An Investigation of Benzo[*g*]pteridine-2,4(3*H*,10*H*)-diones (Flavins) as Antimalarials

A Thesis

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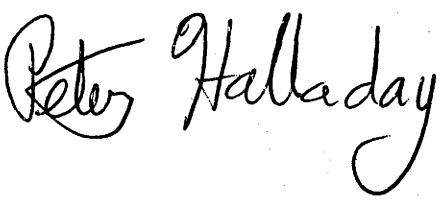
Statement

The work described in this thesis was carried out by the candidate at The Australian National University. Where the work of others was employed or quoted, appropriate acknowledgement and/or references are given.



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Abstract

This thesis describes an investigation of the antimalarial activity of a number of novel 10-phenylflavin analogues and includes a study of the structure activity relationships between several distinct series. This work was undertaken in order to define the structural features required for activity and to find analogues with improved activity and reduced toxicity.

One series of flavins was prepared to help identify the substitution profile in the 10-phenyl moiety associated with the highest biological activity. Another series of compounds was prepared in which the substituents at the 3-N position were varied. This group of flavins gave some insight into the role that lipophility played in activity.

Altering the benzenoid ring-3 of the flavin to produce a series of 7,8,9-substituted 3-methyl-10-(substituted phenyl)flavins and 10-(4'-chlorophenyl)-3-methyl-6,8-diazaflavin showed that changes to the ring-3 were not well tolerated.

Removing the ring-3 of the flavin to produce a series of 3-methyl-8-(substituted phenyl)pteridine-2,4(3*H*,8*H*)-diones showed that the 3-membered ring structure of the flavin was required for *in vivo* activity.

All of the above compounds were tested for antimalarial activity and the results discussed. Some of the above compounds were tested for antibabesial and anti-HIV activity and found to be inactive. The compound 3-ethyl-10-(3'-trifluoromethyl)flavin was tested for anticoccidial activity and found to be active.

The synthesis and some of the spectral properties of the above compounds are described and discussed.

The possibility that these flavins might exert their antimalarial action *via* the inhibition of host glutathione reductase was investigated and discounted.

Publications arising from this thesis

Manuscripts

Halladay PK, Hunt NH, Butcher GA, Cowden WB. Antimalarial action of flavin analogues seems not to be due to inhibition of glutathione reductase of host erythrocytes. *Biochem Pharmacol* 1990; **39**: 1063-1065.

Halladay PK, Cowden WB. 3-Methyl-8-(substituted phenyl)pteridine-2,4(3*H*,8*H*)-diones as potential antimalarials. *Aust J Chem* 1990; **43**: 1449-1454.

Abstracts

Halladay P, Hunt NH, Clark IA, Cowden WB. Identification of analogues of 10-(substituted phenyl)-3-methylflavin with increased antimalarial activity. (SP12). Proceedings of the Australian Biochemical Society, Vol. 20, 1988.

Halladay P, Hunt NH, Clark IA, Cowden WB. Identification of analogues of 10-(substituted phenyl)-3-methylflavin with increased antimalarial activity. (Abstract 431). Chemistry International Conference RACI, 1989.

Nomenclature

In this thesis the following common names have been used; primary benzenamines are referred to as anilines, pyrimidine-2,4(1*H*,3*H*)-diones are referred to as uracils and benzo[*g*]pteridine-2,4(3*H*,10*H*)-diones are referred to as flavins.

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CHAPTER 1

CHAPTER 1 Introduction

1-1 Malaria

1-1.1 The importance of malaria

Malaria is one of the world's major health problems. This is due to the wide distribution of the disease, the severity of its pathology and the resulting hindrance to socioeconomic development.

Malaria is distributed over approximately 100 countries, mostly in the tropical and highly populated areas of Africa, Asia, Central and South America. This means nearly 45% of the world's population live in malaria endemic or potentially endemic areas [1,2]. In 1988 five million cases of malaria were reported world-wide (excluding Africa), though the actual number is probably much higher as many cases go unreported. According to the World Health Organization (WHO) 100 million people are clinically ill with malaria at any given time and around one million die from it each year [2-4]. Malaria is not only a problem confined to countries in which its infected vector is present. The greatly increased amount of travel to infected areas due to tourism or business from malaria free areas has lead to many cases of "imported" malaria in countries such as Britain, the United States, France and Australia [4-6].

1-1.2 Malaria - the disease

Malaria is an infectious disease caused by parasites belonging to the genus *Plasmodium.* The large number of species in this genus have a great variety of hosts including mammals, reptiles and birds. Malaria parasites, to a large extent, are host specific and are only known to parasitize other species when they are closely related to the normal host [7].

Human malaria is caused by four plasmodial species, these are *Plasmodium ovale, P. malariae, P. vivax* and *P. falciparum. P. vivax* and *P. falciparum* are the species of greatest epidemiological importance as they are responsible for most human

malaria. *P. falciparum* is the cause of malignant tertian malaria which often produces a sudden intense disease in the non-immune patient. If untreated the infection may rapidly progress to fatal termination, this is frequently the result of cerebral malaria. *P. vivax*, the cause of benign tertian malaria, is responsible for much debilitating illness though very few deaths. It is the human malaria best known for relapses after treatment. *P. ovale* and *P. malariae*, broadly speaking, are less of a problem as they have a smaller distribution, are usually not fatal and have milder clinical symptoms.

The clinical symptoms of malaria may assume protean forms and a worthwhile review of the clinical features of malaria are given by Harinasuta and Bunnag [5]. Typical textbook cases of malaria involve a series of chronologically regular attacks. Briefly, the attack consists of firstly a feeling of chilliness and shivering followed by a feeling of intense heat. Both these stages of the disease are associated with a rise in temperature. The paroxysm can last for 2 to 6 hours and ends with profuse sweating and a gradual drop in temperature [4,8]. Between each attack patients may be asymptomatic. The periodicity of attacks is dependent upon the species of malaria. The intervals between attacks are less than 48 hours for *P. falciparum*, 48 hours for *P. vivax* and *P. ovale*, and 72 hours for *P. malariae*. Some of the prominent pathological features of the disease are enlargement of the liver and spleen, varying degrees of anaemia, and deposition of malarial pigment (the parasitic digestive residue of haemoglobin) in various organs and tissues of the body.

1-1.3 Life cycle of the parasite

Malaria parasites undergo a complex life cycle alternating between vertebrate and arthropod host. Figure 1-1 displays the life cycle of plasmodia in primates. What follows is a brief description of the generalized life cycle of the parasite in primates. More precise and exhaustive reviews of this subject are given in books by Peters [9a], Garnham [10a], and Peters and Richards [11].

The female Anopheles mosquito is the vector for malaria. Sporozoites are injected into the vertebrate host *via* the saliva during the blood feed of an infected vector.

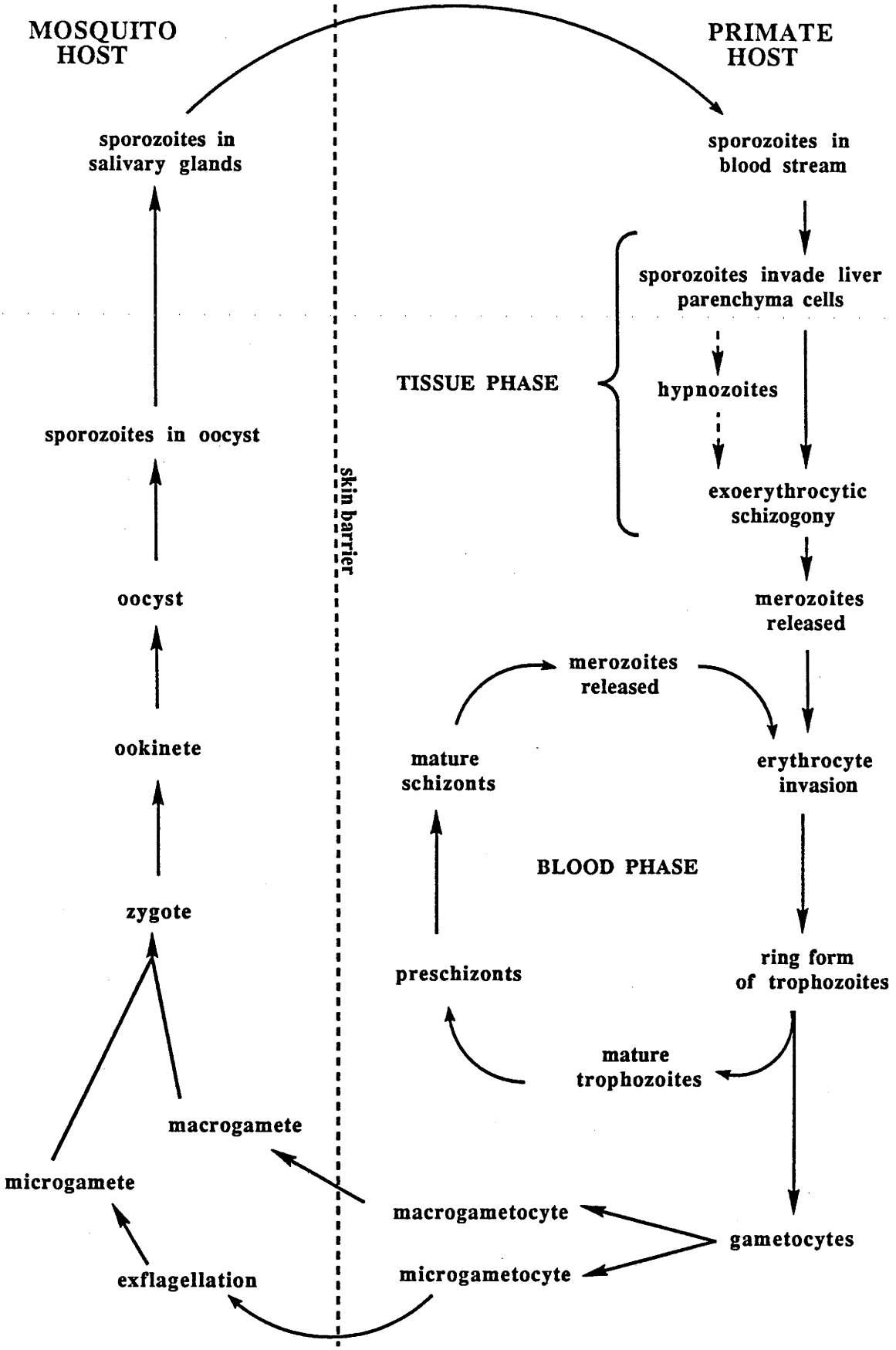


Fig 1-1 Life cycle of plasmodia in primates

Once in the blood stream the sporozoites invade the parenchyma cells of the liver.

In this tissue phase the sporozoite becomes rounded as it develops into a schizont. The schizont's nucleus then undergoes repeated divisions. Once this is complete the cytoplasm segregates to form many merozoites inside the schizont. The now fully developed schizont and liver cell walls rupture to release large numbers of merozoites into the blood stream. This tissue phase of asexual reproduction is known as tissue or exoerythrocytic schizogony. In mammalian malaria only a single generation of exoerythrocytic schizogony occurs. In some species of plasmodia (eg *P. vivax*) not only do the sporozoites undergo tissue schizogony but some also form a latent stage, called hypnozoites, which lie dormant for varying time periods before undergoing exoerythrocytic schizogony. This latter event accounts for the "recurring" nature of *P. vivax* infections.

The blood phase starts with the invasion of erythrocytes by merozoites released from the tissue phase. After entering the cell the invading merozoite feeds on the haemoglobin of the cell. This uninuclear feeding form of the parasite is termed a trophozoite. The parasite now begins to go through another cycle of asexual multiplication. The nuclear material of the parasite increases and undergoes several divisions resulting in a number of nuclei which are situated in the cytoplasmic syncytium. At this stage the parasite is referred to as a preschizont. The preschizont becomes a mature schizont when the cytoplasmic syncytium divides to form completely differentiated merozoites. When the mature schizont bursts it liberates the merozoites (the number of merozoites released is species dependent) into the blood. These merozoites quickly infect other erythrocytes. This cycle of asexual reproduction is known as blood schizogony and is associated with disease symptoms.

After infecting a new erythrocyte the merozoite can either initiate a new cycle of blood schizogony or transform into either a micro (male) gametocyte or macro (female) gametocyte. Blood schizogony continues until either the parasite is completely eradicated by drug treatment, the host's immune system or the death of the host.

The sexual phase in the parasite's life cycle begins when the gametocytes are taken up by a suitable arthropod. In the insect's midgut the gametocytes shed the

erythrocyte membrane and mature into gametes. The macrogametocyte quickly forms the macrogamete with few evident morphological changes, retaining its rounded structure. The microgametocyte on the other hand undergoes a dramatic maturation process termed exflagellation. In this process the nucleus divides three times, forming usually eight nuclei which combine with the cytoplasm to form microgametes. These microgametes move through the mid-gut, with the aid of a flagellum, in search of a macrogamete. Once a microgamete reaches a macrogamete fertilization takes place. The newly formed zygote remains quiescent for a period before it becomes mobile. The latter form, known as an ookinete, actively invades the intestinal ephithelium of the arthropod and lodges itself beneath the basal lamina, forming an oocyst. The diploid nucleus of the oocyst undergoes meiotic division, followed by intensive mitotic divisions, while the cytoplasm maintains its syncytial structure. Finally the cytoplasm divides to form sporozoites. The elongated and mobile sporozoites migrate from the oocyst to the salivary glands of the arthropod host, where they are ready to infect a new vertebrate host.

1-1.4 Chemotherapy of malaria

The chemotherapy of malaria is determined by the therapeutic objective of the treatment and the effectiveness of drugs against different species, strains and life cycle stages of the parasite.

As could be expected by the many forms that the parasite goes through in its life cycle, existing antimalarial drugs have differing levels of effectiveness on different stages in the parasite's life cycle. This has lead to the classification of drugs in terms of the section of the life cycle they effect. The following terms have been used:

Blood schizontocide - refers to a drug which acts upon asexual parasites in the blood;

Tissue schizontocide - refers to a drug which acts upon asexual parasites in the tissues. This includes drugs active against hypnozoites, more specifically referred to as hypnozoitocides;

Gametocytocide - refers to a drug which acts upon the sexual forms of the parasite in the blood; and

Table 1-1: Action of some antimalarial drugs against different life cycle stages of the malaria parasites [a]

Antimalarial	Activity status against different life cycle stages of the human malaria parasites [b,c]			
	Erythrocyctic asexual phase	Tissue phase (excluding hypnozoites)	Hypnozoites	Gametocytes
Chloroquine	active and fast acting	not active	not active	active for *P. vivax* and *P. malariae*, not active for *P. falciparum*
Primaquine	weak activity	weak activity	high activity	high activity
Mefloquine	active and fast acting	probably not active	probably not active	active for *P. vivax* and *P. malariae*, not active for *P. falciparum*
Proguanil	active but slow acting	active against *P. falciparum* and *P. ovale*	not active	highly active
Pyrimethamine	active but slow acting	active	some activity for *P. vivax*	uncertain
Sulfones and Sulfonamides	weak activity	possible action	uncertain	uncertain
Tetracycline	active but slow acting (only known for *P. falciparum*)	active (only known for *P. falciparum*)	not active for *P. vivax*	not active (only known for *P. falciparum*)

[a] Information for this table was obtained mostly from reference [16,16a]. [b] The sporozoites are not included in this table as none of these antimalarials is known to be active against them. [c] As more research in this area is conducted the information in this table is subject to change.

Sporontocide - refers to a drug which acts upon the sporogonic forms in the mosquito after it has fed on the treated host.

In Table 1-1 the differences in activities among some antimalarials against life cycle stages as well as between species are demonstrated.

The interplay between the parasite life cycle and symptomatology of malaria needs to be understood when using drugs to treat malaria. The attacks of malaria occur during blood schizogony whereas no symptoms are noticeable in the tissue schizogony phase. To alleviate symptoms effective blood schizontocides should be used. The relapse phenomenon, in which months or even years after the initial infection has been cleared malaria reappears, is due to the dormant hypnozoites which is characteristic in the human plasmodia species *P. vivax* and *P. ovale*. In infections of this kind the use of hypnozoitocides must be considered.

Treatment regimens for malaria have different therapeutic goals which are related to the species of infecting plasmodia, the chances of reinfection, the severity of the infection and the toxicity of the drug to the individual. The development of drug resistance by plasmodia has lead to further complications in treatment. This means that not only the species of the infecting *Plasmodium* but also its likely drug resistant status should be known before treatment regimens are decided. Different treatment aims have been classified as follows:

Causal prophylaxis - this refers to the use of drugs that exert a lethal effect on malaria parasites during the pre-erythrocytic stages, i.e. the sporozoites and/or the tissue stages. In this way prevention of acute attacks of malaria is effected. This treatment is usually over long periods and accordingly the drug must be well tolerated.

Suppressive treatment - this refers to the use of blood schizontocides to prevent the clinical manifestation of the disease by elimination of asexual parasites from the blood. The treatment as for causal prophylaxis usually occurs over long periods so the drug used should be well tolerated.

Clinical cure - this refers to the termination of a clinical attack of malaria by interrupting blood schizogony with blood schizontocides. Drugs of choice here should be fast acting as the progress of the disease to fatality is often very rapid.

Radical Cure - refers to treatment with drugs that eradicate all asexual stages from the body. There is little point trying to effect a radical cure while still in an endemic area because of possible reinfection.

Suppressive cure - refers to complete elimination of all asexual stages of the parasite by continued suppressive treatment.

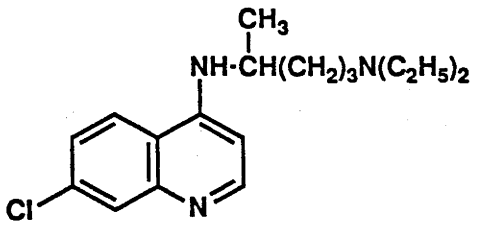
Gametocytocidal therapy - refers to agents which destroy the sexual forms of malarial parasites in human blood and thereby reduce the reservoir of infection.

