and darkness (appendix 1). Hughes *et al.*,[18] by examining RBS [$Fe_4S_3(NO)_7$], sodium nitroprusside (SNP) [$Fe(CN)_5NO$] and the corresponding chromium species [$Cr(CN)_5NO$] have shown that neither iron nor sulphur are essential for the bacteriostatic effects of Roussin's type compounds. The property these compounds have in common is that they contain NO^+. It is proposed that NO^+ is the active species and a relationship may exist between the N-O stretching frequency (a measure of the NO^+ character) and the toxicity of these compounds. The compounds RBS and SNP donate the species NO^+ which has a N-O stretching frequency of 2300 cm^{-1}. RBS was 400 times and SNP 20 times more toxic towards *E. coli* than SNAP and GSNO, which donate NO and have a lower N-O stretching frequency of 1840 cm^{-1}.

SNP was toxic towards *E. coli* in darkness with 50% of colonies killed at 1 x 10^{-3} mol dm^{-3}. Following exposure to light this concentration was not toxic (appendix 1). RBS and SNP are good nitrosating agents towards a variety of substrates including amines, carbanions and thiols.[19]

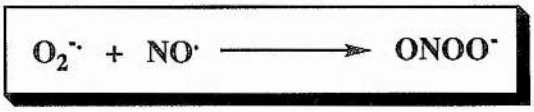
Peroxynitrite was a potent bactericidal agent killing *E. coli* in direct proportion to its concentration with 50% of colonies killed at 1.5 x 10^{-4} mol dm^{-3}. This toxicity increased with contact time (appendix 1). RBS, SNP and peroxynitrite were more toxic towards *E. coli* than the S-nitrosothiols SNAP and GSNO. Dibenzylhydroxyguanidine (DBHG) was the least toxic of the compounds tested with a concentration of 5 x 10^{-2} mol dm^{-3} required to kill 50% of colonies (appendix 1).

The greater effect of RBS, SNP and peroxynitrite may be due more to the transfer of the coordinated nitrosyl group to cellular targets than to the release of nitric oxide. This

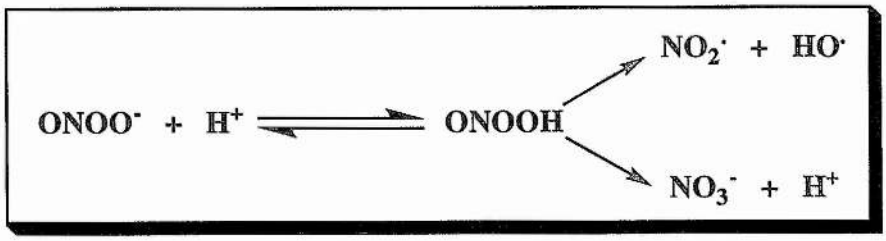
is borne out by the fact that NO is less toxic to *C. sporogenes* than iron nitrosyl complexes.[18]

The site of action of the toxic species is thought to be the cell membrane. Payne *et al.*[20] showed by electron microscopy that cells treated with RBS at lethal concentrations caused the apparent optical density to decrease, indicating cell lysis. Potential targets under investigation include the cell membrane and the cell wall components. It is to be expected that there will be variation in effectiveness resulting from differences in aqueous solubility, stability in the medium, charge and liposolubility. In order to maximise contact between the test compound and cell membrane the cell wall was disrupted using ampicillin and lysozyme. This process increased toxicity slightly for all the compounds tested with the exception of peroxynitrite (appendix 2). The toxic species in the case of peroxynitrite may require the cell wall to be present in order to function optimally.

Because activated macrophages simultaneously synthesise superoxide and nitric oxide, their reaction product peroxynitrite is thought to be a transient bactericidal agent produced by these cells. The reaction of superoxide with nitric oxide is rapid with a rate constant of at least $3.7 \times 10^7\ m^{-1}\ s^{-1}$



Peroxynitrite is a strong oxidant capable of reacting by multiple oxidative mechanisms. The peroxynitrite anion directly oxidises sulphydryl groups at a rate a thousand times faster than hydrogen peroxide. The anion also reacts with metal ions and with superoxide dismutase to form a potent nitrating agent with reactivity of the nitronium ion. Furthermore, peroxynitrite has a pKa of 6.8 so that 20% will be protonated at physiological pH.



It has been proposed that peroxynitrous acid (ONOOH) spontaneously decomposes to form a potent oxidant with the reactivity of a hydroxyl radical without a requirement for transition metal catalysis.[12] It is interesting that while many studies of the nitric oxide/superoxide interaction demonstrate augmented cytotoxicity,[24] others show diminished cytotoxicity.[25]

Zhu *et al.*[13] found that peroxynitrite was highly bactericidal killing *E. coli* in direct proportion to its concentration. Metal chelators did not decrease peroxynitrite mediated killing, indicating that exogenous transition metals were not required for toxicity. The hydroxyl radical scavengers mannitol, ethanol and benzoate did not significantly affect toxicity while dimethyl sulphoxide (DMSO) enhanced peroxynitrite mediated killing. DMSO is a more efficient hydroxyl radical scavenger than the other three and increased the formation of NO_2. The formation of NO_2 may have enhanced the toxicity of peroxynitrite decomposing in the presence of DMSO.

Our studies further confirmed that the hydroxyl radical produced by the decomposition of peroxynitrite did not account for the observed toxicity towards *E. coli*. L-ascorbic acid (a scavenger of HO·) did not significantly decrease the toxicity of peroxynitrite. However, evidence for hydroxyl radical production was presented by ESR (appendix 3). These results indicate that another species produced by peroxynitrite must be responsible for the observed toxicity. Taking into account that peroxynitrite was highly toxic towards *E. coli*, with comparable toxicity to NO^+ donor compounds (RBS, SNP) it is not unreasonable to postulate that NO^+ produced by peroxynitrite decomposition is the toxic species.

SNAP decomposes to form the corresponding disulphide and NO. The effect of copper (II) ions on its decomposition and hence toxicity towards *E. coli* was investigated. It was demonstrated that copper in the form of Cu(II) acetate enhanced the decomposition of SNAP and hence the production of NO resulting in increased toxicity. However at concentrations greater than 5×10^{-3} mol dm^{-3} Cu(II) acetate was toxic (appendix 4).

An attempt was made to produce a mutant strain of *E. coli* using the mutagen 1,2,7,8-diepoxyoctane. Ideally the strain produced would be resistant to RBS and therefore allow investigation of the toxicity mechanisms of test compounds in comparison to RBS. However, the production of a RBS resistant strain was unsuccessful, partly due to the insolubility of RBS in agar-containing medium. However, the resultant strain was tested with RBS and SNAP and further confirmed the greater cytotoxicity of a NO^+ donor (RBS) in comparison with a NO donor (SNAP).

Previous research[26] demonstrated that nitroprusside crosses the erythrocyte membrane. The apparently low transport rate of nitroprusside into red blood cells[27] determined by experiments with carbon-14 labelled nitroprusside can be rationalised by consideration of the toxic effects of nitroprusside on cells. It has been postulated that the greater toxicity of NO^+ donor compounds (RBS, SNP) is attributed to increased membrane solubility. This theory was investigated using octanol to mimic the cell membrane and solubility was determined by spectrophotometry. SNAP and RBS were considerably more soluble in the organic (octanol) layer than in the aqueous layer. However, SNP, although soluble in the organic layer, was slightly more soluble in the aqueous layer (appendix 5). On the basis of these findings the ability to dissolve in the cell membrane alone does not explain the observed antibacterial effect.

A nitric oxide sensor was used to follow the decomposition of SNAP catalysed by Cu(II) acetate (appendix 6), NO evolution was observed reaching a maximum after 22 minutes. This reaction was repeated adding hydrogen peroxide, consequently the amount of NO produced was reduced by 50% and maximum evolution occurred after 10 minutes. In the presence of hydrogen peroxide NO is oxidised to nitrite and nitrate and therefore accounts for the reduced evolution of NO from SNAP decomposition.

The reactions between cysteine/RBS and cysteine/SNP were also studied (appendix 6). Maximum NO evolution from cysteine/RBS was observed after 265 minutes, while maximum NO evolution from cysteine/SNP was observed after 340 minutes. These results suggest that in the presence of a thiol (cysteine), NO was not readily produced from either RBS or SNP. Kaplan *et al.*[28] have shown that NO mediated killing of *S. aureus* was not manifested until about 5 hour exposure had occurred, thus providing further evidence that NO is not the species (or main species) responsible for the observed toxicity of RBS and SNP towards *E. coli* .

From the evidence presented it appears that the toxicity of SNP, RBS and possibly peroxynitrite towards *E. coli* is due to the transfer of NO^+ to cellular targets rather than to the release of NO.