1-1.5 Currently used antimalarial drugs

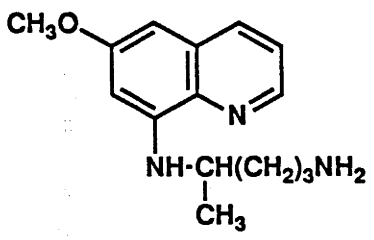
(i) The 4-aminoquinolines

Chloroquine is a member of this group and is the most commonly used and well known of all the antimalarial drugs. Other compounds in this series include hydroxychloroquine and amodiaquine, the structure of chloroquine is shown in Fig 1-2. Chloroquine, the best example of the series, is very effective against the asexual erythrocytic forms of all human plasmodia. It is the drug of choice in the treatment of an acute malarial attack as it rapidly controls parasitemia and thus, clinical symptoms. The good blood schizontocidal activity coupled with chloroquine's low toxicity means it is commonly used for suppressive treatment. It is not, however, effective against hepatic forms of the parasite and is only used to obtain suppressive cures usually in the case of *P. falciparum* where the tissue stage of the parasite does not persist. The spread of chloroquine-resistant plasmodia, especially *P. falciparum,* is increasing dramatically, making this drug less reliable as an effective treatment than it once was.

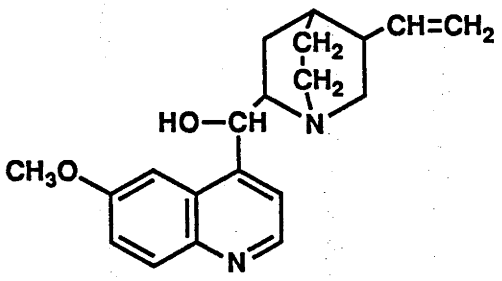
Fig 1-2 Chemical structures of currently used antimalarials



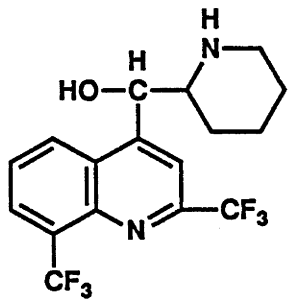
Chloroquine



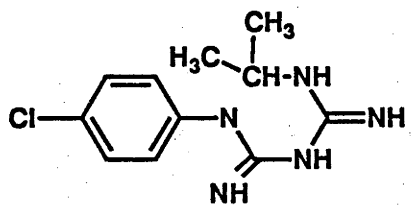
Primaquine



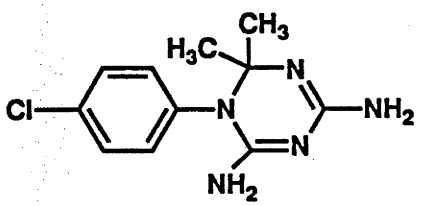
Quinine



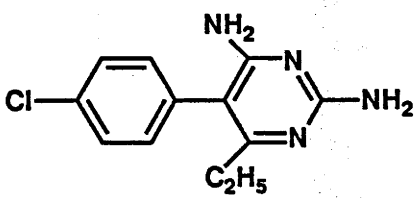
Mefloquine



Proguanil



Cycloguanil



Pyrimethamine

The mechanism of action is as yet uncertain but it appears, in part, to be a result of preferential accumulation in parasitized erythrocytes. Two of the present theories on its mode of action are [12];

(1) chloroquine concentrates inside the parasite's food vacuole and raises the intralysosomal pH, thereby inhibiting digestion of host protein, and

(2) ferriprotoporphyrin IX, a product of the malaria parasite's digestion of haemoglobin, tightly binds chloroquine forming a toxic chloroquine-ferriprotoporphyrin complex.

(ii) The 8-aminoquinolines

Primaquine is the only example of an 8-aminoquinoline in common current clinical use; its structure is shown in Fig 1-2. Other members of this series include pamaquine and quinocide. Primaquine, unlike chloroquine has weak blood schizontocidal activity but is highly active against the exoerythrocytic forms of *P. vivax* and *P. falciparum.* Its great clinical value lies in its hynozoitocidal activity which allows radical cures in the case of *P. vivax.* Primaquine has to be administered with care as it can cause haemolytic anaemia in a significant proportion of the population which are genetically glucose-6-phosphate dehydrogenase deficient. Primaquine is also noted for its good gametocytocidal activity against all human malarias. This is probably responsible for the infrequent observation of primaquine resistance [13]. This is fortunate since the 8-aminoquinolines are the best antimalarial agents for elimination of the latent tissue stages of relapsing malaria. Little is known of the mode of action of the 8-aminoquinolines.

(iii) The 4-quinolinemethanols

The two drugs of major interest from this series are quinine and mefloquine (structures shown in Fig 1-2). The alkaloid quinine was first used as an antimalarial in the sixteen hundreds in the form of a crude extract of the bark of the cinchona tree. Later it was isolated and used directly until early this century when synthetic antimalarials became available.

Although, quinine was superseded by newer less toxic synthetic drugs the advent

and increase of multi-drug resistant strains of malaria has seen it brought back into use as an important alternate treatment. Its primary action is as a rapid blood schizontocide for all human malarias and a gametocytocide for *P. vivax* and *P. malariae*.

Mefloquine is a relatively new derivative (clinical trials began in 1972) of quinine which has been found to be an effective, reasonably well tolerated and safe antimalarial drug. It is highly active against blood forms, with its major use as a treatment for chloroquine-resistant *P. falciparum*. Presently its use is being rigidly controlled in order to prevent the rapid occurrence of mefloquine resistance.

(iv) The biguanides

These drugs of which proguanil (shown in Fig 1-2) is representative, differ considerably in their structure from the already discussed antimalarials. It has been shown that biguanides act as a prodrug and the active form is a dihydrotriazine formed during the metabolism of the biguanides. These dihydrotriazines have themselves been made and used directly as antimalarials, an example of one of these is cycloguanil shown in Fig 1-2. The discovery of proguanil represents an important advance made in the chemotherapy of malaria. It is an effective blood schizontocide for all four human plasmodia and is one of the most innocuous of the antimalarials. These properties make it highly suitable for suppressive treatment. Its use in achieving clinical cures is limited by a slow onset of action compared to the 4-aminoquinolines. Proguanil has gametocytocidal activity against *P. falciparum* and *P. vivax* and has activity against the tissue stages of *P. falciparum* and *P. ovale* but not against the hypnozoites of *P. vivax*. The biguanides exert their antimalarial activity by selectively inhibiting parasitic dihydrofolate reductase, a key enzyme in the folic acid cycle which is necessary for the synthesis of methionine, glycine, thymidine and the purines. The spread of proguanil resistance has been reported in all species of malaria except *P. ovale* [14]. To overcome this resistance potentiating combinations of drugs were introduced. The biguanicides are used in association with sulphones (usually dapsone) and sulphonamides (such as sulfadoxine) themselves mild antimalarials. The sulphones and sulphonamides inhibit another stage of the folic acid cycle of the parasite. Unlike man, plasmodia must

synthesise folic acid *de novo*, the sulphones and sulphonamides inhibit this biosynthesis. These inhibitors of two different steps in the same biochemical pathway when used in combination have a strong synergistic effect.

(v) The diaminopyrimidines

Pyrimethamine (shown in Fig 1-2) and trimethoprim are the two compounds of this group used as antimalarials. Like the biguanides, they function by inhibition of parasitic dihydrofolate reductase and as such have many similar pharmacological properties. Pyrimethamine is active against blood forms of plasmodia generally and as such is used mainly in suppressive treatment. Like proguanil, pyrimethamine is slow acting and well tolerated. Resistance to pyrimethamine developed quickly in many areas of the world [14] and cross resistance to other antifolates meant that pyrimethamine, like the biguanides, is normally used in combination with sulfones or sulphonamides to increase its antimalarial activity. Maloprim, which contains pyrimethamine and dapsone, and Fansidar, which contains pyrimethamine and sulfadoxine, are two widely used examples of such pharmaceutical preparations.

A more complete and extensive review of the chemistry and pharmacology of antimalarials can be found in monographs by Thompson and Werbel [15], and Bruce-Chwatt *et al.* [16].

1-1.6 Drug resistance

A major threat to the effective control of malaria has been the development of drug resistance. Table 1-2 shows when some of the common antimalarials were first introduced and when resistance developed to them. *P. falciparum* has been most prone to develop drug resistance especially to chloroquine. This type of resistance is increasing at an astounding rate both in its intensity and geographically [17,18]. Chloroquine resistance is well established over large parts of South East Asia and the Amazon basin and is now spreading over Africa [18,19].

Table 1-2: The occurrence of drug resistance to common antimalarials [a]

Antimalarial drug	Year in which clinical use began	Year and location in which resistance was reported for *P. falciparum*
Quinine	*ca* 1630	1910 Brazil
Primaquine	1951	1963 Colombia
Chloroquine	1945	1960 Venezuela
Proguanil	1948	1949 Liverpool
Pyrimethamine	1951	1952 Gambia
Sulfadoxine	1964	1968 Cambodia
Dapsone	1965	1968 Cambodia

[a] Information obtained from reference [9b].

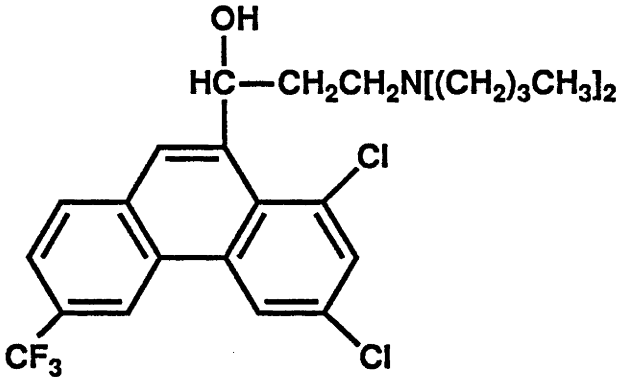
Furthermore, the appearance of strains of *P. falciparum* which are resistant to other drugs and even combinations of drugs have compounded the problems of increased virulence and drug resistance. Drug resistance has occurred as a result of wide-spread, indiscriminate, inappropriate or incomplete treatment and failure to combat transmission. These conditions are favourable for the occurrence of genetic changes in the parasite which allow it to develop tolerance to particular compounds [20].

A number of strategies have been introduced to overcome and slow the spread of resistance. These include developing new antimalarial agents, using combinations of drugs in treatment and more thoughtful dispensing practices for existing drugs.

The development of new drugs effective against malaria is a long slow process, only a few have become available over the last twenty years. The most valuable of the new compounds are those that have a unique mode of action; these avoid problems of cross resistance with the existing antimalarials.

In an endeavour to find new drugs to treat malaria some useful candidates have been identified including mefloquine, halofantrine, artemisinin and some antibiotics.

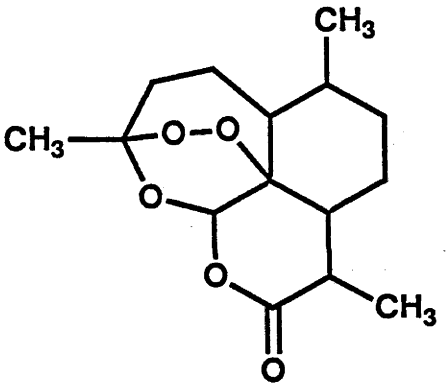
Mefloquine (discussed in section 1-1.5(iii)) has been made available for clinical use and acts as a potent blood schizontocide and is active against multi-drug resistant falciparum malaria. Mefloquine's structural similarity to quinine lead to concerns about the possibility of cross resistance occurring. This was borne out by reports of mefloquine resistance occurring in areas where mefloquine had not yet been used. The first of these were from Thailand where quinine-resistant strains were already wide-spread [21]. Halofantrine (structure shown below), though not at the same stage of development as mefloquine, has schizontocidal activity against chloroquine-resistant strains of *P. falciparum* [21]. Even though it is based on the phenanthrene ring, and not the quinoline ring structure, it is suspected that it has a similar mode of action to quinine and mefloquine, again raising the strong possibility of cross resistance occurring.



Halofantrine

Some antibiotics such as chloramphenicol and tetracycline have been shown to be antimalarials and have been used mostly to treat drug-resistant strains of malaria. Tetracycline has been shown to have some activity against liver stages and marked activity against blood stages of the parasite [9c]. The onset of activity of the antibiotics is slow so they are often used in combination with other fast acting antimalarials. The advantages of these antibiotics are that they probably have a different mode of action from other antimalarials and they are already in clinical use, thus reducing the need for extensive pretesting. The most exciting of the new antimalarials are compounds based on artemisinin (qinghaosu), a natural product first isolated and characterised in 1972 from the wormwood, *Artemisia annua Linnaeus*. Extracts of this plant were used

historically as a Chinese folk remedy to treat malaria and other fever-associated diseases, references to it date back to the middle of the fourth century [22]. Artemisinin has a structure (shown below) unlike all presently used antimalarial drugs. It is a sesquiterpene lactone consisting of four rings and contains a peroxide bridge. This novel compound has been shown to have a different mechanism of action from both chloroquine and the antifolate drugs [22]. The necessity of the endoperoxide bridge for activity [22] and the apparent interference to membrane structures of the parasite [23] have lead some authors to suggest that the mode of action may be due to free oxygen radical-induced stress [22,24,25]. Artemisinin and its derivatives are quick acting blood schizontocides with low toxicity, and low cross resistance to chloroquine and other antimalarials. Two reviews on artemisinin have been published by Luo and Shen [22], and Anand *et al.* [26].



Artemisinin

The use of appropriately selected drug combinations is a necessary practise in the treatment of malaria to retard the spread of drug resistance. Use of a single compound may lead to resistance by causing genetic alteration of some aspect of the parasite. The basis of combination drug therapy in overcoming drug resistance is that the development of genetic changes in the parasite allowing it to combat two or more drugs at once is far less likely.

It is possible with different combinations of the existing antimalarials to produce different effects. There are combinations of drugs which have complementary, additive and potentiating effects. Complementary combinations are those which may act on different stages of the parasite's life cycle such as a combination of chloroquine and

primaquine. Chloroquine acts on the blood schizonts whereas primaquine eliminates the hypnozoites and the gametocytes. Combinations such as chloroquine and pyrimethamine have an additive effect when used together whereas the combination of antifolates in Maloprim and Fansidar are found to be synergistic. The use of combination drug therapy has been applied to slow the development of mefloquine resistance by the triple combination of mefloquine, sulfadoxine and pyrimethamine know collectively as Fansimef. Care must be taken not to use a drug combination in areas where malaria resistance to one of the drugs in the combination already exists, as this lessens the antimalarial activity of the combination and defeats the purpose of the combination therapy in slowing the development of resistance [27].

The realization that drug resistance is still likely to grow, even with the advent of new antimalarials and drug combinations, has prompted careful dispensing practices to be undertaken. These practices were stated by WHO [28 [a]] and include;

1) the cessation of mass distribution of antimalarial drugs and chemotherapeutic targeting to defined high risk groups or the control of epidemics,

2) use of new antimalarials should be tightly controlled and limited to cases involving drug resistant malaria,

3) knowledge of the drug resistant status of the parasite to allow the appropriate use of existing, new and combinations of antimalarials to minimize development of resistance, and

4) that a complete course of therapy be used to effect a successful treatment and prevent transmission.

The above points aim to reduce drug resistance by lowering the rate of contact or sub-therapeutic contact between the plasmodia and antimalarials or by lowering the chances of transmission of parasite populations which have been exposed to drugs.

A complete work on drug resistance in malaria has been written by Peters [9]. A recent review by Björkman and Phillips-Howard [29] deals with the current epidemiology of drug resistant malaria.

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[a] Originally from, *Advances in malaria chemotherapy*, WHO Technical report series No. 711, WHO, Geneva, 1984.

1-1.7 Malaria control and vaccine

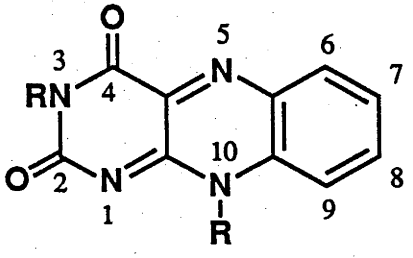
Other methods of combating malaria besides the use of chemotherapy include the traditional method of vector control and the revived hope of a malaria vaccine. Vector control includes among other methods the use of insecticides, destruction of mosquito breeding areas, bed nets impregnated with insecticides, house screening and introduction of specific mosquito pathogens [4].

The hope first generated by the idea of a malaria vaccine was high, however, it has become apparent in the last few years that the high degree of antigenic polymorphism of the parasite makes vaccine development difficult [3,30]. This has been reflected in the disappointing trails of candidate vaccines against sporozoite and asexual blood stages of malaria [31].

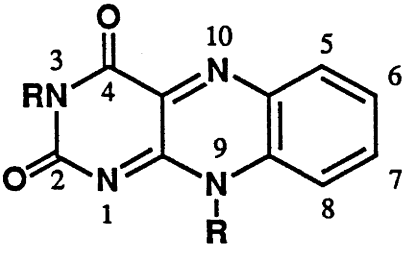
1-2 Flavins

1-2.1 Structure and nomenclature

The trivial though common names of benzo[*g*]pteridine-2,4(3*H*,10*H*)-dione are isoalloxazine and flavin. The new I.U.P.A.C. numbering system of benzo[*g*]pteridine-2,4(3*H*,10*H*)-dione, shown below in structure **1.1** replaces the original German numbering system shown in structure **1.2**. For brevity benzo[*g*]pteridine-2,4(3*H*,10*H*)-dione will be referred to as flavin throughout the rest of this work and the numbering system in formula **1.1** will be used.



1.1



1.2

1-2.2 The history of flavins as antimalarials

The association between flavins and malaria began in 1944 with the observation of Seeler and Ott [32] that riboflavin deficiency in chicks infected with *Plasmodium lophurae* had the effect of depressing parasitemia during the course of the disease.

Thirty-nine years later this phenomenon was renoted by Thurnham *et al.* [33] who found there was a relationship between riboflavin status in Papua New Guinean infants and their susceptibility to malaria. It was found that the number of infants with malaria and normal riboflavin levels was significantly higher than would be expected statistically compared to those infants with riboflavin deficiency. This suggested riboflavin deficiency may be malaria protective. To confirm this finding a similar experiment to that of Seeler and Ott was conducted by Kaikai and Thurnham [34]. Using *Plasmodium berghei* in rats they showed that riboflavin deficiency depressed parasite counts and that the depression was inversely related to the riboflavin status.

In an effort to determine the minimal nutritional requirements of *P. falciparum* in culture Divo *et al.* [35] found that the supply of exogenous riboflavin was not necessary for normal parasite growth over a 96 hour period. This is a similar finding to that of Siddiqui and colleagues [36] who, in an *in vitro* study, found no effect on the growth of *P. knowlesi* after 24 hours of riboflavin deficiency. These findings seemingly conflict with the *in vivo* results above. However, the nutrients necessary for parasite success cannot always be identified by depleting them from the culture medium as there may be intraerythrocytic stores of nutrients that are sufficient for parasite viability. Divo *et al.* admits this in their paper and to quote William Trager [37], one of the pioneers in *in vitro* parasitology, "Only in such medium (an axenic parasite culture in which the host is excluded) can all of the nutritional requirements of an organism be determined ...".

Geary, Divo and Jenson [38] undertook an antimalarial screen of antimetabolite drugs based on analogues of riboflavin, nicotinamide, pyridoxine and thiamin using the minimal medium developed by Divo *et al.* [35] in order to enhance any parasiticidal activity. This study showed *P. falciparum* growth inhibition was most profound for riboflavin analogues (antagonists) over 96 hours in the minimal medium.

Dutta *et al.* [39] in 1985 put forward the hypothesis that interference with riboflavin metabolism could possibly provide protection against malaria. They suggest that metabolic inhibitors of riboflavin metabolism should be investigated for their therapeutic potential as antimalarial agents. They state [40] "investigation of the possible chemotherapeutic efficacy of metabolic inhibitors of riboflavin which may have selective antagonistic effects on *Plasmodium* is vital".

Cowden *et al.* [41] reported the results of an *in vivo* antimalarial screen using a range of riboflavin analogues. In that work they found 10-(4'-chlorophenyl)-3-methylflavin (Fig 1-3) to have significant activity against *P. vinckei vinckei* in mice and against *P. falciparum* in culture. They also showed that 10-(4'-chlorophenyl)-3-methylflavin was well tolerated at the doses needed to obtain cures. In a continuation of this work a number of analogues based on 10-(4'-chlorophenyl)-3-methylflavin were synthesised and tested by Cowden *et al.* [42]. In that paper they were able to undertake a limited structure activity relationship (SAR) study, the main conclusions of which were;

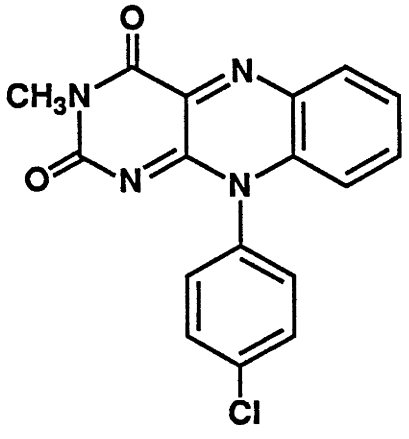
(1) for compounds with 4'-halo substituents the order of antimalarial activity was Br > Cl > F,

(2) 2'-substituents lowered or abolished activity,

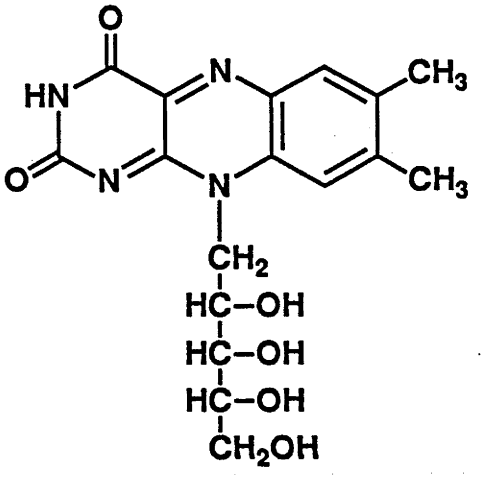
(3) the 3-methyl group was required for activity, and

(4) the halophenyl group had to be directly attached to the N-10 position to maintain activity.

Fig 1-3 Chemical structures of riboflavin and its antimalarial analogue 10-(4'-chlorophenyl)-3-methylflavin



10-(4'-chlorophenyl)-3-methylflavin



riboflavin

1-3 Scope of this thesis

10-(4'-Chlorophenyl)-3-methylflavin represents a structurally novel antimalarial compound. The spread of drug resistance has heightened the need for new malarial drugs. With this in mind the present study was undertaken, primarily to investigate the structure activity relationship between analogues of the lead compound and their antimalarial activity. This was done to define the structural features required for activity and to find analogues with improved activity and reduced toxicity. Other aspects dealt with and discussed are the syntheses and some of the physical characteristics of these compounds, the electrophilic substitution of the parent structure (10-phenylflavin) and an investigation of a possible mode of action of these compounds.

In Chapters 2 and 3 the effects of varying the substituents on the 10-phenyl ring, and at the 3-N position and the 10-phenyl ring together are examined.

In Chapters 4 and 5 the effect of altering ring-3 of the flavin moiety by adding substituents, making a 6,8-diaza analogue and removing the ring to produce a series of pteridine-2,4(3*H*,8*H*)-diones are examined.

In these chapters the results of antimalarial testing in the *P. vinckei vinckei* mouse model were the main measure of antimalarial activity. A number of the compounds were also tested for activity against chloroquine-sensitive and chloroquine-resistant *P. falciparum* in culture as well as for anti-HIV, anticoccodial and antibabesial activity.

In Chapter 6 the possibility that these flavins may be exerting their antimalarial action *via* the inhibition of host glutathione reductase is investigated and discounted.

Finally, the experimental details of the organic syntheses and most of biological testing of the earlier chapters are collated in Chapter 7.

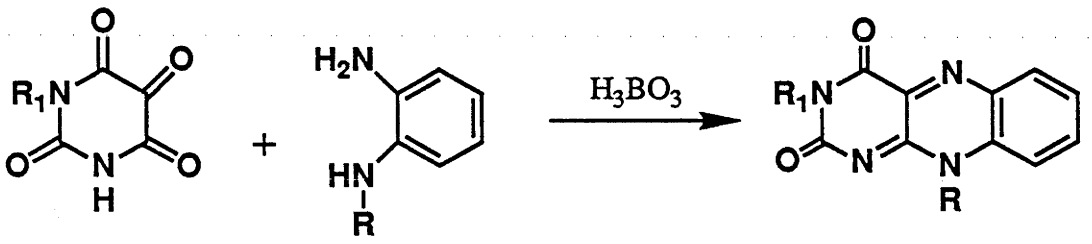
CHAPTER 2

CHAPTER 2 Syntheses and antimalarial activity of some 3-methyl-10-(substituted phenyl)flavins

2-1 Introduction

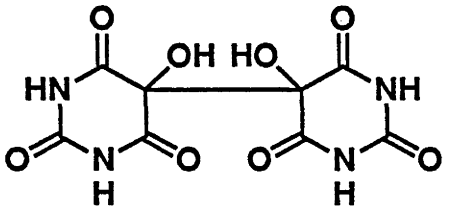
In view of the antimalarial activity of the lead compound 10-(4'-chlorophenyl)-3-methylflavin and the highly variable response achieved by alterations made on the 10-N-phenyl ring [42] it was decided to undertake a systematic study of substituents in this position (including a quantitative structure-activity relationship study). In this chapter the synthesis and antimalarial activity of the 3-methyl-10-(substituted phenyl)flavins are discussed. The ^{1}H n.m.r., ^{13}C n.m.r. and ultraviolet and visible (UV) spectra of this class of compounds are described and discussed. Some of the compounds produced were screened for activity against, drug resistant *P. falciparum*, *Babesia microti* and HIV and the results discussed. Additionally some of the compounds were submitted for *in vitro* testing against *Giardia intestinalis* and a number of human tumour cell lines.

Scheme 2-1

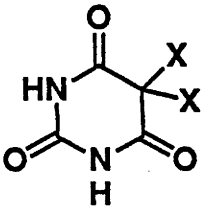


N-substituted alloxan

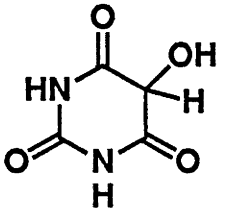
Other pyrimidines that have been used instead of alloxan



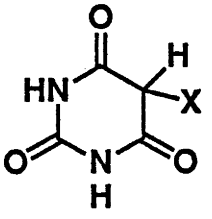
alloxantin



5,5-dihalobarbituric acid



dialuric acid



5-halobarbituric acid

2-2 Syntheses

2-2.1 Introduction

The large number of synthetic routes known for the production of the flavin ring system has mainly stemmed from the interest in the production of the important vitamin riboflavin and closely related derivatives. Kuhn and co-workers reported the first flavin synthesis in 1934 as part of the effort to characterise the structure of riboflavin [43,44]. The synthesis involved the condensation of 4,5-dimethyl-N-methyl-1,2-benzenediamine and alloxan to produce 7,8,10-trimethylflavin (lumiflavin).

The synthesis and chemistry of flavins are reviewed by Lambooy [43], and Ohta, Wrigglesworth and Wood [45]. A brief review of several methods of flavin synthesis found in the literature and their potential usefulness in regard to the aim of producing 3-methyl-10-(substituted phenyl)flavins is outlined in the following sections.

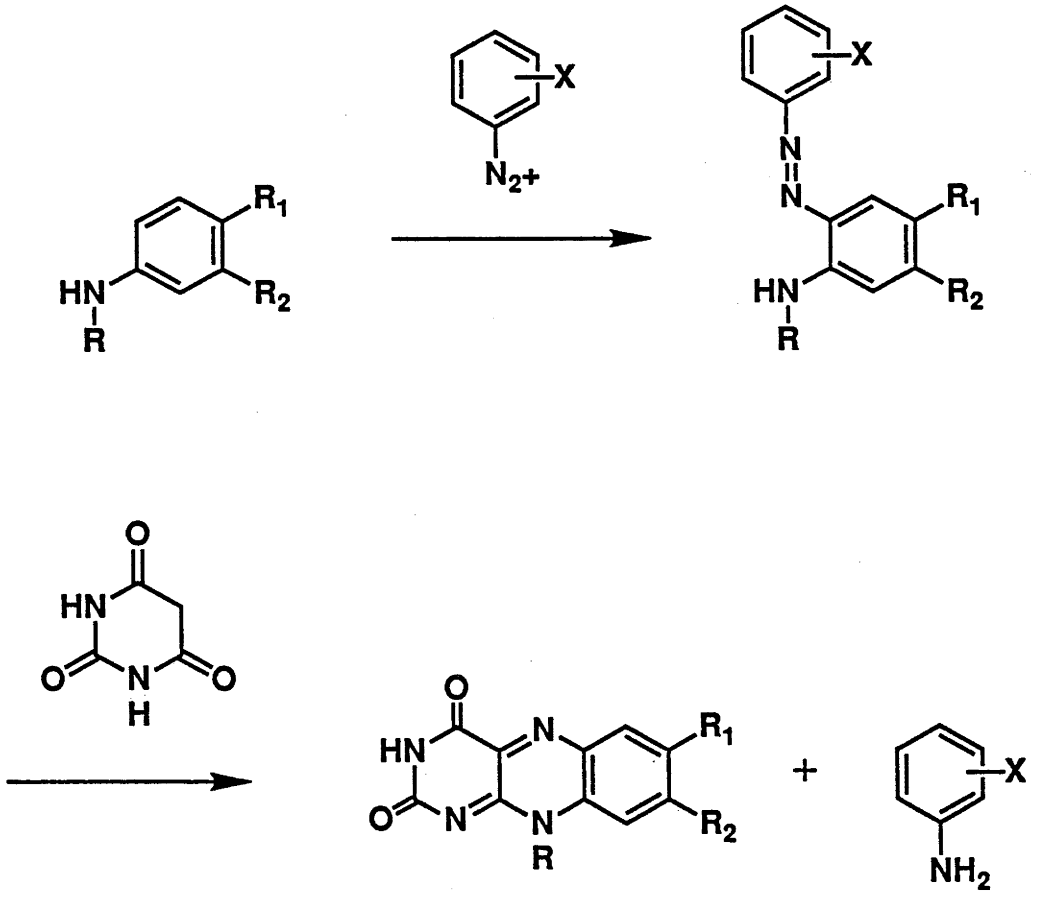
2-2.2 Literature methods

(i) The condensation of N-substituted 1,2-benzenediamine with various pyrimidines as depicted in Scheme 2-1 involves the formation of the central pyrazine ring and has been the most commonly used synthesis of flavins [43,46,47]. The various pyrimidines that can be used in this condensation include alloxan, N-substituted alloxans, alloxantin, dialuric acid, 5-halo and 5,5-dihalobarbituric acids. Varying degrees of success have been obtained with all of these pyrimidines though the most favoured seems to be alloxan. Boric acid has been found to catalyse this reaction. The major difficulty associated with this reaction is preparing the desired 1,2-benzenediamine starting material.

(ii) The reaction of 2-arylazobenzenamine with barbituric acid illustrated in Scheme 2-2 was shown by Tishler *et al.* [48] to be a convenient high yielding method of producing flavins, not requiring the less accessible and unstable 1,2-benzenediamine starting material used in method (i) above.

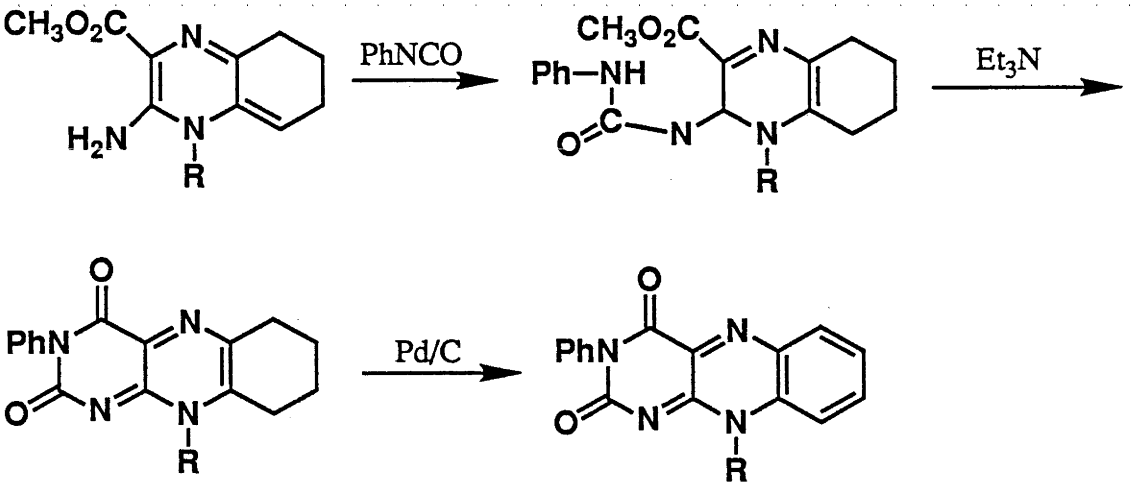
The production of the 2-arylazobenzenamine starting material is achieved by the coupling of an aryl diazonium salt to an N-substituted benzenamine. It is necessary to block the activated 4-position of the N-substituted benzenamine with a substituent to ensure the electrophilic aromatic substitution occurs at the 2-position. Experience has shown that 3,4-substituted N-substituted benzenamines usually give the best yields in this reaction [43]. The practical implication of this limitation in preparing the starting material for this synthetic route is that it is restricted to producing 7,8-disubstituted flavins.

Scheme 2-2

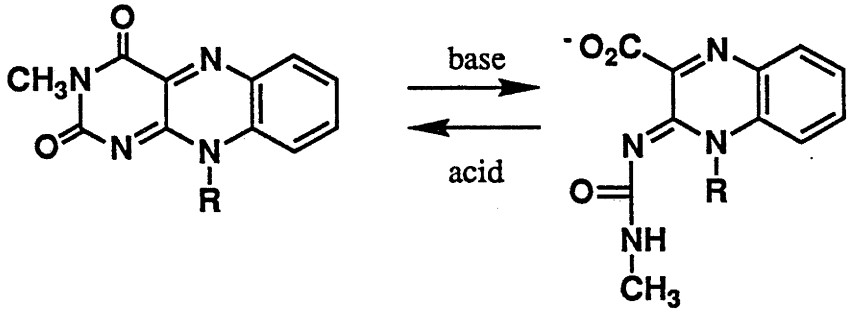


Scheme 2-4

(a)

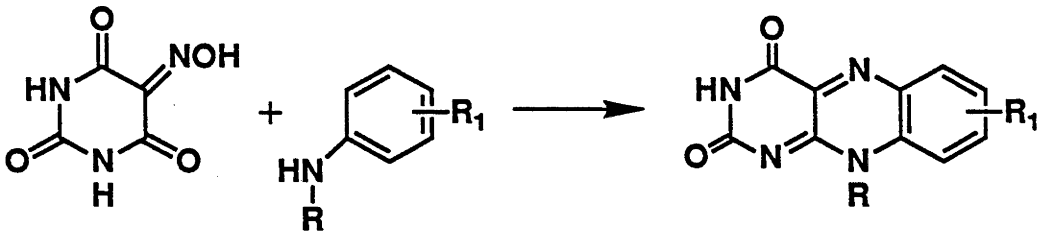

CH3O2C
N
H2N
N
R
PhNCO
CH3O2C
Ph–NH
C
O
N
N
R
Et3N
O
PhN
N
O
N
N
R
Pd/C
O
PhN
N
O
N
N
R

(b)


O
CH3N
N
O
N
N
R
base
acid
-O2C
N
N
N
O
NH
CH3

(iii) The condensation of 5-nitrosobarbituric acid (violuric acid) with an N-substituted benzenamine is a method of producing flavins from very readily available starting materials. Since this condensation shown in Scheme 2-3 can take place *via* either the 2 or 6 position of the N-substituted benzenamine the possibility exists for the formation of two isomers, depending upon the pattern of substitution in the benzene ring of the N-substituted benzenamine.