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Appendices

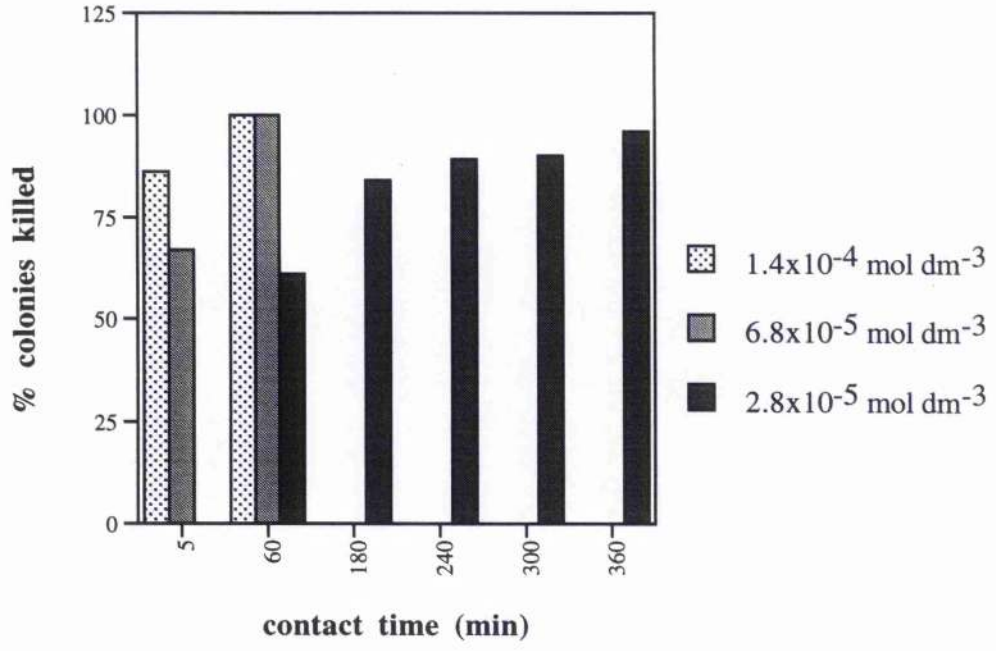
Appendix 1

i) Toxicity Studies

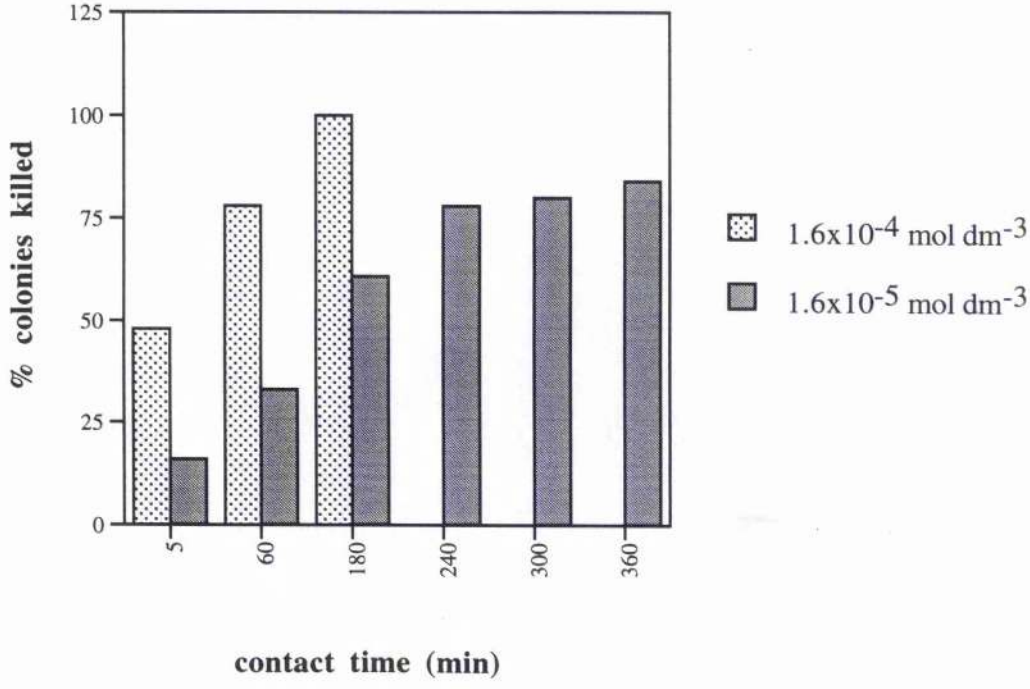
IC_{50} values of test compounds - the concentration at which 50% of colonies are killed relative to control.

compound	concentration (mol dm^{-3})
RBS	5.0×10^{-5}
$ONOO^-$	1.5×10^{-4}
SNP	1.0×10^{-3}
SNAP	2.0×10^{-2}
GSNO	2.5×10^{-2}
DBHG	5.0×10^{-2}

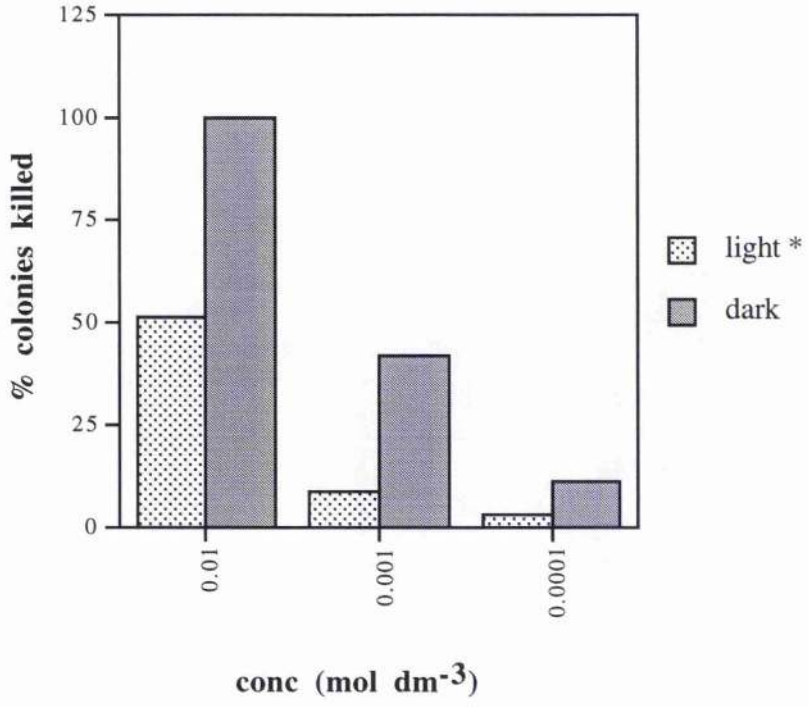
The toxicity of RBS: variation with contact time



The toxicity of peroxynitrite: variation with contact time



The Effect of Light on SNP Toxicity



* 200 Watt bulb at a distance of 30 cm

Appendix 2

ii) Disruption of the Cell Wall of *E. coli* With Ampicillin (100 μg/cm^3 culture)

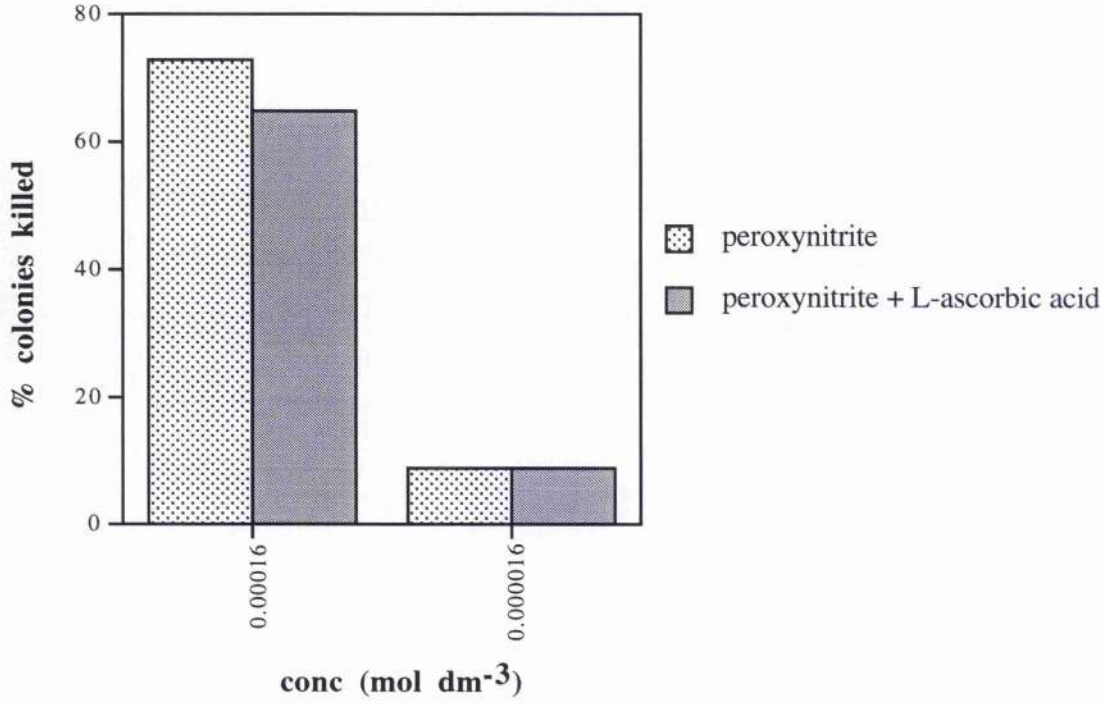
compound	*conc (mol dm^{-3})*	*% colonies killed*	*% colonies killed (ampicillin)*
ONOO$^-$	1.6 x 10^{-4}	73	72
	1.6 x 10^{-5}	9	9
RBS	3.8 x 10^{-4}	100	100
	4.0 x 10^{-5}	42	62
SNAP	1.8 x 10^{-2}	53	62
	4.5 x 10^{-3}	4	13
GSNO	1.8 x 10^{-2}	42	61
	4.5 x 10^{-3}	0	0

Disruption of the Cell Wall of *E. coli* With Lysozyme (100 μg/cm^3 culture)

compound	*conc (mol dm^{-3})*	*% colonies killed*	*% colonies killed (lysozyme)*
ONOO$^-$	1.6 x 10^{-4}	65	48
	1.6 x 10^{-5}	8	1
RBS	1.9 x 10^{-4}	100	100
	9.5 x 10^{-5}	61	57
SNAP	1.1 x 10^{-2}	35	48
	8.5 x 10^{-3}	12	16
GSNO	1.1 x 10^{-2}	29	35
	9.2 x 10^{-3}	7	0

Appendix 3

iii) The Effect of L-Ascorbic Acid on Peroxynitrite Toxicity

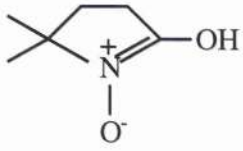


ESR Studies: Peroxynitrite

The following compound was detected:

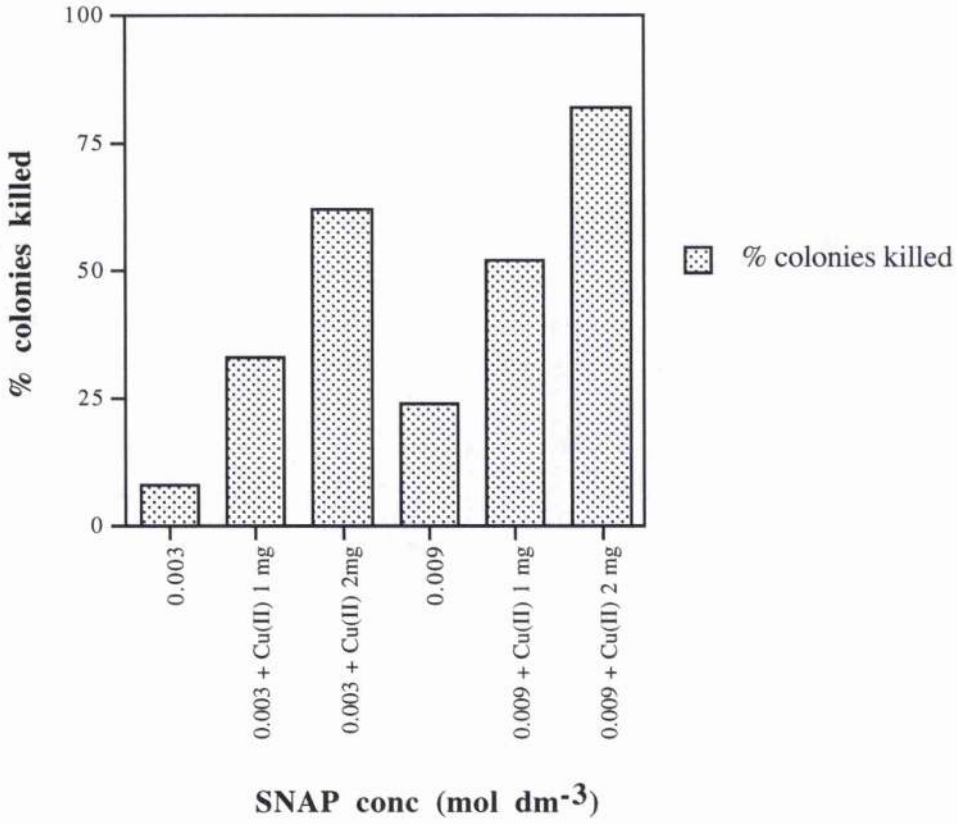
a (1H) 1.4 G

a (N) 16G



Appendix 4

iv) SNAP Decomposition, the Effect of Cu(II)



Appendix 5

Distribution Studies

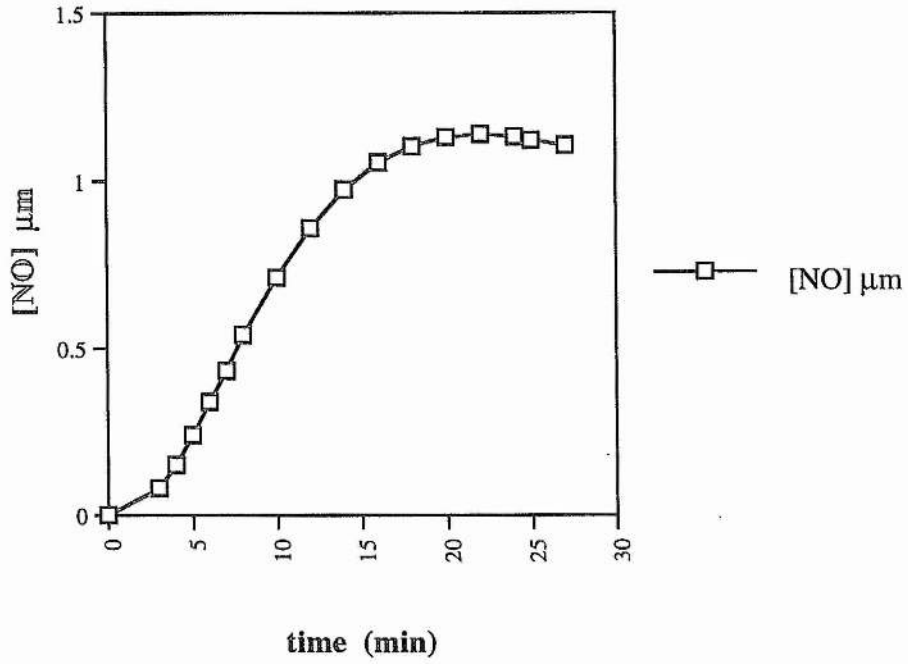
compound	**layer**	**λ (nm)**	**Abs (A)**
SNAP	organic	342.7	1.750
	aqueous	339.2	0.404
RBS	organic	351.0	1.634
	aqueous	353.8	0.703
SNAP	organic	199.7	2.196
	aqueous	192.3	2.996

Appendix 6

Reactions monitored using a NO sensor

i) The decomposition of SNAP by Cu(II)

SNAP 9.1 x 10^{-6} mol dm^{-3} + Cu(II) 4.76 x 10^{-6} mol dm^{-3}

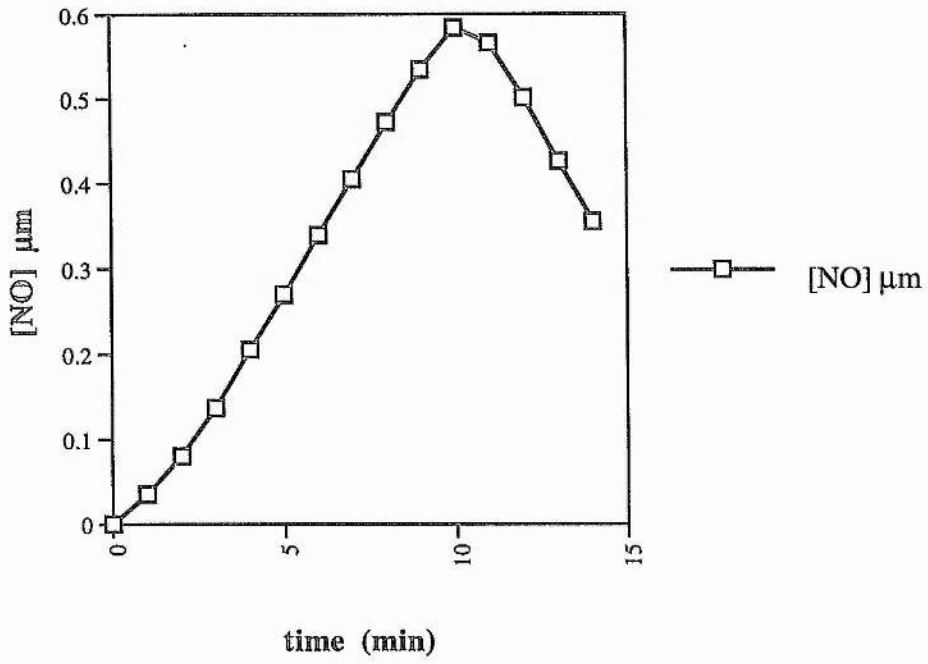


ii) The decomposition of SNAP by Cu(II)

- the effect of hydrogen peroxide

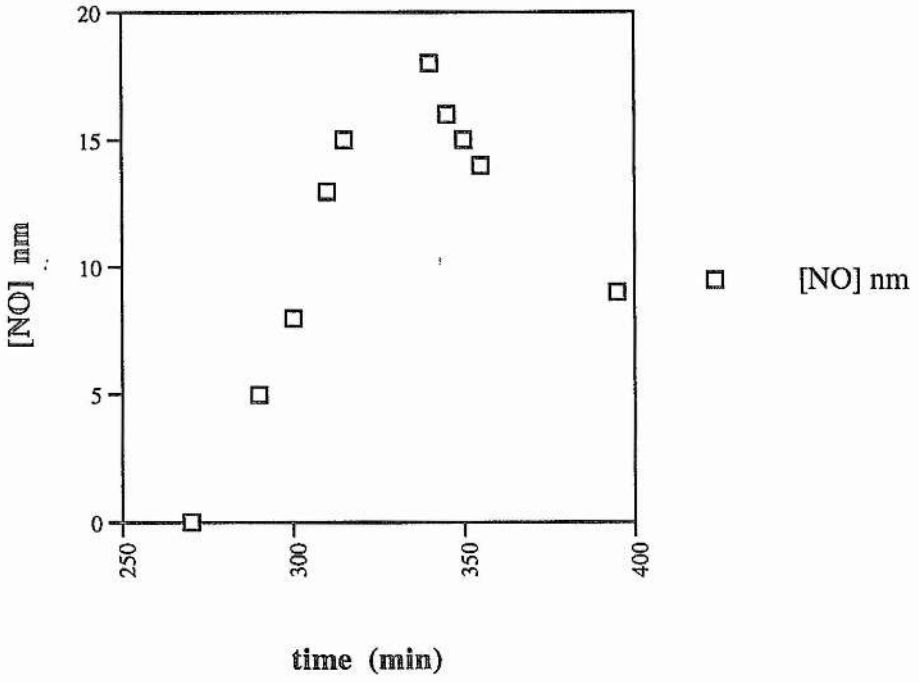
SNAP 9.1 x 10^{-6} mol dm^{-3} + Cu(II) 4.76 x 10^{-6} mol dm^{-3}