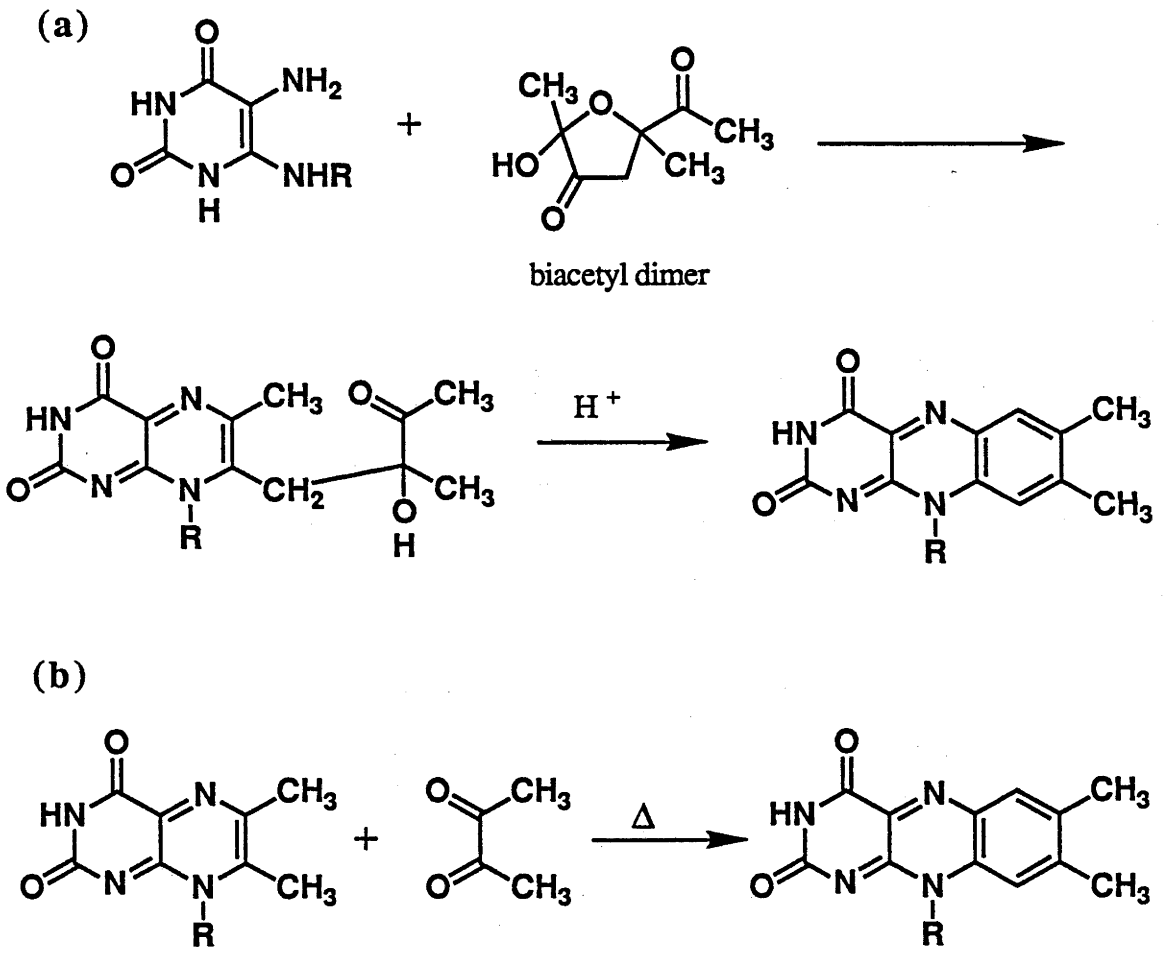
Scheme 2-3



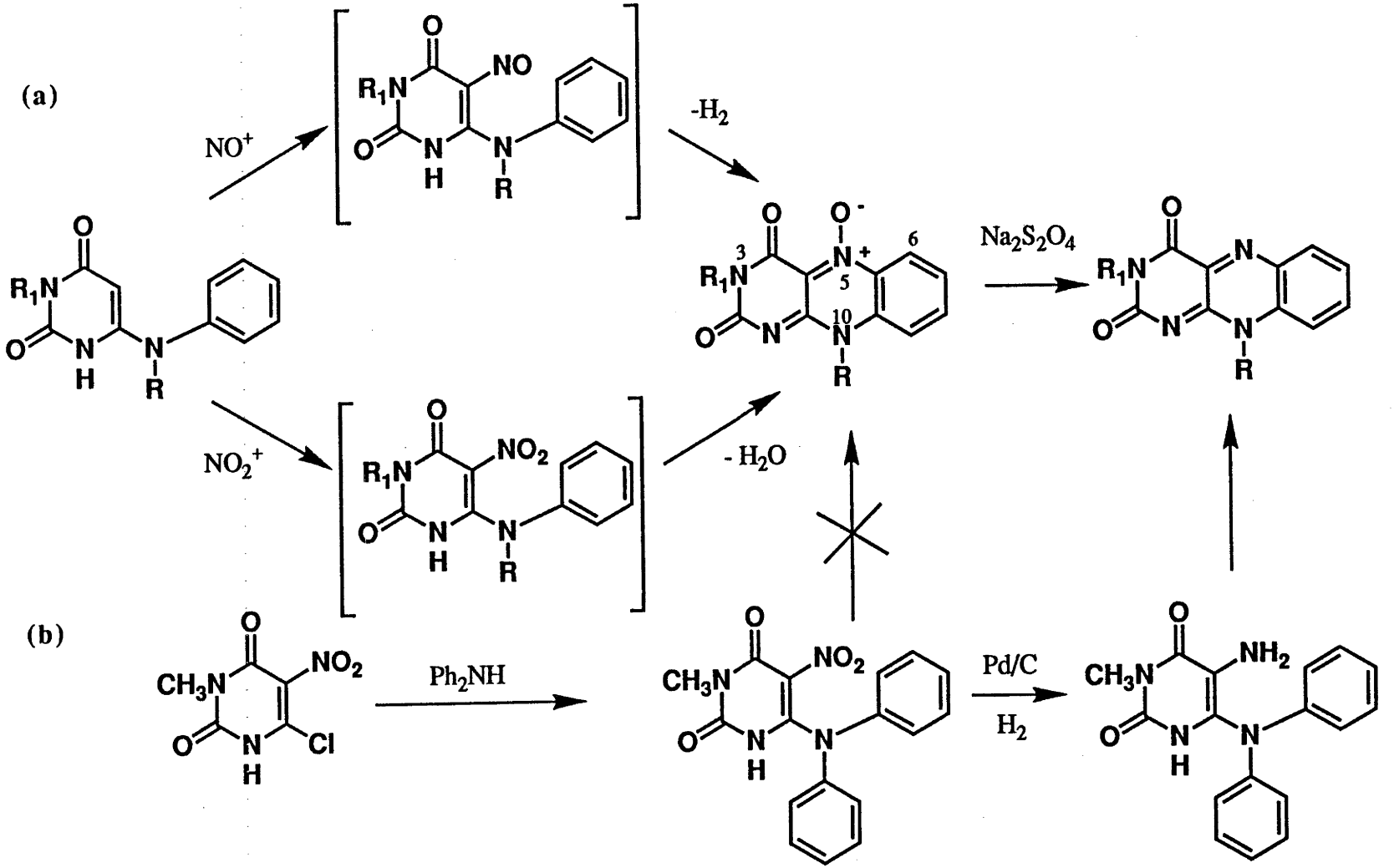
(iv) The preparation of flavins from quinoxalines involves the construction of the pyrimidine ring. There are only a few reports in the literature using this method of producing flavins. A novel route (Scheme 2-4a) used by Clerin *et al.* [49] involved the preparation of a 10-substituted 3-phenylflavin from the intermediate methyl 1-alkyl-2-amino-1,5,6,7-tetrahydro-3-quinoxaline carboxylate by reaction with phenylisocyanate, followed by cyclization in the presence of triethylamine and subsequent dehydrogenation. Smith and Bruice [50] showed that the alkaline hydrolysis of a flavin (Scheme 2-4b) lead to the formation of a 2-(N-methylureido)-1-substituted quinoxaline-3-carboxylic acid. This reaction was reversible under acidic anaerobic conditions. This reaction shows that the appropriate quinoxaline intermediate for the synthesis of flavins would probably require a 3-carboxylic acid functionality. The synthesis of such quinoxalines usually involves a 1,2-benzenediamine starting material [51].

(v) Birch and Moye [52] found they could produce flavins by reacting 5-amino-6-(N-substituted amino)uracil with the biacetyl dimer as its hemiacetal. This, as shown in Scheme 2-5a, leads to the formation of a pteridine-2,4(3*H*,8*H*)-dione intermediate that readily undergoes cyclization to give the corresponding flavin. A variation on this procedure, depicted in Scheme 2-5b, involves the condensation of monomeric biacetyl with a 6,7-dimethylpteridine-2,4(3*H*,8*H*)-dione to form a 7,8-dimethylflavin [43,53].

Scheme 2-5

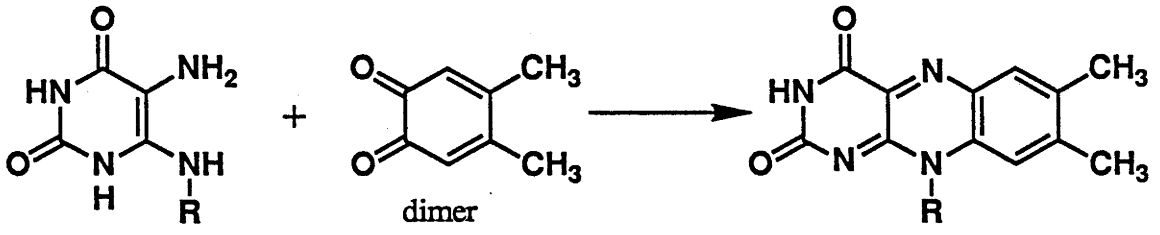


Scheme 2-7



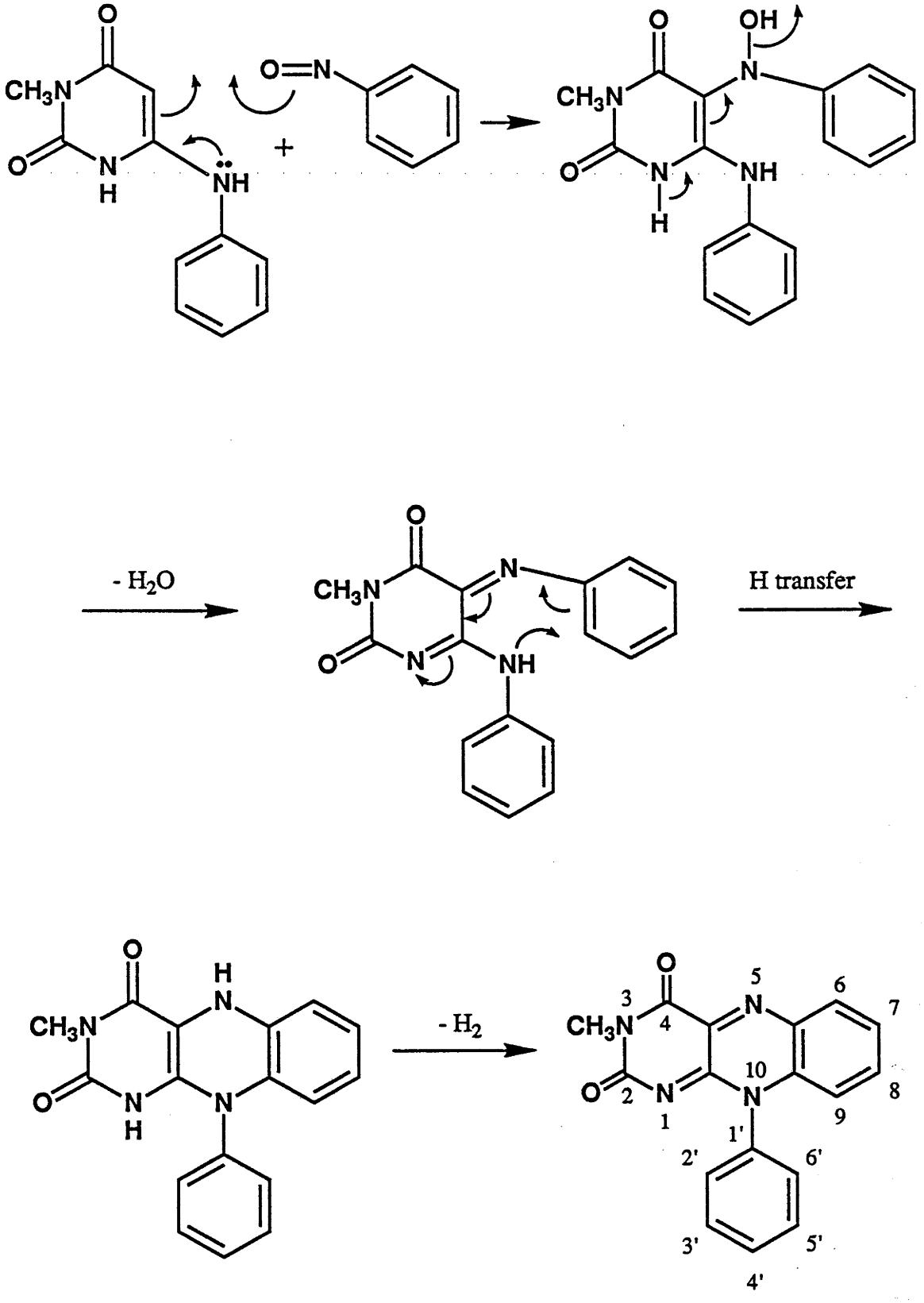
(vi) The condensation of 5-amino-6-(N-substituted amino)uracils with the dimer of 3,4-dimethyl-1,2-benzoquinone has produced flavins in reasonable yields [54]. However, this reaction, shown in Scheme 2-6, seems to be restricted to the production of 7,8-disubstituted flavins as 1,2-benzoquinone is reported [43,55] not to react with 5,6-diaminouracils to form flavins. This could possibly be due to oxidation of the diaminouracils by the monomeric 1,2-benzoquinone [43,56].

Scheme 2-6



(vii) Yoneda *et al.* [57] have shown that 6-(N-substituted anilino)uracils, upon nitrosation or nitration of the 5 position and subsequent cyclization, give 10-substituted flavin 5-N-oxides which are easily reduced to the corresponding flavins (Scheme 2-7a). Sakuma *et al.* [58], using a similar method, unsuccessfully attempted to produce 3-methyl-10-phenylflavin by cyclization of 3-methyl-5-nitro-6-diphenylaminouracil. However, the expected flavin was obtained by reduction of the 5-nitro group to 5-amino followed by autoxidation (Scheme 2-7b). The 3-methyl-5-nitro-6-diphenylaminouracil used in this reaction was produced by condensation of 6-chloro-3-methyl-5-nitrouracil with N-phenylbenzenamine. This route was used probably as a consequence of the reported failure of the nucleophilic substitution of the less reactive 6-chlorouracils by N-phenylbenzenamines [59,60].

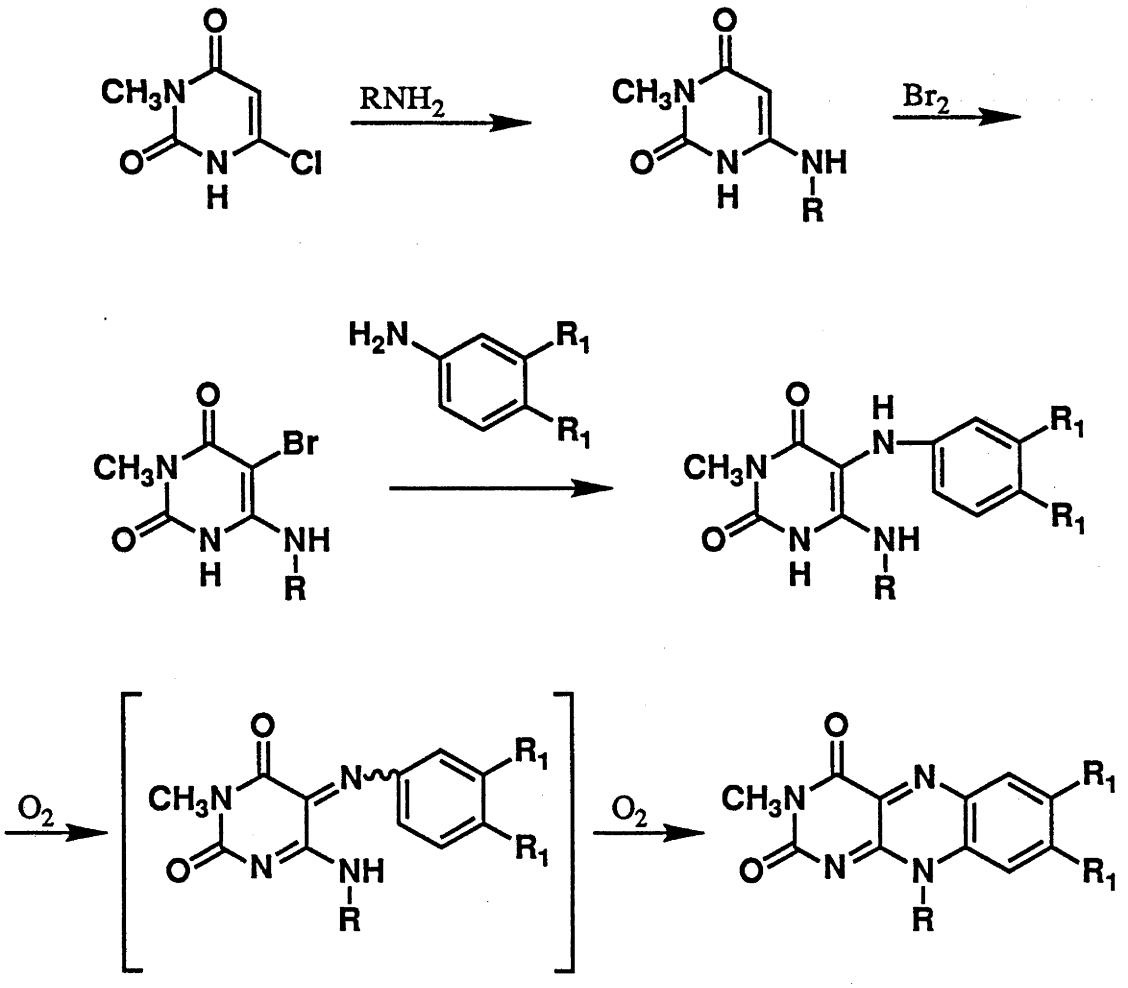
Scheme 2-8



(viii) A procedure pioneered by Yoneda *et al.* [60,61] specifically designed for the production of 10-arylflavins, involves the condensation of a 6-anilino-3-methyluracil with nitrosobenzene. The mechanism of the reaction suggested by Yoneda *et al.* [60] is shown in Scheme 2-8. It involves the formation of a 5-hydroxylamino intermediate followed by dehydration and cyclization to a 1,5-dihydroflavin, which is dehydrogenated with excess nitrosobenzene to the corresponding flavin.