+ H_2O_2 9.3 x 10^{-6} mol dm^{-3}



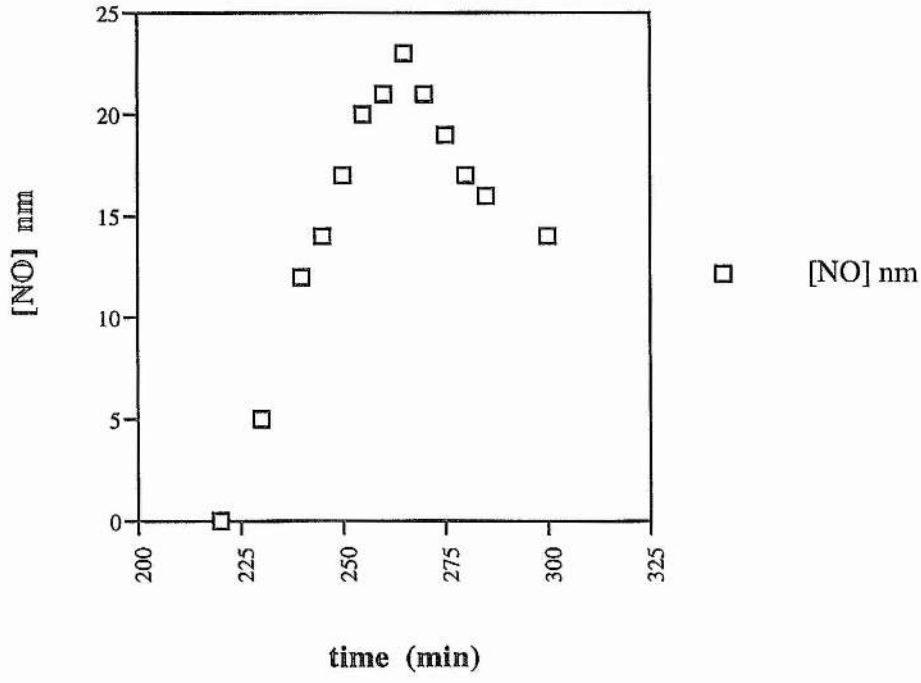
iii) Reaction betweeen cysteine and SNP

cysteine 1 x 10^{-3} mol dm^{-3} + SNP 1.25 x 10^{-3} mol dm^{-3}



iv) Reaction between cysteine and RBS

cysteine 1 x 10^{-3} mol dm^{-3} + RBS 1.25 x 10^{-3} mol dm^{-3}



Chapter 6

Leishmaniasis, a Model for Malaria?

6.1 Introduction

Leishmaniasis refers to a spectrum of diseases caused by parasitic protozoa belonging to the genus *Leishmania*. All forms of leishmaniasis begin with a bite from a sandfly vector. The amastigote form of the parasite is ingested by the sandfly during a blood meal and develops into the promastigote form in the sandfly gut. The insect deposits the extracellular promastigote into mammalian skin. The parasite subsequently undergoes phagocytosis whereupon it converts to the amastigote form. Thus their means of entry into macrophages and their resistance to macrophage microbicidal mechanisms are critical for the organism's subsequent survival.

Leishmania replicate only within macrophages of an infected host. Elimination of the parasite and resolution of disease must invoke extraordinary metabolic changes in the infected macrophage. These changes shift the intracellular environment of the parasite from one that is supportive of replication to one that is hostile to survival. Both *in vitro* and *in vivo* studies convincingly demonstrate that the intracellular persistence of *L. major* depends on the differentiate state of the macrophage at the onset of infection.

The intracellular conversion of the sandfly-adapted promastigote into the amastigote form is instrumental to the survival and propagation of the *leishmania* species. Unlike the promastigote of *L. major* the amastigote is quite resistant to the antimicrobial mechanisms of the unstimulated macrophage. For instance, attachment and entry of either the promastigote or the amastigote into macrophages elicits a superoxide synthesising respiratory burst which the amastigote readily survives. Unlike the promastigote, *L. major* amastigotes are also less sensitive to cytokine-induced, oxygen dependent antimicrobial mechanisms such as hydrogen peroxide.[1]

6.1.1 The Antiparasitic Nature of Nitric Oxide

The precise nature of the metabolic changes in the amastigote induced by nitrogen oxides that result in death of the parasite are unknown. Several effects of L-arginine catabolism in other systems, however, may shed light on the intracellular fate of the amastigote.

Iyengar *et al.*[2] speculated that N-guanido-hydroxylated arginine, a postulated intermediate in the conversion of L-arginine into NO and L-citrulline, is a potent inhibitor of DNA synthesis. In as much as N-hydroxylated guanidines possess antitumour and microbicidal activity, it is possible that such derivatives inhibit DNA synthesis and are the proximal cause of amastigote cytostasis in IFN-γ treated macrophages.

The generation of NO by activated macrophages also induces the haem-dependent activation of guanylate cyclase with the subsequent stimulation of the secondary messenger cGMP, which may in turn serve as a signal for monokine production. Another possibility is that NO may be the actual effector molecule that causes stasis and/or lysis of the intracellular parasite. This could explain intracellular iron loss and inhibition of enzymes with iron-sulphur prosthetic groups that are important in a number of metabolic pathways and in certain microorganisms.[3] Cytotoxic activated macrophages inhibit two oxidoreductases of the mitochondrial electron transport chain in tumour targets,[4] the citric acid cycle enzyme aconitase and DNA replication.[5] Each of the enzymes have a catalytically active iron linked to a sulphur group that could be degraded by NO and released as an iron-nitrosyl complex. NO gas mimics macrophage-mediated tumour cytotoxicity, inhibition of mitochondrial aconitase activity and respiration. *Leishmania* infected macrophages may cause iron efflux, followed by iron-dependent enzyme inhibition in the amastigote.[6]

6.1.2 The Effect of Qinghaosu and its Derivatives on *Leishmania*

The artemisinin family of compounds are characterised by having an endoperoxide bridge within the molecule. Whether the endoperoxide bridge is essential for antileishmanial activity as it is for antimalarial activity is unknown. Under *in vivo* conditions metabolism of the endoperoxide linkage of qinghaosu and its derivatives by parasites may generate oxygen radicals which are detrimental to parasite macromolecules.

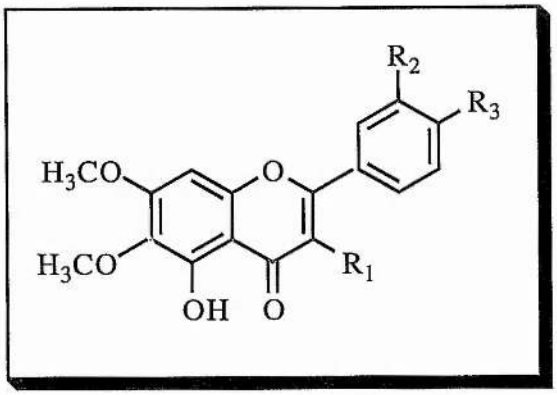
The effect of qinghaosu and its ether derivative artemether on *L. major* replication *in vitro* and on the disease development in mice infected with *L. major* have been investigated.[7] BALB/c mice infected in the footpad with *L. major* developed significantly smaller lesions and parasite loads when treated with these compounds. Intra-lesion injection was the most effective route, intramuscular and oral routes were also effective, but to a lesser extent.

Qinghaosu and its derivatives have been shown to be effective against *Schistosoma mansoni* [8] and *Clonorchis sinensis*.[9] Therefore, this group of compounds may have broad antiparasitic potential. These compounds have no effect on the viability of macrophages and the activity of lymphocytes to proliferate *in vitro*. Given this low toxicity *in vitro*, their reported safety profile *in vivo* [10] and their effectiveness when administered orally, this series of compounds are potentially useful therapeutic agents in the treatment of clinical leishmaniasis.

6.2 The Effect of Flavonoids on the Antimalarial Activity of Qinghaosu

Most natural antioxidants are from plants. The most common are flavonoids (flavanol, isoflavones, flavones, catchins and flavanones). Natural antioxidants may function as reducing agents, free radical scavengers, as complexes of pro-oxidant metals and as quenchers of the formation of singlet oxygen.[11] Many flavones are present in the Chinese herb *Artemisia annua* . For centuries malaria has been treated with a decoction from a combination of herbs including *A. annua*. These herbs were boiled in water and strained before drinking. This decoction proved very effective. It appears that small amounts of qinghaosu in the plant are more effective when unrefined. Therefore, it is possible that a synergistic effect occurs in herbal combinations.

6.2.1 Major Flavones of *Artemisia annua* Plants and Cell Cultures



comparison of levels of major flavones in plant and cultures of *A. annua*

		% DW of plant	% DW of suspension
artemetin	$R_1=R_2=R_3=OCH_3$	0	0.07
casticin	$R_1=R_3=OCH_3$, $R_2=OH$	0.01	0
chrysoplenetin	$R_1=R_2=OCH_3$, $R_3=OH$	0.04	0.06
chrysoplenol-D	$R_1=OCH_3$, $R_2=R_3=OH$	0.10	0.06
cirsilineol	$R_1=H$, $R_2=OCH_3$, $R_3=OH$	0.01	0.05
eupatorin	$R_1=H$, $R_2=OH$, $R_3=OCH_3$	0.02	0

(DW= Dry Weight)

Cell suspension cultures developed from *A. annua* exhibited antimalarial activity *in vitro* with *P. falciparum* in both the n-hexane extract of the plant cell culture medium and in the chloroform extract of the cells. Trace amounts of qinghaosu account for the activity of the n-hexane fraction but only the methoxylated flavones artemetin, chrysoplenetin, chrysoplenol-D and cirsilineol can account for the activity of the chloroform extract.