(ix) The most recent new synthesis of flavins is reported by Sako *et al.* [62]. It involves the oxidative cyclization of 6-(substituted amino)-5-anilinouracils by heating in the presence of oxygen as seen in Scheme 2-9. The virtue of this method lies in the fact that the 10-N substituents and ring-3 of the flavin are introduced into the molecule in the form of primary amines and anilines, respectively, of which many substituted forms are commercially available. This allows a great variety of substituents to be introduced into these positions.

Scheme 2-9



2-2.3 Literature summary

The overall synthetic goal outlined in this chapter was to produce a large number of 3-methyl-10-(substituted phenyl)flavins. Of the literature methods discussed above, methods (ii), (v) and (vi) were deemed unsuitable as they are apparently restricted to the production of 7,8-disubstituted flavins. For 10-(substituted phenyl)flavin synthesis, methods (iii) and (vii) would require the often difficult to make N-(substituted phenyl)benzenamine starting material. Here, the appropriately substituted benzene is required to form the 10-phenyl ring of the flavin while the unsubstituted benzene ring forms the ring-3 of the flavin, however, the reverse of this can also occur, generating isomers for both routes. These two methods, therefore, were deemed not worthy of pursuit. Methods (i) and (iv) would require the difficult and time consuming synthesis of N-(substituted phenyl)-1,2-benzenediamine starting materials for each 10-(substituted phenyl)flavin produced. Methods (i) and (iv) were therefore considered impractical because of the large number of flavins planned for synthesis. From Schemes 2-8 and 2-9 it can be seen the remaining two methods (viii) and (ix) would use the common intermediate 6-(substituted anilino)-3-methyluracil. Method (viii) was considered superior to method (ix) for the present series because method (ix) has an additional two synthetic steps in the production of the flavins. Furthermore, method (viii) was considered the most appropriate reaction for the following reasons:

1) This reaction has been proven to be reliable in the production of 3-methyl-10-(substituted phenyl)flavins in reasonable yields [42].

2) The 10-substituted phenyl group can be introduced into the reaction sequence usually in the form of a commercially available aniline.

3) The site of variation in the molecule is added at the second last synthetic step allowing the use of a common starting material until late in the reaction sequence.

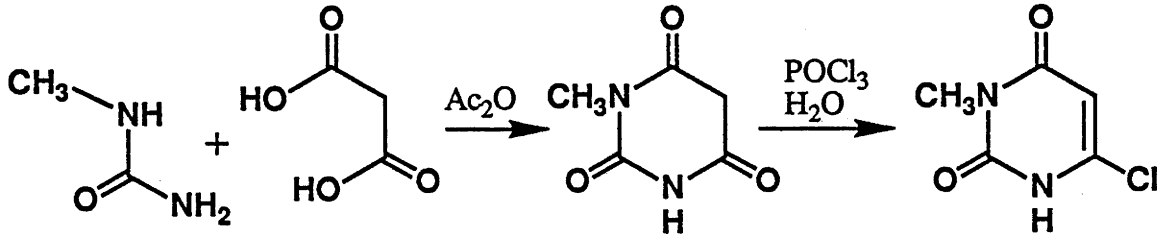
2-2.4 Synthesis description

The majority of the 3-methyl-10-(substituted phenyl)flavins (**2.5**) described in this chapter were formed by the condensation of 6-(substituted anilino)-3-methyluracil (**2.4**) with three equivalents of nitrosobenzene in the presence of acetic anhydride, essentially according to the method of Yoneda *et al.* [60]. The starting materials, methylurea, malonic acid, substituted anilines (excepting **2.3u** and **2.3w**) and nitrosobenzene, for the four step sequence outlined in Schemes 2-10 and 2-11 are commercially available.

Methylurea and malonic acid were cyclized to N-methylbarbituric acid (**2.1**) by the slow addition of the condensing agent, acetic anhydride, at 60° as described by Stein *et al.* [63]. This method was used in preference to the more common preparation of barbituric acids of condensation of malonic esters with urea or alkylated urea in the presence of sodium alkoxide. As this latter method has been reported to be less satisfactory in the preparation of N-methylbarbituric acid [63,64 [a]].

The monochlorination at the 6-position of N-methylbarbituric acid (**2.1**) was achieved by refluxing the barbituric acid in a mixture of phosphorus oxychloride and water [65,66].

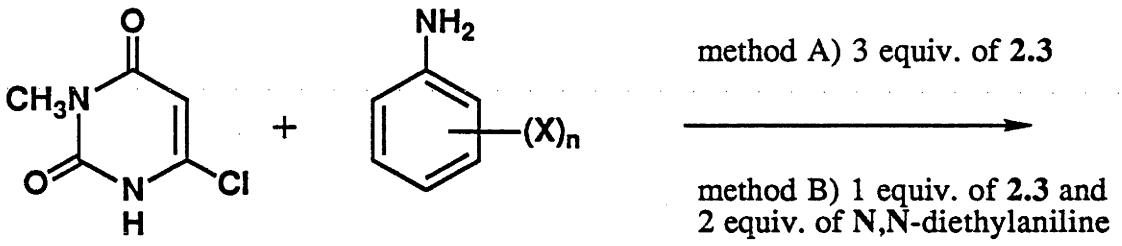
Scheme 2-10



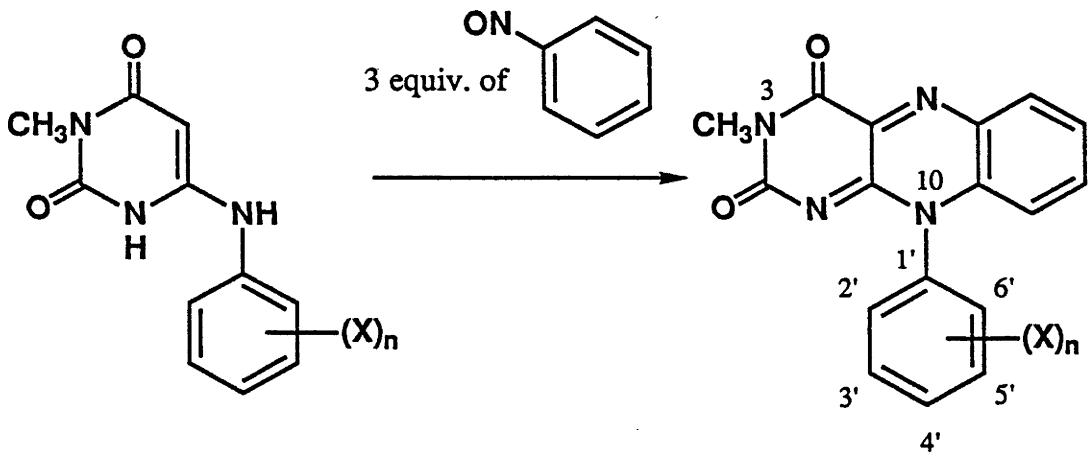
methylurea malonic acid **2.1** **2.2**

[a] Dr W. Cowden personal communication.

Scheme 2-11



2.2 2.3



2.4 2.5

2.3 - 2.5	$(X)_n$	2.3 - 2.5	$(X)_n$	2.3 - 2.5	$(X)_n$
a	H	k	4-Et	gg	3,5-$(OMe)_2$
b	2-Me	t	3-Cl,4-Me	hh	4-OH
c	3-Me	u	3-Cl,5-Me	ll	4-CF_3
e	2,4-Me_2	w	4-Cl,3-Me	nn	3-SMe
f	3,4-Me_2	x	4-Cl,2-F	oo	4-SMe
i	2-Et	dd	3,4-F_2	qq	4-CN

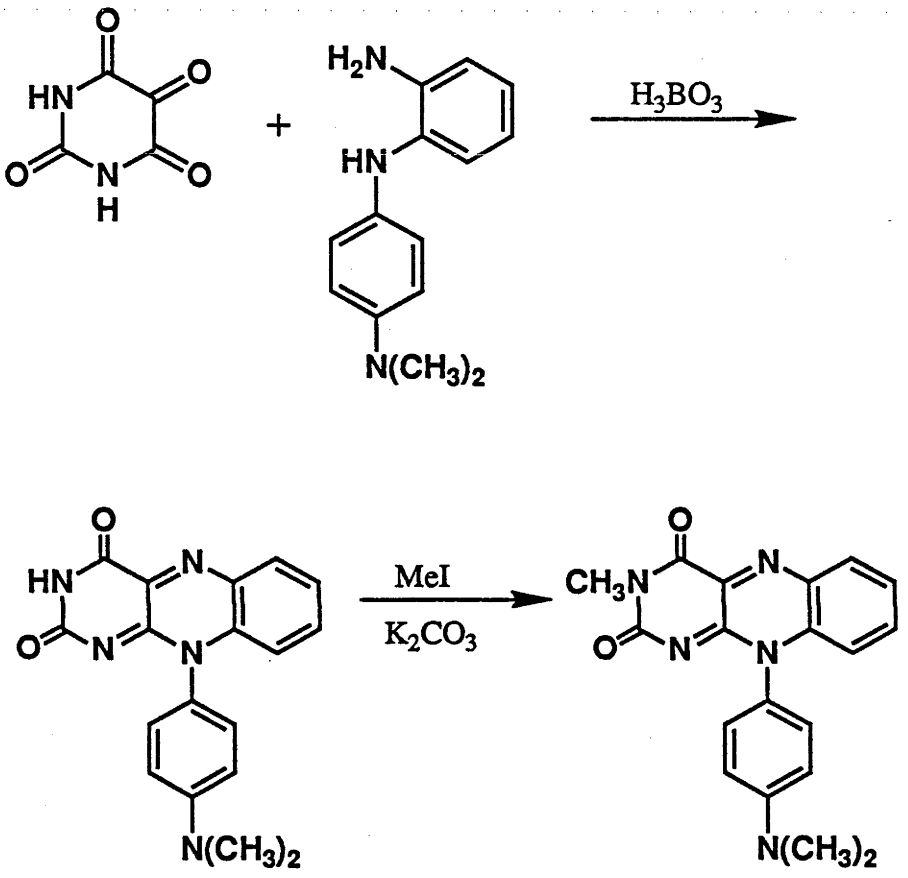
Initially some of the 6-(substituted anilino)-3-methyluracils were prepared by heating three equivalents of the appropriate aniline with 6-chloro-3-methyluracil (**2.2**). This method, which was described previously [42,60,67,68], called for an excess of the aniline to be used. This procedure, in synthetic terms is satisfactory, however some of the aniline starting materials were either expensive or less readily available synthetically, therefore a new method was devised to conserve them. The latter consisted of reacting molar equivalents of the chlorouracil **2.2** and the appropriate aniline nucleophile **2.3** in the presence of two molar equivalents of N,N-diethylaniline. This method proved satisfactory, giving equivalent yields and fewer coloured by-products than the original method.

4-Chloro-3-methylaniline (**2.3w**) and 3-chloro-5-methylaniline (**2.3u**) were required reagents that were not available commercially. The 4-chloro-3-methylaniline was prepared according to a literature procedure [69] in which 2-methyl-4-nitroaniline is converted, *via* the Sandmeyer reaction to 2-chloro-5-nitrotoluene and the latter chemically reduced to the desired aniline. The 3-chloro-5-methylaniline was prepared according to the method of Browne and Dyson [70]. In that paper the starting material is misnamed as 5-nitro-*o*-toluidine instead of 4-nitro-*o*-toluidine (2-methyl-4-nitroaniline). The method of preparation involved the chlorination of 2-methyl-4-nitroaniline with potassium chlorate to give 2-chloro-6-methyl-4-nitroaniline. The latter was converted to the diazonium salt, the diazonium group was replaced with hydrogen by the action of sulfuric acid to give 3-nitro-5-chlorotoluene which was then chemically reduced to give the required aniline.

A number of the 3-methyl-10-(substituted phenyl)flavins were not synthesized by the method above. These were the 4'-NMe_2 (**2.5jj**), 2',6'-Me_2 (**2.5h**), 3'-NO_2 (**2.5ii**), 4'-CO_2H (**2.5rr**) and 4'-SO_2Me (**2.5pp**) substituted compounds.

Attempts to condense 6-(4'-dimethylaminoanilino)-3-methyluracil with nitrosobenzene failed to give the expected product, possibly due to the activation of the 6-anilino ring to electrophilic substitution by the N,N-dimethylamino substituent. An alternate route (see sections 2-2.2(i), 3-2 and Scheme 2-12) involving the reaction of the N-(2'-aminophenyl)-N',N'-dimethyl-1,4-benzenediamine and alloxan with boric acid as

Scheme 2-12



2.5jj

a catalyst was undertaken. This gave the 10-(4'-dimethylaminophenyl)flavin which was then methylated with methyl iodide to give 10-(4'-dimethylaminophenyl)-3-methylflavin (**2.5jj**).

The 4'-CO_2H (**2.5rr**), 4'-SO_2Me (**2.5pp**) and 3'-NO_2 (**2.5ii**) substituted 10-phenylflavins were derived from existing flavins. The 4'-carboxylate **2.5rr** was prepared in good yield by acid hydrolysis of the corresponding nitrile **2.5qq**. The 4'-methylsulfonyl analogue **2.5pp** was readily prepared by the action of peroxyacetic acid on the 4'-methylthio compound **2.5oo**. The 3'-nitro compound **2.5ii** was prepared from 10-phenylflavin by nitration with a mixture of nitric and sulfuric acid (nitronium ion) to give the 10-(3'-nitrophenyl)flavin. Methylation of this flavin gave 3-methyl-10-(3'-nitrophenyl)flavin (**2.5ii**). The electrophilic substitution of 10-phenylflavin is discussed in more detail in section 3-3.

The nucleophilic substitution of 6-chloro-3-methyluracil by 2,6-dimethylaniline failed to occur under the conditions used. This may be due to the steric hindrance occasioned by the two methyl groups *ortho* to the attracting amino group. The 2',6'-Me_2 compound **2.5h** used in this study was a generous give from Dr F. Yoneda [b]. Two different syntheses for this compound have been reported by Sako *et al.* [62] and Main, Kasperek and Bruice [47].

Some flavins used in this study were made by Dr W. B. Cowden and are acknowledged as such.

The experimental details of the above section can be found in section 7-2.

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[b] Faculty of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606, Japan.

2-3 Physical properties

2-3.1 General

All the flavins are yellow high melting crystalline solids which are highly fluorescent under long wave ultraviolet light (*ca* 365 nm).

2-3.2 Nuclear magnetic resonance spectra

(i) ^{1}H Nuclear magnetic resonance

The signal due to the 3-N methyl group of this series appears as a singlet located between 3.24-3.27 ppm (Table 2-1). This methyl peak appears typically downfield because of the electron withdrawing effects of the neighbouring nitrogen and the two adjacent β carbonyl groups.

The hydrogen atoms on the benzenoid ring-3 of the flavin differ enough in their electronic environments to allow accurate assignment of peaks. The peak for H 6 appears downfield between 8.20-8.28 ppm as a doublet, the signal for H 7 is a triplet with a shift range of 7.62-7.68 ppm, the signal from H 8 also occurs as a triplet between 7.76-7.80 ppm and the signal due to H 9 is a doublet occurring considerably upfield at 6.70-7.00 ppm. The spectral data of these hydrogen atoms are presented in Table 2-1. The resonance from H 9 has the largest range of chemical shifts of these four hydrogen atoms as it is in closest proximity, and most subject to effects of the 10-phenyl ring and the various electron withdrawing and donating groups present there.

The simple splitting pattern (apparently first order) that occurs in this spin system results from the almost equal coupling constants $J_{6,7} = J_{7,8} = J_{8,9} = 8$ Hz, the small $J_{6,8}$ and $J_{7,9}$ coupling constants of approximately 1 Hz and the relative spread of chemical shifts of the peaks. The simulated splitting pattern [c] based on the above information about peak positions and coupling constants for 10-(3',5'-dimethylphenyl)-3-methylflavin (**2.5g**) and assuming an ABCD spin system is shown in Fig 2-1b.

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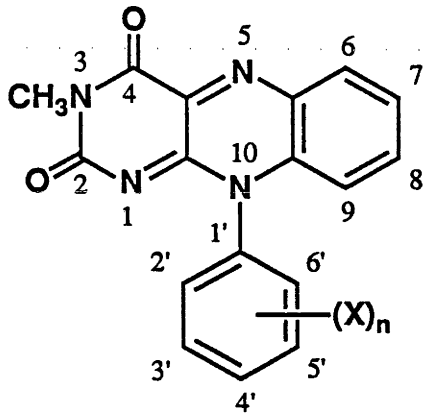
[c] Kindly run by Ms P. T. Culnane, University Nuclear Magnetic Resonance Centre, The Australian National University, using a Gemini 300 spin simulator.

Table 2-1: **^{1}H n.m.r. spectral data (δ)[a] for 3-methyl-10-(substituted phenyl)flavins**



[a] Chemical shifts reported as parts per million (δ) in CD_3SOCD_3. [b] Peaks appear as singlets with integration of 3H. [c] Peaks appear as doublets with integration of 1H, $J_{6,7}$ and $J_{8,9}$ are 8.0 Hz. [d] Peaks appear as triplets with integration of 1H, $J_{7,8}$ is 8.0 Hz. [e] Peak obscured by hydrogen atom peaks of the 10-phenyl ring. [f] Compound provided by Dr W. B. Cowden. [g] Also synthesised in Chapter 3. [h] Compound provided by Dr F. Yoneda.

Compound no.	$(X)_n$	3-NMe [b]	H 6 [c]	H 7 [d]	H 8 [d]	H 9 [c]
2.5a [f,g]	H	3.25	8.24	e	e	6.78
2.5b	2-Me	3.24	8.24	e	e	6.70
2.5c	3-Me	3.26	8.24	e	e	6.82
2.5d [f]	4-Me	3.26	8.23	7.63	7.78	6.84
2.5e	2,4-$(Me)_2$	3.25	8.24	7.63	7.76	6.74
2.5f	3,4-$(Me)_2$	3.24	8.22	7.62	7.76	6.84
2.5g [f]	3,5-$(Me)_2$	3.26	8.23	7.64	7.78	6.84
2.5h [h]	2,6-$(Me)_2$	3.27	8.28	7.67	7.80	6.70
2.5i	2-Et	3.25	8.24	e	e	6.71
2.5j [f]	3-Et	3.27	8.22	7.64	7.78	6.80
2.5k	4-Et	3.25	8.23	7.63	7.77	6.81
2.5l [f]	4-*n*-butyl	3.25	8.22	7.62	7.77	6.79
2.5m [f]	2-Cl	3.25	8.28	e	e	6.90
2.5n [f]	3-Cl	3.25	8.25	e	e	6.98
2.5o [f]	4-Cl	3.25	8.24	7.64	e	6.88
2.5p [f]	2,4-Cl_2	3.25	8.28	e	e	6.90
2.5q [f]	2,5-Cl_2	3.25	8.28	e	e	6.91
2.5r [f]	3,4-Cl_2	3.26	8.25	7.66	7.79	6.99
2.5s [f]	3,5-Cl_2	3.26	8.26	e	7.81	7.00
2.5t	3-Cl,4-Me	3.26	8.25	e	e	6.91
2.5u	3-Cl,5-Me	3.25	8.24	7.65	7.79	6.89
2.5v [f]	4-Cl,2-Me	3.25	8.25	e	e	6.79
2.5w	4-Cl,3-Me	3.25	8.23	7.63	e	6.89
2.5x	4-Cl,2-F	3.24	8.25	e	e	e
2.5y [f]	4-Cl,3-CF_3	3.26	8.26	e	e	6.97
2.5z [f]	3-Br	3.25	8.24	e	e	6.86
2.5aa [f]	4-Br	3.25	8.24	7.64	7.77	6.87
2.5bb [f]	3-F	3.25	8.24	e	e	6.87
2.5cc [f]	4-F	3.25	8.23	7.63	7.77	6.85
2.5dd	3,4-F_2	3.25	8.24	e	e	6.96
2.5ee [f]	3-OMe	3.25	8.23	e	7.78	6.86
2.5ff [f]	4-OMe	3.25	8.21	7.62	7.77	6.87
2.5gg	3,5-$(OMe)_2$	3.26	8.22	7.64	7.79	6.94
2.5hh	4-OH	3.24	8.20	7.62	7.77	6.89
2.5ii	3-NO_2	3.26	8.27	7.66	7.77	6.91
2.5jj	4-NMe_2	3.25	8.21	7.62	7.77	e
2.5kk [f]	3-CF_3	3.26	8.26	e	e	6.81
2.5ll	4-CF_3	3.25	8.21	e	e	6.83
2.5mm [f]	3,5-$(CF_3)_2$	3.26	8.28	7.68	7.80	6.96
2.5nn	3-SMe	3.26	8.24	e	7.80	6.86
2.5oo	4-SMe	3.25	8.23	7.63	7.77	6.88
2.5pp	4-SO_2Me	3.25	8.25	e	e	6.82
2.5qq	4-CN	3.25	e	e	e	6.83
2.5rr	4-CO_2H	3.25	e	7.64	7.76	6.82

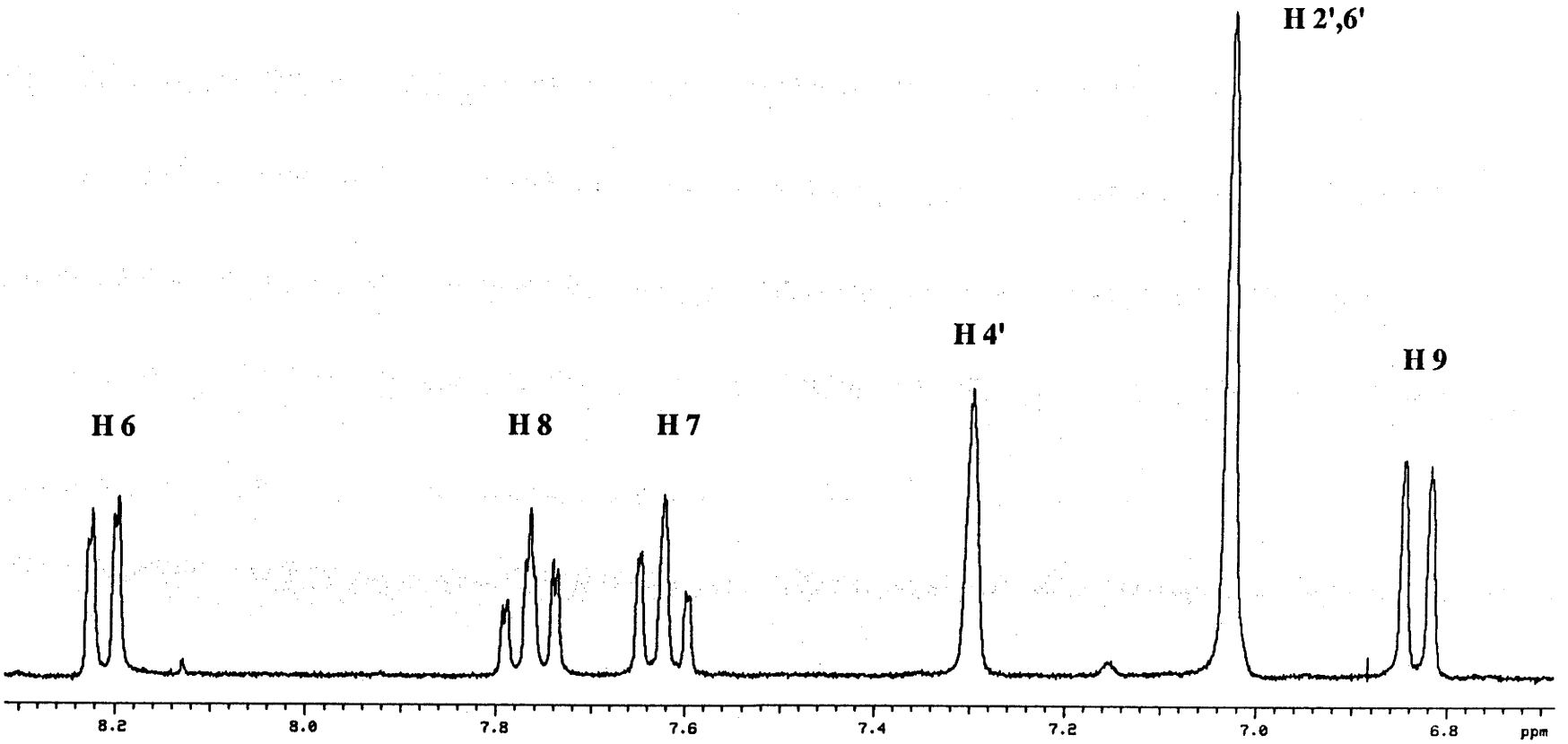


Figure 2-1a The aromatic region of the ^{1}H n.m.r. spectrum of 10-(3',5'-dimethylphenyl)-3-methylflavin (2.5g)

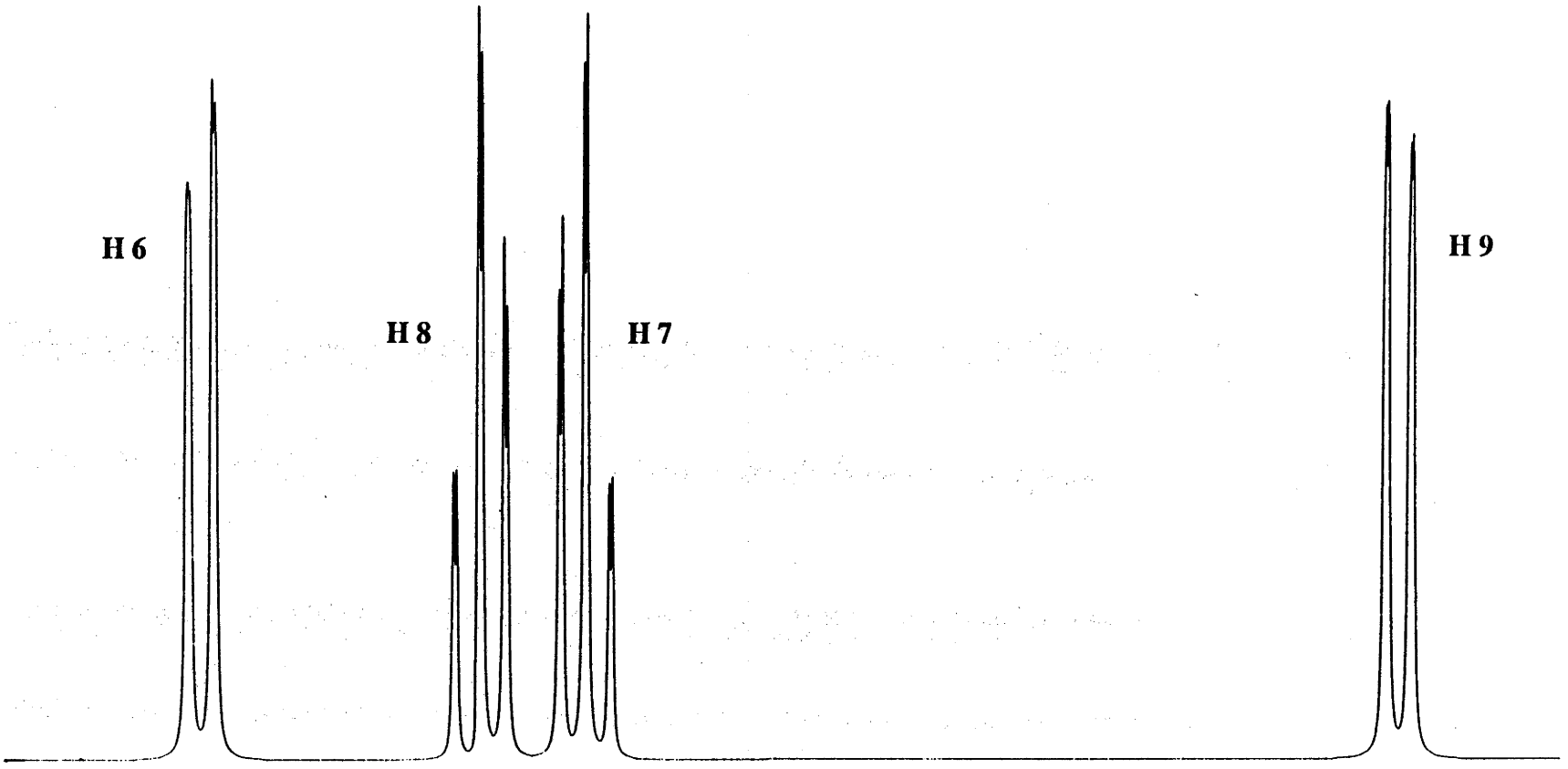


Figure 2-1b The simulated splitting pattern of the ring-3 hydrogen atoms of 10-(3',5'-dimethylphenyl)-3-methylflavin (2.5g)

This simulated pattern verifies the assignment of the two doublets and two triplets in the observed spectrum (Fig 2-1a). In the better resolved spectra the fine structure due to the long range hydrogen atom coupling can usually be seen and is similar to that in the simulated splitting pattern shown in Fig 2-1b.

The above assignments of peaks for the ring-3 hydrogens are in agreement with those made by Grande *et al.* [71] in their ^{1}H n.m.r. study of methyl substituted flavins. Furthermore, the ^{1}H n.m.r. spectra of flavins substituted in the 7, 8 and 9 positions (discussed in section 4-3.1) are consistent with the assignments given. A decoupling experiment on 10-(2',6'-dimethylphenyl)-3-methylflavin (**2.5h**) in which the peak from H 6 was decoupled, resulted in the collapse of the H 7 triplet to a doublet while the decoupling of the peak from H 9 resulted in the collapse of the H 8 triplet to a doublet. This further confirms the assignment of the signals and splitting pattern of the spin system due to H 6, 7, 8 and 9.

The coupling and shifts of the variously substituted 10-phenyl rings are presented in Table 2-2. The chemical shifts, splitting patterns and integration of the 10-phenyl hydrogen atom peaks are consistent with the electronic nature of their substituents and substitution pattern. The peaks due to substituents ($(X)_n$) containing hydrogen atoms are also in agreement with their assigned structure.

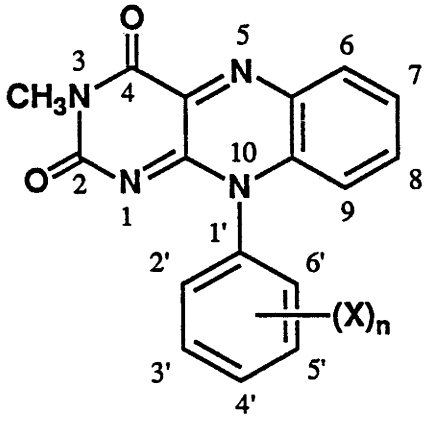
The multiplicity and integration of the most upfield peak in the 10-phenyl spin system of the following compounds indicate these upfield peaks are due to H 2' (when present) and H 6':

(1) **2.5a** (unsubstituted) in which it appears as a doublet that integrates for 2H;

(2) **2.5g** (3',5'-Me_2), **2.5s** (3',5'-Cl_2), **2.5gg** (3',5'-$(OMe)_2$) and **2.5mm** (3',5'-$(CF_3)_2$) in which it appears as a singlet that integrates for 2H; and

(3) **2.5b** (2'-Me) and **2.5i** (2'-Et) in which it appears as a doublet that integrates for 1H.

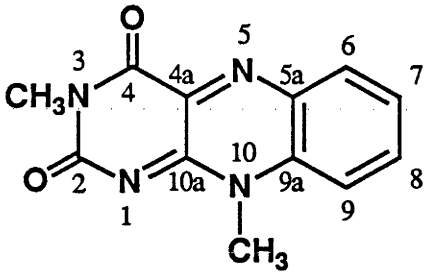
Table 2-2: **^{1}H n.m.r. spectral data (δ)[a] for 3-methyl-10-(substituted phenyl)flavins**



[a] Chemical shifts reported as parts per million (δ) in CD_3SOCD_3. [b] Data are presented in the following form; chemical shift, multiplicity, coupling constants (where appropriate) and integration. The following abbreviations were used: s (singlet); d (doublet); t (triplet); q (quartet); qu (quintet); sex (sextuplet); com (complex); and in cases where the coupling constants are the same in a spin system it is presented lastly. [c] For some compounds the hydrogen atom signals of the H 6, 7, 8 and/or 9 are inseparable from signals from the 10-phenyl hydrogen atoms. In these cases those signals which were not included in Table 2-1 are incorporated in this column and are apparent by integration. [d] Also synthesised in Chapter 3. [e] Compound provided by Dr W. B. Cowden. [f] Compound provided by Dr F. Yoneda. [g] Two symmetrical doublets seem to be present within this complex splitting pattern.