6.2.2 The Inhibition of [^{3}H] Hypoxanthine into *P. falciparum in vitro* by Extracts of Cell Suspension of *A. annua*

solvent extract	***in vitro* antiplasmodial activity** IC_{50} ($\mu g/cm^3$)
n-hexane (medium)	15.6
chloroform	14.5
methanol (cells)	500

Trace amounts of qinghaosu detected in the n-hexane fraction were responsible for inhibiting the incorporation of [^{3}H] hypoxanthine, causing parasite death. However, no qinghaosu was detected in the chloroform extract of the cells. This latter extract contained methoxylated flavonoids some of which are known to be cytotoxic.[12]

These purified flavones have IC_{50} values at 2.4 - 6.5 x 10^{-5} mol dm^{-3} against *P. falciparum in vitro* compared with an IC_{50} value of 3 x 10^{-8} mol dm^{-3} for purified qinghaosu. At concentrations of 5 x 10^{-6} mol dm^{-3} these flavones were not active against *P. falciparum* but did have a marked and selective potentiating effect on the antiplasmodial activity of qinghaosu.[13]

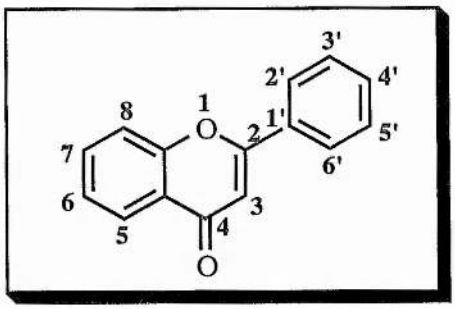
6.2.3 The Inhibitory Effect of Flavones Alone or with Qinghaosu

-incorporation of [^{3}H] hypoxanthine into *P. falciparum* -

	IC_{50}	
	flavonoid alone ($M \times 10^{-5}$)	qinghaosu ($M \times 10^{-8}$) + flavonoid (5 μM)
qinghaosu	-	3.3
artemetin	2.6	2.6
casticin	2.4	2.6
chrysoplenetin	2.3	2.25
chrysosplenol-D	3.2	1.5
cirsilineol	3.6	1.6
eupatorin	6.5	3.0

To some extent all the methoxylated flavonoids except eupatorin had a comparable potentiating effect on the *in vitro* antiplasmodial activity of qinghaosu. Since the flavones have little effect on the dose response curve of chloroquine at concentrations which significantly enhance the antimalarial activity of qinghaosu, this suggests a different mechanism of action of these compounds.[13]

6.2.4 Structure-Related Activity[12]



The flavones studied were all methoxylated at C-6 and C-7; however, except for eupatorin where only slight potentiation of qinghaosu activity was observed, variations in methoxyl-hydroxyl substitution at C-3, C-3' and C-4' appeared to have a limited effect on antimalarial activity. This suggests that for potentiating activity, if there is no substitution at C-3 in eupatorin or cirsilineol, then unsubstituted oxygen at C-4' is required as in cirsilineol.

If C-3 is substituted with a methoxyl the ring β may have either C-3', C-4' dihyroxyl (chrysoplenol-D); C-3' hydroxyl, C-4' methoxyl (casticin); C-3' methoxyl, C-4' hydroxyl (chrysoplenetin) or C-3', C-4' dimethoxyl (artemetin) for potentiating activity to be observed. Why eupatorin has so little effect was not clear from the results. Sufficient flavonoids have not been tested biologically in order to ascertain the structural requirement of flavonoids which potentiate the activity of qinghaosu towards *P. falciparum*. Furthermore, the activity of qinghaosu towards a number of parasites including *leishmania* may be enhanced when combined with flavonoids.

6.3 Properties of Flavonoids

6.3.1 Antioxidant and Iron-Chelating Activities

Flavonoids have been reported to exert multiple biological effects, including antioxidant and free radical-scavenging abilities.[14] Their anti-radical property is directed towards highly reactive species implicated in the initiation of lipid peroxidation.[15] Flavonoids are soluble chain-breaking inhibitors of the peroxidation process, scavenging intermediate peroxyl and alkoxyl radicals.[16] Phenolic compounds also have been suggested to present a strong affinity for iron ions[17] which are known to catalyse many processes leading to the formation of free radicals. Thus, the antiperoxidative capability of flavonoids may be due to concomitant activities of scavenging free radicals and chelating iron.

6.3.2 Flavonoids as Scavengers of Nitric Oxide (NO)

Flavonoids are clinically used in diseases of the vascular wall involving inflammation and endothelial damage.[18] Venorutin, a semi-synthetic hydroxyethylrutoside mixture is used to reduce capillary permeability in chronic venous insufficiency and to protect the endothelial layer of blood vessels in diabetes mellitus.[19] In endothelial damage and inflammation free radicals play a major role. Most radicals possess both beneficial as well as undesirable effects.[20] This holds especially true for NO. On one hand NO is a key mediator in various physiological processes and on the other NO is toxic e.g. as a precursor of peroxynitrite.[21] Studies have shown that flavonoids are excellent NO scavengers. Their activity exceeds that of glutathione 10 to 1000 times.[22]

As mentioned previously NO has both beneficial as well as undesirable effects. NO is a mediator of inflammation[23] and plays a role in the defence mechanism of macrophages against micro-organisms.[24] NO generated by inflammatory cells is toxic,

probably after reaction with the superoxide anion which gives rise to peroxynitrite. Once protonated peroxynitrite may decompose to form the reactive HO· and the stable NO_2· as suggested by Beckman *et al.* [25] Scavenging NO radicals contributes to the therapeutic effect of flavonoids.

In blood vessels NO is produced by endothelial cells, upon stimulation of muscarinergic receptors. The NO diffuses into the vascular smooth muscle where NO-induced cGMP formation leads to muscle relaxation. Scavenging of this NO leads to vasoconstriction and possibly vascular damage. Flavonoids appear to accumulate between the endothelial layer and the vascular smooth muscle cells where a high concentration is reached.[26] This would favour the scavenging of the endothelial derived NO which is responsible for vasodilation. However at the site where flavonoids accumulate arteriosclerosis begins in a process where undoubtedly NO radicals display an unfavourable role.

A correlation between the intake of flavonoids and a low incidence of cardiovascular disease has been found.[27] Therefore, it is tempting to speculate that the NO· scavenging properties of flavonoids play an important role in their therapeutic effect. If indeed NO is one of the major protective features of flavonoids, how does the scavenger discriminate between the wanted and deleterious NO? Probably the local distribution of the flavonoid has to be taken into consideration.

These findings demonstrate the importance of flavonoids in many disease states and in the enhanced performance of qinghaosu towards the malarial parasite *Plasmodium falciparum.*

Leishmaniasis is often used as a model for malaria since malarial parasites (*plasmodia*) are extremely difficult to culture. By comparison with *Plasmodia* it is not unreasonable to expect enhanced activity of qinghaosu towards *leishmania* when combined with flavonoids.

6.4 Research programme

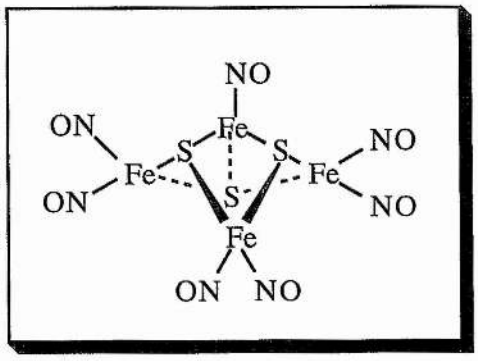
The previous discussion highlights the importance of NO in microbicidal activity including death of the intracellular parasite. Consequently, the antiparasitic activity of the NO donor compounds SNP, SNAP, GSNO and RBS were tested *in vitro* with *Leishmania mexicana* amastigotes.

The artemisinin family of compounds are potent antimalarials which show broad antiparasitic potential. They have been tested *in vivo* and *in vitro* with *Leishmania major* [7] and were found to exhibit antileishmanial activity. In this study qinghaosu, artemether and arteether were tested with *L. mexicana* amastigotes *in vitro*.

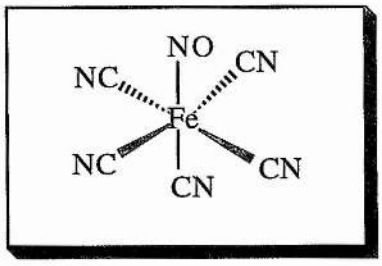
It has been shown[12] that many flavones are present in *A. annua* (the Chinese herb from which qinghaosu is extracted). Flavones are natural antioxidants, reducing agents, free radical scavengers (including NO) and are capable of chelating iron. They have been shown to potentiate the antimalarial activity of qinghaosu and therefore may enhance the activity of qinghaosu towards *L. mexicana*. In order to produce a range of methoxylated flavones the concept of combinatorial chemistry was employed. Combinatorial chemistry is a novel and innovative way of rapidly generating a number of related compounds. This idea has been adapted by the pharmaceutical industry over the last decade and has meant that a large number of compounds can be screened rapidly for biological activity.[28]

Test Compounds

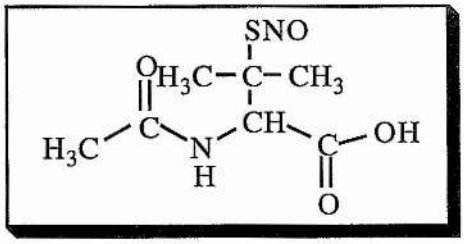
Roussin's Black Salt (RBS)



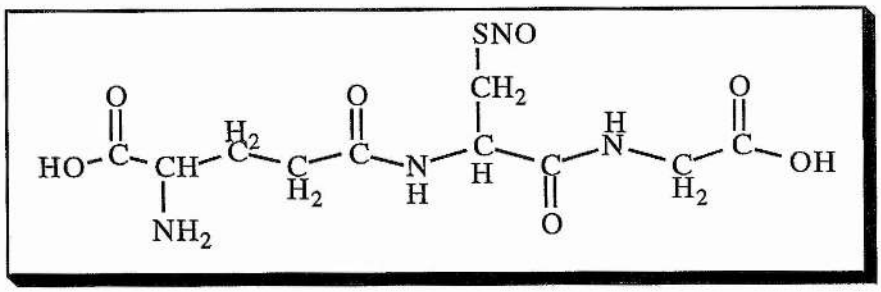
Sodium nitroprusside (SNP)



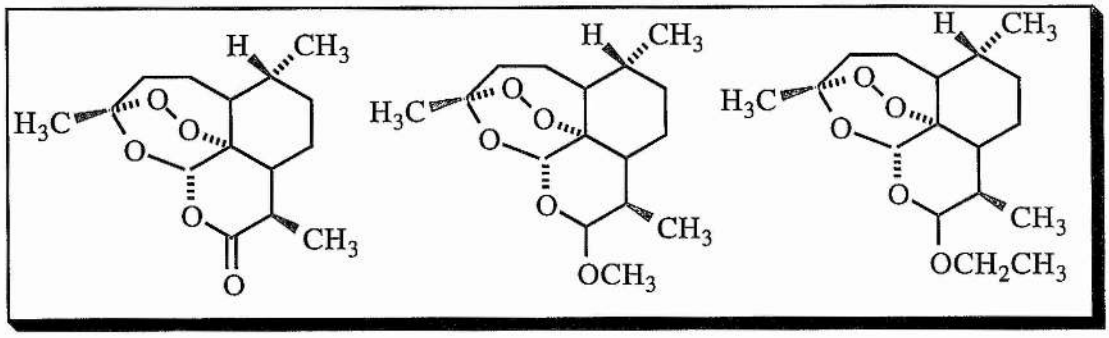
S-nitroso-N-acetylpenicillamine (SNAP)



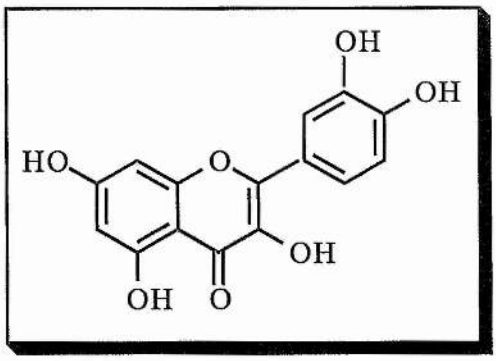
S-nitrosoglutathione (GSNO)



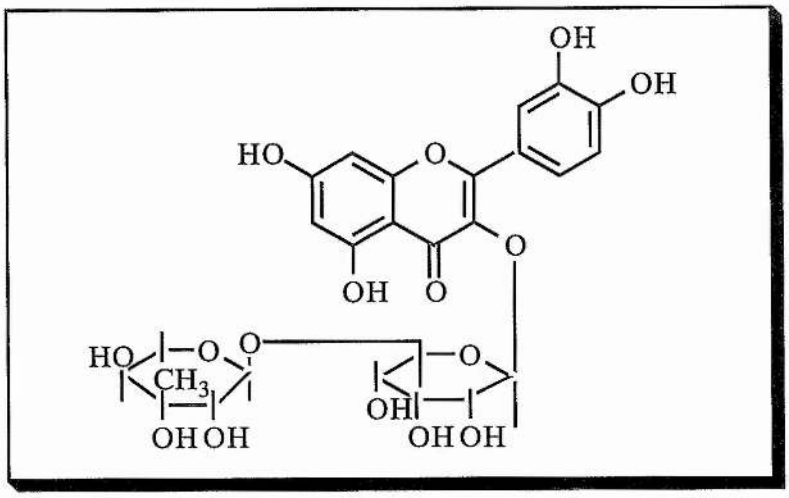
Qinghaosu **Artemether** **Arteether**



Quercetin



Rutin



A mixture of methylated quercetin products (some hydroxyl groups were replaced with methoxyl groups).

6.5 Experimental

Instrumentation and General Techniques

NMR spectra were recorded on a Varian Gemini spectrometer operating at 200 MHz for ^{1}H and 50.3 MHz for ^{13}C. Mass spectra were generated on an A.E.I. MS-50 spectrometer. Plates were harvested using a Wallac Pharmacia 1295-004 Betaplate 96 well harvester. In order to quantify the incorporation of radioactivity a Wallac Pharmacia 1205 Betaplate liquid scintillation counter was used.

Test Compounds

RBS, SNAP and GSNO were prepared as described in chapter 5.
Qinghaosu, artemether and arteether were gifts from the World Health Organisation.
Quercetin and Rutin were purchased from Sigma and SNP from BDH.

Preparation of Methylated Quercetin products[29]

Quercetin (2.03 g; 6 mmol) in acetone (9 cm^3) and pulverised sodium hydroxide (3.84 g; 0.1 mol) were warmed to 45°C with stirring, dimethyl sulphate (5.1 g; 0.04 mol) was added dropwise. This solution was warmed to 50°C for 90 minutes then 55-60°C for 3h. The solution was diluted with water and extracted with dichloromethane (3 x 18 cm^3), washed with water, 1 M hydrochloric acid and saturated sodium bicarbonate solution. The organic layer was dried over magnesium sulphate and the solvent removed under vacuum. The product was recrystallised from ethanol. Analysis confirmed, that upon methylation quercetin decomposed to form a mixture of products. At this stage no attempt was made to separate or characterise these products as the concept of combinatorial chemistry was being investigated.

Preparation of Culture Medium

Culture medium was prepared using Schneiders drosophila medium (SDM); Gibco BRL lot 28F5745, foetal calf serum (FCS); Labtech 206 and gentamicin sulphate.

In order to prepare media (200 cm^3); SDM (160 cm^3) was combined with FCS (40 cm^3) and gentamicin sulphate (25 $\mu g/cm^3$). The pH of the resultant solution was adjusted to 5.5 with 1 M hydrochloric acid.

Preparation of Amastigotes for Culture from a Lesion

A BALB/c mouse infected with *Leishmania mexicana* was killed by breaking its neck. It was placed in a rubber glove filled with 70% ethanol and soaked thoroughly. The lesion was dissected, cut into small pieces and pushed through a wire gauze into medium (10 cm^3). This suspension was taken up through a 19G needle into a syringe and pushed out through a $26G^{3}/_{8}$ needle to break open the mouse cells. The preparation was centrifuged at 1600g for 5 minutes, the supernatant was discarded and the pellet resuspended in medium (5 cm^3). The amastigotes were counted using a haemocytometer and resuspended to a concentration of 4×10^6 mol dm^{-3} per cm^3 medium.

Preparation of Test Compounds

The compounds to be tested were prepared at 2×10^{-2} mol dm^{-3} or at 7×10^{-2} mol dm^{-3} in medium and filter sterilised before being plated in 96 well flat-bottomed microculture plates and supplemented with medium. The amastigotes were resuspended to 4×10^6 mol dm^{-3} per cm^3 medium and pulsed with 1 μCi/well [^{3}H] thymidine then plated. Plates were incubated in triplicate at 33°C and harvested after 24 h. The incorporation

of radioactivity was determined using a β scintillation counter. The results were expressed as counts per minute (cpm).

Determination of the Antileishmanial Activity of NO Donor Compounds

The test compounds SNP, SNAP and GSNO were prepared at a concentration of 1 x 10^{-2} mol dm^{-3} in medium and plated as previously described in "preparation of test compounds".

Determination of the Antileishmanial Activity of Qinghaosu and Derivatives

Qinghaosu, artemether and arteether were prepared at a concentration of 1 x 10^{-2} mol dm^{-3} in medium and plated as previously described.

The Antileishmanial Activity of Qinghaosu and Derivatives

In a control experiment the test compounds qinghaosu, quercetin, rutin and a mixture of methylated products were prepared at a concentration of 7 x 10^{-4} mol dm^{-3} and plated as previously described.

The Effect of Flavones on the Antileishmanial Activity of Qinghaosu

Each of the compounds to be tested ; quercetin, rutin and methylated quercetin products were prepared at a concentration of 7 x 10^{-4} mol dm^{-3} and plated with qinghaosu also prepared at 7 x 10^{-4} mol dm^{-3} in medium.

6.6 Discussion

There has been much interest in the biological role of NO as a widespread transduction mechanism leading to a variety of functions in different cells. Activated macrophages form nitrite and nitrate from the terminal guanidino nitrogen atoms of L-arginine by a process known to proceed by via the formation of NO. This pathway is responsible for the cytotoxic action of macrophages, NO may disrupt iron-dependent enzymatic pathways vital to the survival of amastigotes in macrophages. Antileishmanial activity was suppressed in a dose dependent fashion by L-NMMA (a competitive inhibitor of NO from L-arginine). Excess L-arginine added to infected macrophage cultures reversed the inhibitory effects of L-NMMA.[6]

Of the NO donor compounds tested with *L. mexicana* amastigotes; RBS was insoluble in SDM (the medium crucial for parasite survival). Attempts were made to solubilise RBS in ethanol and in dimethyl sulphoxide (DMSO) and then supplement these solutions with SDM. However, in each case addition of medium caused RBS to come out of solution. Each solution (RBS in; SDM, DMSO/SDM and ethanol/SDM) was filtered and tested, but exhibited no toxicity towards *L. mexicana*, in contrast with the high toxicity of RBS observed with *E. coli* (chapter 5). It is likely that insufficient RBS dissolved in order to reproduce the toxicity previously observed.