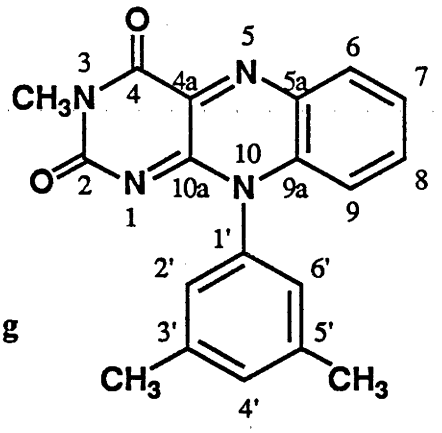
Cmpd no.	$(X)_n$	$(X)_n$ [b]	10-Substituted phenyl [b,c]
2.5a [d,e]	H	-	7.43 d 8.0 Hz 2H, 7.60-7.81 com 5H
2.5b	2-Me	1.95 s 3H	7.30 d 7.0 Hz 1H, 7.46-7.84 com 5H
2.5c	3-Me	2.44 s 3H	7.20-7.84 com 6H
2.5d [e]	4-Me	2.47 s 3H	7.32 d 2H, 7.53 d 2H 8.2 Hz
2.5e	2,4-$(Me)_2$	1.90 s 3H, 2.42 s 3H	7.14-7.35 com 4H
2.5f	3,4-$(Me)_2$	2.31 s 3H, 2.36 s 3H	7.11-7.49 com 4H
2.5g [e]	3,5-$(Me)_2$	2.39 s 6H	7.04 s 2H, 7.31 s 1H
2.5h [f]	2,6-$(Me)_2$	1.88 s 6H	7.36-7.52 com 4H
2.5i	2-Et	0.98 t 3H, 2.28 q 2H 7.6 Hz	7.29 d 8.0 Hz 1H, 7.46-7.84 com 5H
2.5j [e]	3-Et	1.25 t 3H, 2.75 q 2H 7.6 Hz	7.24-7.55 com 4H
2.5k	4-Et	1.23 t 3H, 2.77 q 2H 7.6 Hz	7.33 d 2H, 7.55 d 2H 8.2 Hz
2.5l [e]	4-*n*-butyl	0.95 t 3H, 1.40 sex 2H,1.68 qu 2H, 2.73 t 2H 7.6 Hz	7.32 d 2H, 7.53 d 2H 8.2 Hz
2.5m [e]	2-Cl	-	7.56-7.92 com 6H
2.5n [e]	3-Cl	-	7.42-7.84 com 6H
2.5o [e]	4-Cl	-	7.48 d 2H, 7.81 d 3H 8.6 Hz
2.5p [e]	2,4-Cl_2	-	6.60-8.13 com 5H
2.5q [e]	2,5-Cl_2	-	7.64-7.97 com 5H
2.5r [e]	3,4-Cl_2	-	7.50 d 1H, 7.84 s 1H, 8.03 d 1H 8.5 Hz
2.5s [e]	3,5-Cl_2	-	7.65 s 3H, 8.02 s 1H
2.5t	3-Cl,4-Me	2.50 s 3H	7.33-7.83 com 5H
2.5u	3-Cl,5-Me	2.43 s 3H	7.25 s 1H, 7.40 s 1H, 7.61 s 1H
2.5v [e]	4-Cl,2-Me	1.96 s 3H	7.56-7.83 com 5H
2.5w	4-Cl,3-Me	2.43 s 3H	7.31 d 7.8 Hz 1H, 7.44 s 1H, 7.79 d 8.2 Hz 2H
2.5x	4-Cl,2-F	-	6.24-7.98 com 6H
2.5y [e]	4-Cl,3-CF_3	-	7.62-7.86 com 3H
2.5z [e]	3-Br	-	7.47-7.92 com 6H
2.5aa [e]	4-Br	-	7.41 d 2H, 7.94 d 2H 8.8 Hz
2.5bb [e]	3-F	-	7.30-7.86 com 6H
2.5cc [e]	4-F	-	7.46-7.60 com 4H
2.5dd	3,4-F_2	-	7.31-7.92 com 5H
2.5ee [e]	3-OMe	3.80 s 3H	6.98-7.67 com 5H
2.5ff [e]	4-OMe	3.88 s 3H	7.24 d 2H, 7.35 d 2H 9.4 Hz
2.5gg	3,5-$(OMe)_2$	3.78 s 6H	6.21 s 2H, 6.79 s 1H
2.5hh	4-OH	-	7.03 d 2H, 7.20 d 2H 8.8 Hz
2.5ii	3-NO_2	-	7.61-8.11 com 6H
2.5jj	4-NMe_2	3.02 s 6H	6.94 d 3H, 7.17 d 2H 9.0 Hz
2.5kk [e]	3-CF_3	-	7.61-8.11 com 6H
2.5ll	4-CF_3	-	7.65-8.16 com 6H [g]
2.5mm [e]	3,5-$(CF_3)_2$	-	8.33 s 2H, 8.55 s 1H
2.5nn	3-SMe	2.51 s 3H	7.19-7.71 com 5H
2.5oo	4-SMe	2.59 s 3H	7.35 d 2H, 7.57 d 2H 8.4 Hz
2.5pp	4-SO_2Me	2.92 s 3H	7.60-8.10 com 6H
2.5qq	4-CN	-	7.60-8.27 com 7H [g]
2.5rr	4-CO_2H	-	7.56 d 2H, 8.26 d 3H 8.6 Hz

Table 2-3: **Comparison of the chemical shift (ppm) of the carbon atoms in 3,10-dimethylflavin [a] and (3',5'-dimethylphenyl)-3-methylflavin**



3,10-dimethylflavin

2.5g



Carbon	3,10-dimethyflavin [b]	**2.5g** [c]
C 2	156.6	155.1
C 4	160.3	159.3
C 4a	d	138.4
C 5a	136.2	134.8
C 6	133.5	131.3
C 7	127.2	126.0
C 8	136.4	134.8
C 9	115.8	116.9
C 9a	133.8	133.9
C 10a	149.7	150.1
C 1'	-	135.7
C 2',6'	-	124.9
C 3',5'	-	139.8
C 4'	-	131.2
NCH_3	28.8	28.0
3,5-$(CH_3)_2$	-	20.8
10-CH_3	32.3	-

[a] Spectral data for 3,10-dimethylflavin obtained from reference [74]. [b] Spectrum obtained in the solvent mixture $CDCl_3/CD_3OD$ on a Varian XL 100 spectrometer. [c] Spectrum obtained in CD_3SOCD_3 on a Varian VXR 300 spectrometer. [d] Not observed.

Most of the 4'-substituted 10-phenyl hydrogen atom peaks have a splitting pattern of two symmetrical doublets characteristic of *para* disubstituted benzene (either an AA'BB' or AA'XX' system).

(ii) ^{13}C Nuclear magnetic resonance

A carbon-13 spectrum was run on 10-(3',5'-dimethylphenyl)-3-methylflavin (**2.5g**) as a representative of this series. The spectrum revealed three carbon peaks at low field (159.3, 155.1 and 150.1 ppm), eleven peaks between 116 and 140 ppm and, two high field peaks at 28.0 and 20.8 ppm (Fig 2-2a,b).

The peak at 28.0 ppm was assigned to the deshielded 3-N methyl carbon and the intense peak at 20.8 ppm was assigned to the two equivalent carbons of the 3',5'-dimethyl groups on the 10-phenyl ring.

The tertiary carbons were unambiguously assigned with the aid of a two dimensional heteronuclear one-bond correlations plot (HETCOR) [d] . Fig 2-3 shows the HETCOR spectrum of the aromatic region of the hydrogen and carbon spectra making it possible to relate the assigned hydrogen spectrum to the carbon spectrum. The assignments made using the HETCOR spectrum for carbons 6, 7, 8, 9, 2', 6' and 4' are shown in Table 2-3.

A hydrogen-detected carbon-13 heteronuclear multiple bond correlation (HMBC) experiment was also run on this compound [d]. The HMBC was used to detect long range carbon hydrogen atom coupling connectivities while suppressing the one bond coupling [72]. The aromatic region of this spectrum shown in Fig 2-4 consisted of a number of cross peaks (the intensity of which reflects the size of the long-range coupling) which are mostly due to three bond carbon-hydrogen coupling. These cross-peaks confirmed the assignment of the above tertiary carbons and allowed the assignment of the quarternary

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[d] Kindly run by Ms P. Simmonds, University Nuclear Magnetic Resonance Centre, The Australian National University, using a Varian VXR 300 spectrometer.

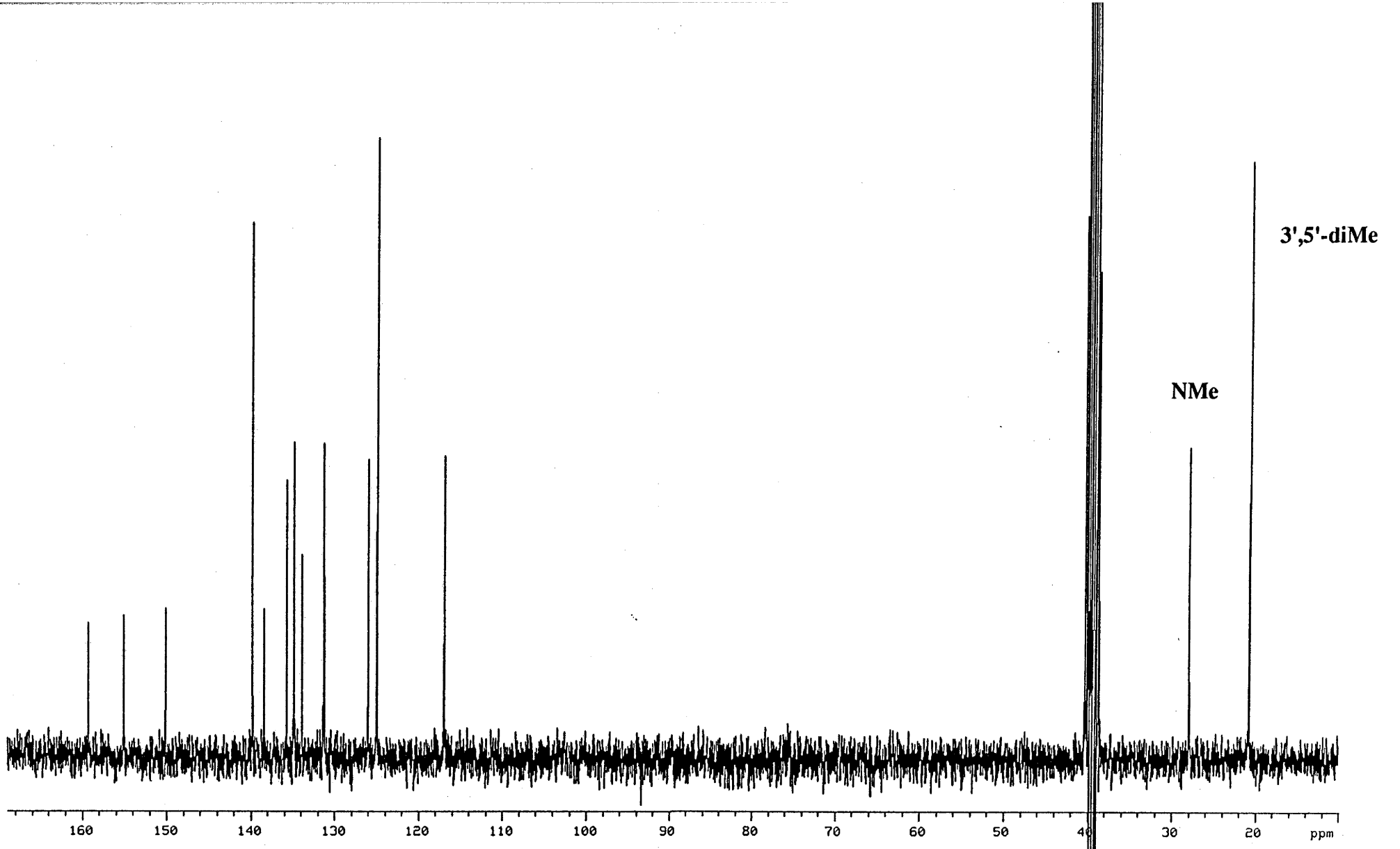


Figure 2-2a ^{13}C N.m.r. spectrum of 10-(3',5'-dimethylphenyl)-3-methylflavin (2.5g)

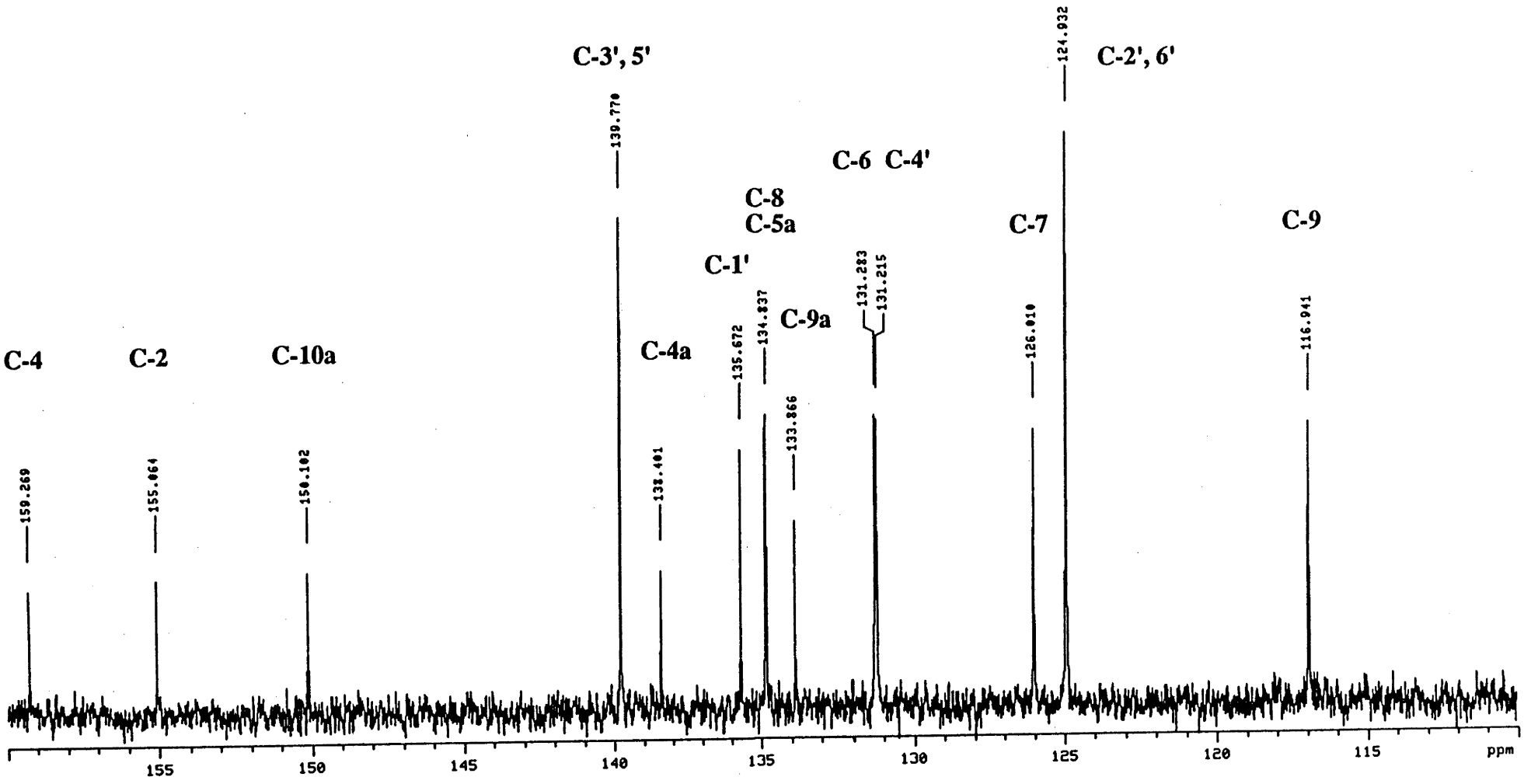


Figure 2-2b Expanded ^{13}C n.m.r. spectrum (110-160 ppm) of 10-(3',5'-dimethylphenyl)-3-methylflavin (2.5g)

Figure 2-3 Two dimensional HETCOR spectrum of 10-(3',5'-dimethylphenyl)-3-methylflavin (2.5g)

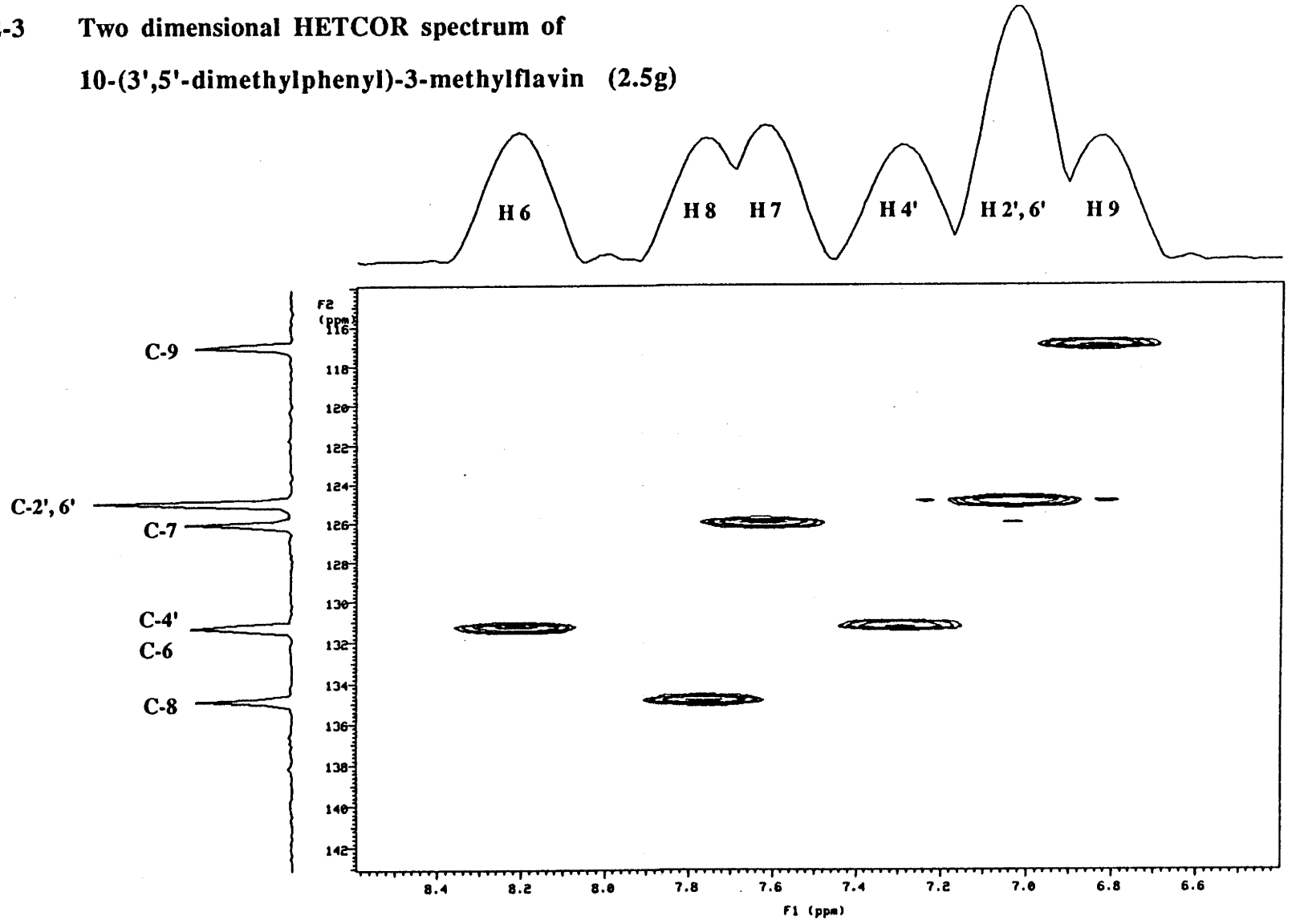
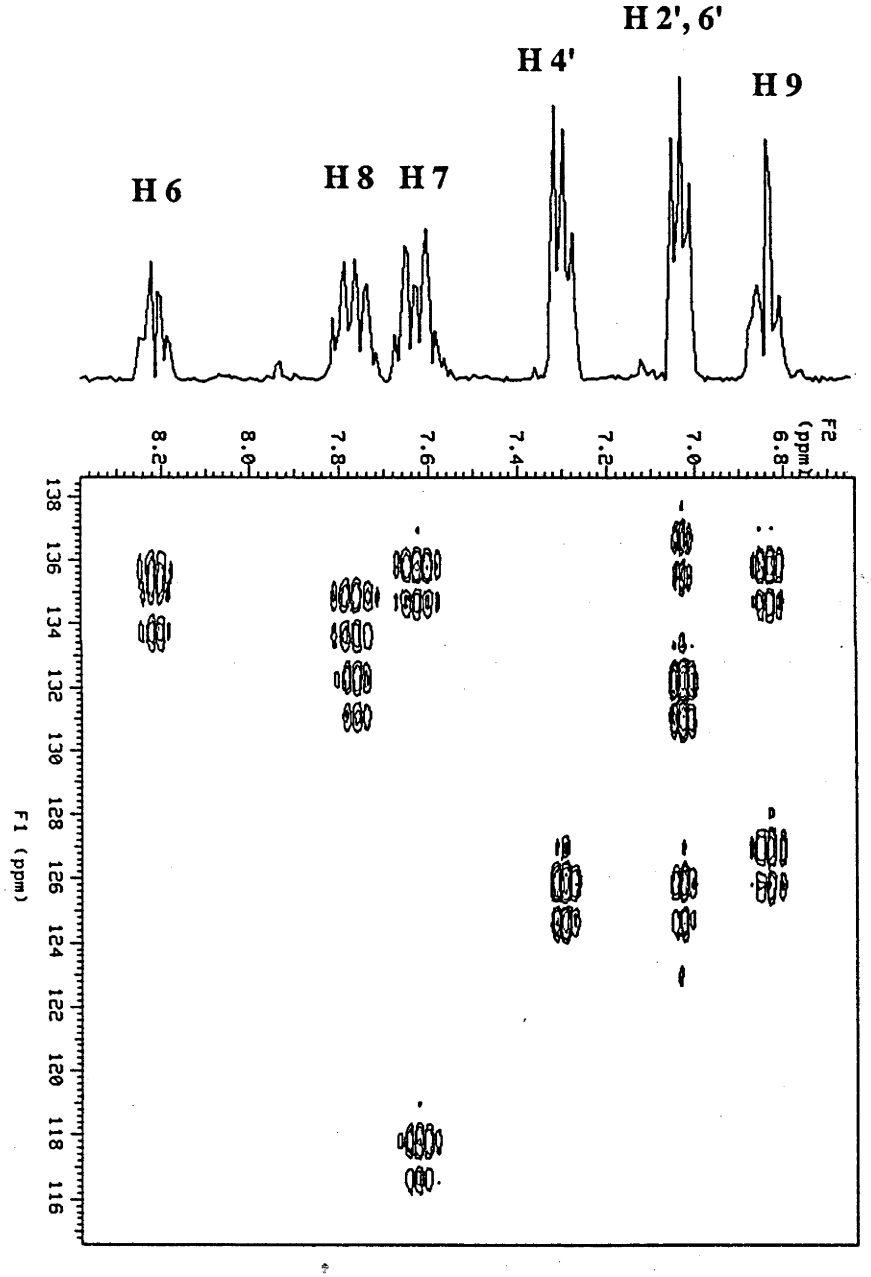