SNP was more toxic than SNAP and GSNO at concentrations lower than 3.70×10^{-4} mol dm^{-3}. At higher concentrations (1×10^{-2} - 3×10^{-3} mol dm^{-3}) antileishmanial activity was comparable (appendix 1).

The greater toxicity exhibited by SNP at lower concentrations could be due to the transfer of NO^+ to cellular targets rather than to the release of NO.[30] The S-nitrosothiols SNAP and GSNO are NO donors and were less toxic than SNP towards *E. coli* (chapter 5). Previous research found that a saturated solution of NO did not inhibit parasite growth.[31]

Nitric oxide can react rapidly with superoxide to generate the peroxynitrite anion which is a more powerful oxidant than NO.[25] Peroxynitrite can decompose yielding hydroxyl radicals and it has been proposed that these radicals may be involved in tissue damage and killing attributable to NO. Assreuy *et al.*[32] have shown that authentic peroxynitrite failed to induce any cytotoxic effect towards *L. major* even at a high concentration.

Yang *et al.*[7] studied the effect of qinghaosu on *L. major* promastigotes *in vitro* and found that the parasites decreased progressively with increasing concentrations of qinghaosu and no viable parasites were detected at around 10^{-5} mol dm^{-3} by radioactivity incorporation. In addition, qinghaosu and artemether were capable of killing intracellular parasites with IC_{50} values of 3×10^{-5} and 3×10^{-6} mol dm^{-3} respectively.

The results show that at concentrations of 3.70×10^{-4} mol dm^{-3} and higher, arteether was approximately 50% more effective at killing *L. Mexicana* amastigotes than qinghaosu and artemether *in vitro* (appendix 2). The artemisinin family of compounds are characterised by having an endoperoxide bridge within the molecules. Whether the endoperoxide bridge is essential for their antileishmanial activity as it is for antimalarial activity is at present unclear. However, under *in vivo* conditions, metabolism of the endoperoxide linkage of qinghaosu and its derivatives may generate oxygen radicals which are detrimental to parasite macromolecules.

In contrast with the antileishmanial activity of NO donor compounds (appendix 1), qinghaosu and its derivatives were significantly less toxic towards *L. mexicana* amastigotes at concentrations greater than 3.70×10^{-4} mol dm^{-3}, indicating the superior toxicity of NO/NO^+ in comparison with metabolised qinghaosu species.

Previous work demonstrated the potentiating effect of methoxylated flavones on the antimalarial activity of qinghaosu.[13] It was considered that this potentiating activity may apply to a range of parasites including *leishmania*. Thus, methoxylated flavones may also potentiate the antileishmanial activity of qinghaosu.

The antileishmanial activity of qinghaosu was compared with the activity of flavones; rutin, quercetin and a mixture of methoxylated flavones. The activity of qinghaosu was consistent with the previous study (appendix 2) with the percentage initial [^{3}H] thymidine uptake 36.5% at 7.09 x 10^{-4} mol dm^{-3}. Quercetin was tested at concentrations 7.09 x 10^{-4} - 8.75 x 10^{-6} mol dm^{-3} and showed negligible antileishmanial activity. Rutin, tested at concentrations 7.0 x 10^{-4} - 8.64 x 10^{-6} mol dm^{-3} also had negligible antileishmanial activity. However, the mixture of methylated quercetins was toxic and killed *L. mexicana* amastigotes with IC_{50} between 7.78 x 10^{-5} - 2.59 x 10^{-5} mol dm^{-3} (appendix 3).

Qinghaosu was combined with each of the flavones mentioned previously in order to observe the effect on antileishmanial activity. Rutin and the mixture of methylated quercetin products were prepared at concentrations 1.75 x 10^{-4} - 5.47 x 10^{-6} mol dm^{-3} and plated with qinghaosu 1.75 x 10^{-4} - 2.75 x 10^{-6} mol dm^{-3}. Quercetin was prepared at 1.77 x 10^{-4} - 5.53 x 10^{-6} mol dm^{-3} and plated with qinghaosu as above. However, no potentiating effect was observed for any flavone, even although the mixture of methylated quercetin products tested in the absence of qinghaosu was toxic towards *L. mexicana*. This advantage was completely lost upon combination with qinghaosu and only slight antileishmanial activity was observed. This activity was comparable to that exhibited by qinghaosu alone.

Methoxylated flavones have been shown to exhibit antimalarial activity[13] and to some extent antileishmanial activity (appendix 3). However, upon combination with qinghaosu they are selective in potentiating the antimalarial activity of qinghaosu.

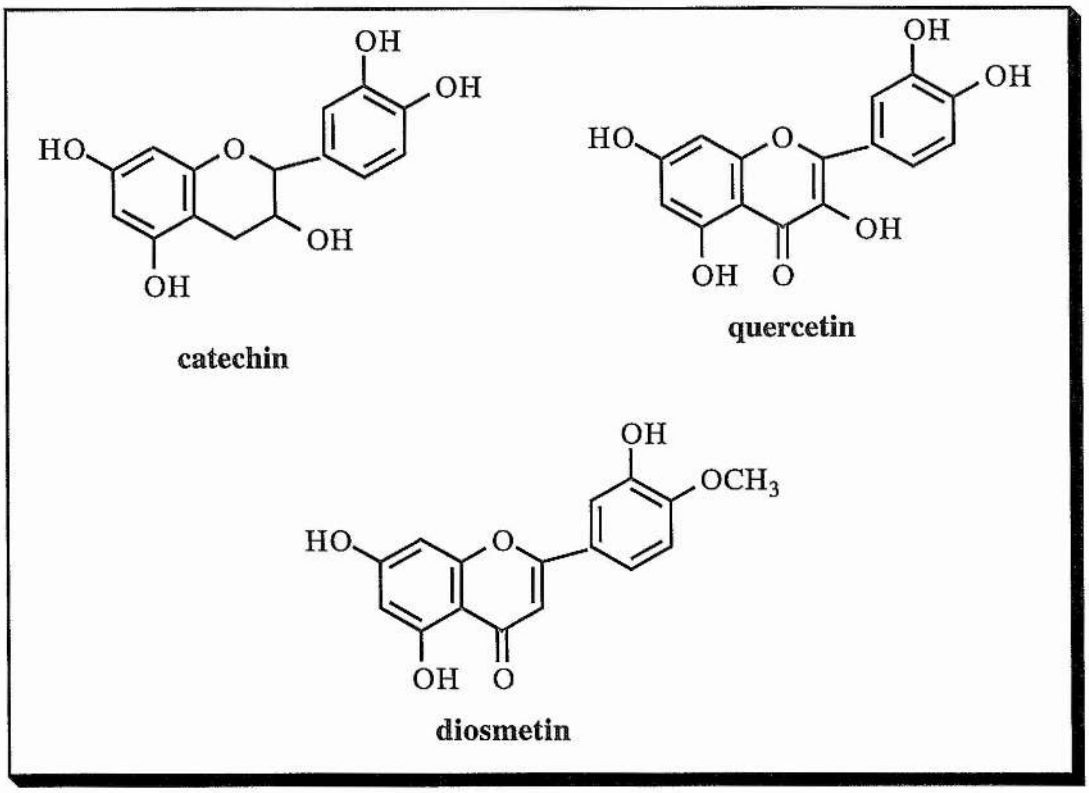
The results obtained may be explained by iron complexation. Qinghaosu and flavones complex iron. Iron is essential for the antimalarial activity of qinghaosu and there are many examples in the literature of iron chelators which exhibit antimalarial activity. Hershko *et al.*[33] explored the antimalarial effects of 3-hydroxypyridin-4-ones, a family of bidentate orally effective iron chelators and the iron chelator desferrioxamine (DFO)

in vivo and *in vitro*. DFO is the standard iron chelator in clinical use, it is a naturally occurring trihydroxamic acid derived from cultures of *Streptomyces pilosus.*

All 3-hydroxypyridin-4-ones were found to be more effective than DFO, which was relatively ineffective as an intraerythrocytic iron chelator. Hence, depriving the parasite of a metabolically important source of iron may represent a novel approach to antimalarial drug development.

Morel *et al.*[34] investigated the iron chelating ability of three flavonoids; catechin, quercetin and diosmetin using iron loaded rat hepatocyte cultures. They classified iron chelating ability as follows;

catechin > quercetin > diosmetin (which was quite devoid of effect)



DFO which was used as a reference remained the most effective agent.

The test compounds were poor iron chelators, with diosmetin (the methoxylated compound) being least effective. Therefore, the synergistic antimalarial effect observed when qinghaosu was combined with methoxylated flavones may be due to factors other than increased iron chelation.

All experiments using *L. mexicana* parasites were performed in an iron rich medium, which is crucial for parasite viability. When the flavones quercetin and rutin were tested with *L. mexicana* parasites no significant toxicity was observed. However, this was not the case for the mixture of methylated quercetin products which proved to be very toxic indeed. If iron chelation is a measure of toxicity then the results obtained conflict with those of Morel *et al* [34] whereby the methoxylated flavone (diosmetin) was a poor iron chelator. Therefore, the observed toxicity may be due to a mechanism distinct from iron chelation.

When qinghaosu was combined with the mixture of methylated quercetin products the previously observed toxicity was lost. Qinghaosu may complex iron, reducing the amount available to sustain parasite viability. In addition, the reaction between qinghaosu and iron results in the production of destructive free radicals which may cause parasite death. Both processes may account for antileishmanial activity. However, it is possible that these processes occur simultaneously and the antileishmanial activity observed is less than that of each process occurring singularly. In effect, negative synergism may explain our observations. Alternatively iron may be displaced by another metal, thus increasing its availability to sustain parasite viability, and consequently poor antileishmanial activity results.

In conclusion, it has been shown that qinghaosu (alone or combined with flavones) had no significant effect on *Leishmania mexicana* amastigotes *in vitro*, and consequently for the requirements of this study leishmaniasis proved to be a poor model for malaria.

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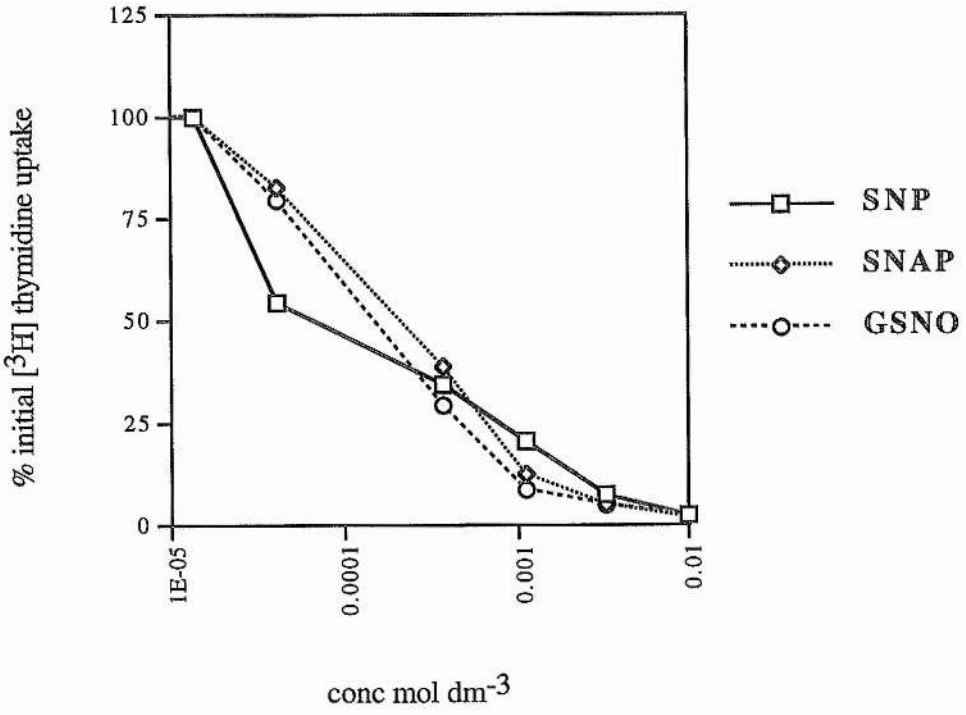
Appendices

Appendix 1

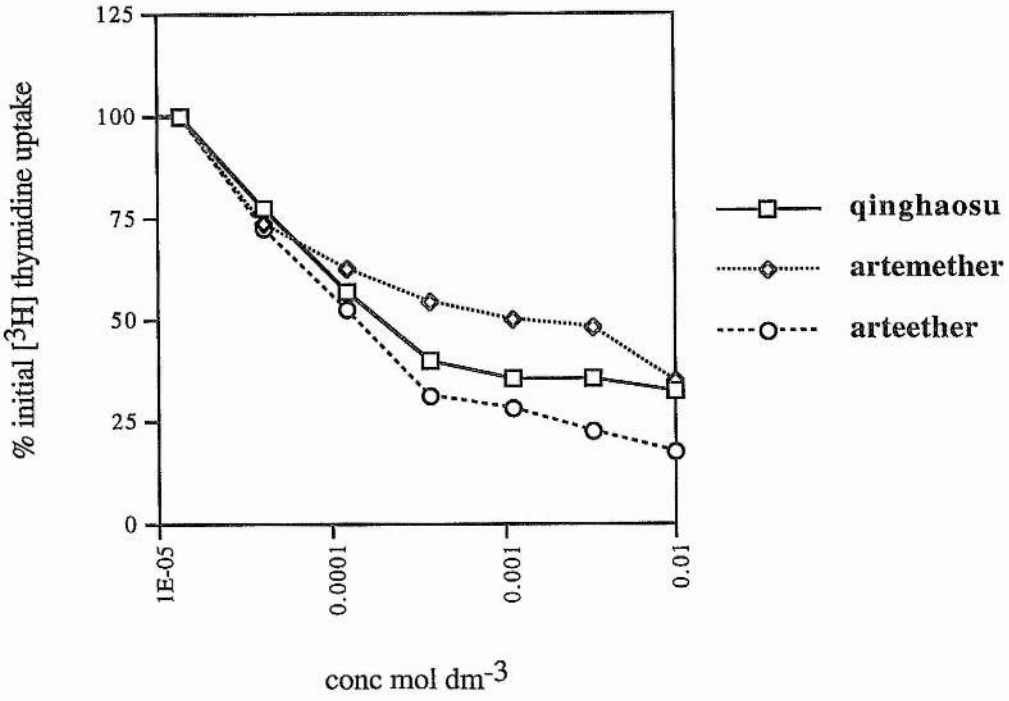
The Leishmanicidal Activity of SNP, SNAP and GSNO



conc (mol dm^{-3})	SNP	SNAP	GSNO
	(% initial [^{3}H] thymidine uptake)		
0	100	100	100
4.10×10^{-5}	54.1	81.9	79.1
3.70×10^{-4}	34.2	38.4	28.9
1.11×10^{-3}	20.2	12.2	8.4
3.33×10^{-3}	7.5	4.6	4.4
1.00×10^{-2}	2.1	1.4	2.5

Appendix 2

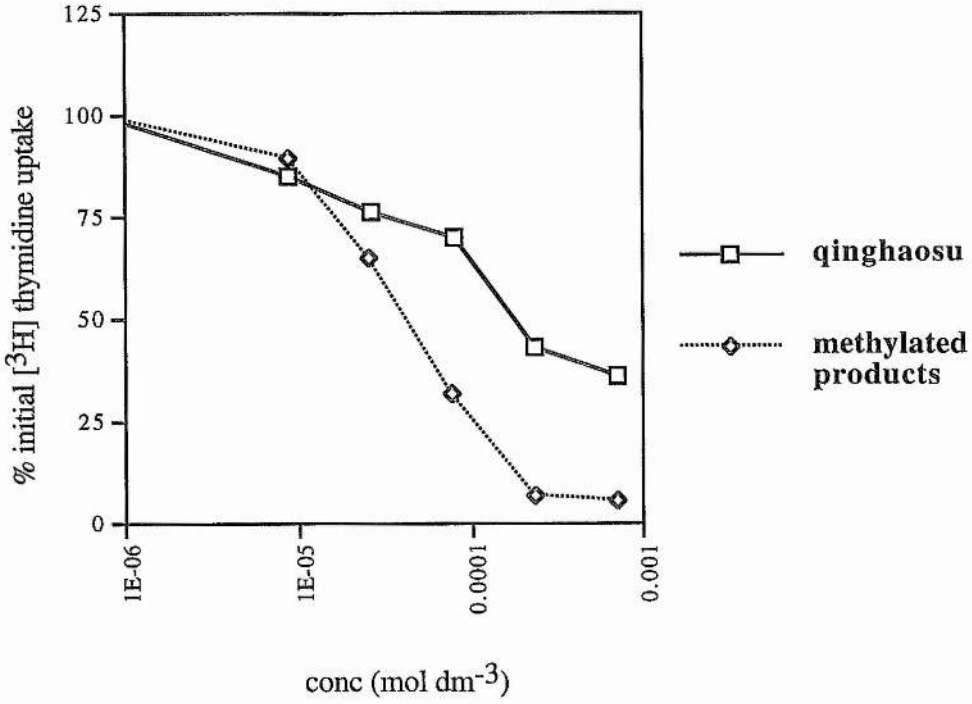
The Leishmanicidal Activity of Qinghaosu, Artemether and Arteether



conc (mol dm^{-3})	qinghaosu	artemether	arteether
	(% initial [^{3}H] thymidine uptake)		
0	100	100	100
4.10×10^{-5}	77.0	78.7	72.5
1.23×10^{-4}	56.7	62.3	52.3
3.70×10^{-4}	39.8	53.9	31.2
1.11×10^{-3}	35.3	49.5	27.7
3.33×10^{-3}	35.3	47.8	22.5
1.00×10^{-2}	32.1	34.8	17.2

Appendix 3

The Leishmanicidal Activity of Qinghaosu; a Comparison with Flavones



conc (mol dm^{-3})	**qinghaosu**	**quercetin**
	(% initial [^{3}H] thymidine uptake)	
0	100	100
8.75×10^{-6}	85.0	100
2.63×10^{-5}	76.1	100
7.88×10^{-5}	70.0	99.6
2.36×10^{-4}	43.2	99.2
7.09×10^{-4}	36.5	99.0

conc (mol dm^{-3})	**rutin**	**methylated products**
	(% initial [^{3}H] thymidine uptake)	
0	100	100
8.64×10^{-6}	100	89.3
2.59×10^{-5}	99.8	65.0
7.78×10^{-5}	99.6	31.6
2.33×10^{-4}	99.3	7.1
7.00×10^{-4}	95.8	5.4