Figure 2-4 **Two dimensional HMBC spectrum of 10-(3',5'-dimethylphenyl)-3-methylflavin (2.5g)**



carbons 5a and 9a (Table 2-3). Carbon C-1' was assigned on the basis of the weak cross peak between the resonance at 135.7 ppm and the hydrogen atom peak assigned to H 2', 6'. This cross-peak is due to two-bond coupling and agrees with the fact that two-bond coupling constants are usually smaller than three-bond coupling constants in aromatic systems [73].

The carbon peak at 139.8 ppm which integrated for 2 carbons was assigned to the equivalent quarternary carbons C-3' and C-5'.

Of the remaining four unassigned peaks carbons 2, 4 and 10a were assigned on the basis of the assignments made by Grande *et al.* [74] who used ^{13}C enrichment techniques to study 3,10-dimethylflavin. By the process of elimination the resonance at 138.4 ppm was assigned to C-4a. For comparison the assignments made by Grande *et al.* are given in Table 2-3. It can be seen that even though a different solvent system was used and the substituent at the 10-position is different there is good agreement between the two sets of peak positions.

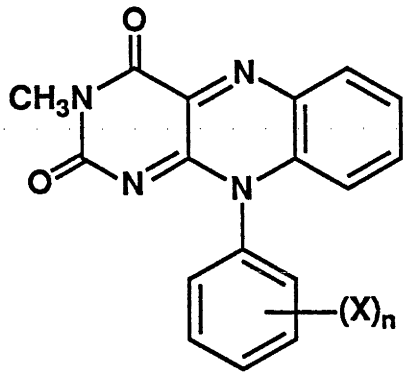
2-3.3 Ultraviolet and visible spectra

The ultraviolet and visible (UV) spectral data for the 3-methyl-10-(substituted phenyl)flavins series are recorded in Table 2-4. The spectra consist of an intense broad peak at 263-270 nm (log ε 4.27-4.69) and two well separated broad peaks at 326-340 nm (log ε 3.60-4.00) and 436-442 nm (log ε 3.65-4.05). These spectra are typical of the flavin chromophore and compare well with the ultraviolet and visible spectra of similar compounds reported by Yoneda *et al.* [60].

2-3.4 Mass spectra

The mass spectrum of 10-(4'-chlorophenyl)-3-methylflavin (**2.5o**) is discussed in section 3-4.2.

Table 2-4: Ultraviolet and visible maxima of the 3-methyl-10-(substituted phenyl)flavins [a]



[a] Experimental details are given in section 7-1. [b] Also synthesised in Chapter 3.
[c] Compound provided by Dr W. B. Cowden. [d] Compound provided by Dr F. Yoneda.
[e] Data taken from reference [42] and included for comparison and completeness.

Compound no.	$(X)_n$	λ max nm (log ε) (EtOH)		
2.5a [b,c]	H	270 (4.27)	332 (3.60)	439 (3.65)
2.5b	2-CH_3	269 (4.55)	334 (3.94)	439 (4.03)
2.5c	3-CH_3	269 (4.48)	330 (3.85)	439 (3.89)
2.5d [c]	4-CH_3	269 (4.50)	335 (3.96)	441(3.96)
2.5e	2,4-$(CH_3)_2$	269 (4.49)	334 (3.83)	439 (3.94)
2.5f	3,4-$(CH_3)_2$	270 (4.49)	334 (3.84)	440 (3.93)
2.5g [c]	3,5-$(CH_3)_2$	269 (4.52)	334 (3.86)	440 (3.96)
2.5h [d]	2,6-$(CH_3)_2$	269 (4.53)	334 (3.94)	437 (3.99)
2.5i	2-CH_2CH_3	269 (4.54)	336 (3.97)	439 (4.03)
2.5j [c]	3-CH_2CH_3	270 (4.44)	333 (3.85)	439 (3.81)
2.5k	4-CH_2CH_3	270 (4.50)	334 (3.87)	440 (3.94)
2.5l [c]	4-n-butyl	270 (4.61)	334 (3.97)	440 (4.05)
2.5m [e]	2-Cl	269 (4.48)	338 (3.90)	438 (4.00)
2.5n [e]	3-Cl	269 (4.43)	338 (3.85)	438 (3.93)
2.5p [e]	2,4-Cl_2	268 (4.38)	339 (3.86)	437 (3.96)
2.5q [e]	2,5-Cl_2	269 (4.33)	340 (3.78)	438 (3.89)
2.5r [e]	3,4-Cl_2	268 (4.30)	340 (3.72)	439 (3.85)
2.5s [e]	3,5-Cl_2	267 (4.36)	339 (3.78)	438 (3.90)
2.5t	3-Cl,4-CH_3	269 (4.51)	336 (3.91)	439 (3.99)
2.5u	3-Cl,5-CH_3	269 (4.50)	333 (3.90)	438 (3.97)
2.5v [e]	4-Cl,2-CH_3	269 (4.39)	339 (3.78)	438 (3.89)
2.5w	4-Cl,3-CH_3	269 (4.53)	333 (3.93)	439 (4.00)
2.5x	4-Cl,2-F	267 (4.52)	336 (4.00)	437 (4.05)
2.5y [c]	4-Cl,3-CF_3	267 (4.25)	332 (3.68)	437 (3.73)
2.5z [e]	3-Br	269 (4.40)	338 (3.80)	439 (3.90)
2.5aa [e]	4-Br	268 (4.41)	337 (3.85)	440 (3.93)
2.5bb [e]	3-F	268 (4.46)	336(3.88)	436 (3.94)
2.5cc [e]	4-F	269 (4.36)	339 (3.74)	438 (3.86)
2.5dd	3,4-F_2	269 (4.47)	334 (3.93)	437 (3.97)
2.5ee [c]	3-OCH_3	269 (4.55)	333 (3.89)	440 (3.97)
2.5ff [c]	4-OCH_3	270 (4.54)	335 (3.89)	441 (3.98)
2.5gg	3,5-$(OCH_3)_2$	269 (4.54)	334 (3.89)	440 (3.99)
2.5hh	4-OH	270 (4.50)	334 (3.86)	442 (3.92)
2.5ii	3-NO_2	265 (4.56)	334 (3.93)	438 (4.00)
2.5jj	4-$N(CH_3)_2$	265 (4.69)	326 (3.86)	437 (3.95)
2.5kk [c]	3-CF_3	269 (4.49)	333 (3.90)	437 (3.98)
2.5ll	4-CF_3	269 (4.50)	333 (3.88)	438 (3.95)
2.5mm [c]	3,5-$(CF_3)_2$	263 (4.43)	333 (3.90)	437 (3.98)
2.5nn	3-SCH_3	264 (4.59)	333 (3.88)	440 (3.95)
2.5oo	4-SCH_3	265 (4.52)	334 (3.74)	440 (3.98)
2.5pp	4-SO_2CH_3	267 (4.50)	335 (3.88)	438 (3.99)
2.5qq	4-CN	265 (4.48)	334 (3.90)	437 (3.96)
2.5rr	4-CO_2H	269 (4.51)	336 (3.87)	440 (3.97)

2-4 *In vivo* antimalarial testing

2-4.1 Introduction

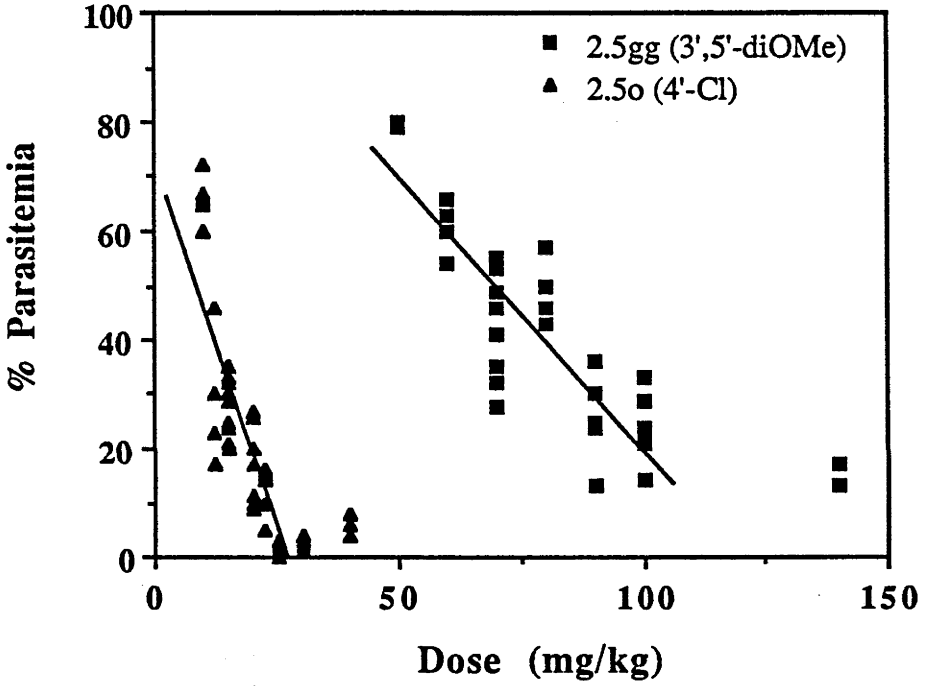
The host specificity of the malaria parasite precludes the direct experimental study of human species of *Plasmodium in vivo*. The most commonly used *in vivo* models are the murine malarias as they are easily and relatively inexpensively maintained in the laboratory. The *P. vinckei vinckei* parasite was selected as an appropriate malaria model as it causes a virulent, fulminating and lethal infection, characteristics which make it suitable for chemotherapeutic studies. *P. vinckei vinckei* was first isolated from Shaba, Zaire and adapted to mice in 1952. Since then it has been used successfully in numerous malaria studies [10b]. This model provides a simple convenient method of monitoring blood schizontocial activity which has been shown to correlate well with antimalarial activity in primates [75].

2-4.2 Method

In this model the infection (after inoculation of CBA mice) was allowed to reach 15 to 35% parasitemia when a single dose of the test compound was injected intraperitoneally. The groups of mice at each dose usually consisted of 4 to 6 animals. The three criteria used to judge the activity of compounds were the per cent of animals cured, the increase in mean survival time compared to controls and the percentage parasitemia on day two after treatment. A control was provided by injecting vehicle alone, here there was a 100% mortality three days after treatment. The parasitemia on day two after treatment of the control animals was $79 \pm 6\%$ ($n = 40$). A test compound was considered toxic if it either reduced mean survival time or treated animals died even though parasite counts on day two were low enough to indicate that the mice should have been cured of the infection.

The experimental details of the murine model are given in section 7-3.1(i).

Figure 2-5 **Dose response curves for compounds 2.5gg (3',5'-$(OMe)_2$) and 2.5o (4'-Cl)**



Each datum point represents the parasitemia of a single mouse on day two after treatment. The dose response curves shown here are for illustrative preposes only. See section 7-3.1(i) for experimental details.

2-4.3 Results

Tables 2-5 and 2-6 show the average per cent survival and the average mean extension in lifespan at doses ranging from 10 to 140 mg/kg for this flavin series (**2.5a-2.5rr**). Some of the flavins in these tables have been previously tested by Cowden *et al.* [42] and are indicated as such; these compounds in Table 2-5 were retested to allow direct comparison of biological activity between the new compounds and the previously reported compounds. Table 2-6 contains all those flavins with a 2'-substituent; the biological data of the compounds with substituents 2',4'-Cl_2 (**2.5p**) and 2'-CH_3, 4'-Cl (**2.5v**) from the previous study are included for comparison.

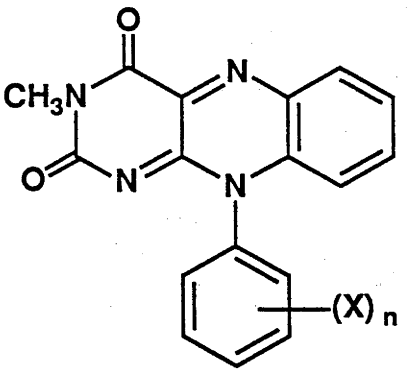
Two examples of dose-response curves of the flavins **2.5gg** and **2.5o** are shown in Fig 2-5, in which the response is per cent parasitemia on day two after treatment.

The ED_{40} value is the effective dose in mmoles/kg required to obtain a parasitemia of 40% on day two. The ED_{40} values were used as a measure of the relative schizontocidal activity of the active flavins. These values were estimated by interpolation from linear regression analysis of the logit biological response (log p/(100-p), where p is the parasitemia of individual mice on day 2) *versus* dose. The regression was restricted to the linear part of the plot corresponding to the change in activity. Alternate ED_{40} values were also obtained using a similar technique except that logit biological response *versus* log dose were used. These values were similar to the other ED_{40} values, however, the fits were slightly poorer and, therefore, the logit biological response *versus* dose derived ED_{40} values were used in subsequent discussion and calculations. The ED_{40} values, as well as their upper and lower 95% confidence limits, are presented in Table 2-7 for all the compounds in Table 2-5 which were active excepting those compounds where ED_{40} determination was not possible because of toxicity or a lack of data due to activity only being detected in the upper part of the dose range tested.

Table 2-5: Antimalarial activity of 3-methyl-10-(substituted phenyl)flavins against *P. vinckei vinckei* in mice [a]

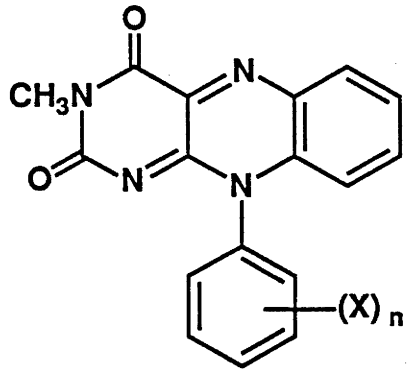
Cmpd no.	$(X)_n$	Per cent cured and increase in mean survival (days) at dose, mg/kg [b]				
		10	15	20	25	30
2.5a	H	0 (0)	-	-	-	0 (0)
2.5c	3-Me	0 (0.8)	0 (0)	0 (0.5)	0 (0)	0 (0)
2.5d	4-Me	0 (0)[d]	-	-	-	0 (0.2)
2.5f	3,4-$(Me)_2$	0 (0.3)	-	0 (-0.2)	-	0 (0.8)
2.5g	3,5-$(Me)_2$ [c]	0 (0)	60 (1.0)	100	100	100
2.5j	3-Et	-	0 (0)	0 (0)	0 (0)	0 (0)
2.5k	4-Et	0 (0.2)	-	0 (0.3)	-	0 (0)
2.5l	4-*n*-butyl	-	-	-	-	0 (0)
2.5n	3-Cl [c]	-	-	-	-	-
2.5o	4-Cl [c]	20 (0.25)	80 (4.0)	100	100	100
2.5r	3,4-Cl_2 [c]	-	-	-	-	0 (0)
2.5s	3,5-Cl_2 [c]	100	100	100	100	100
2.5t	3-Cl,4-Me	-	-	-	-	0 (0)[d]
2.5u	3-Cl,5-Me	0 (0.2)	60 (0.5)	80 (1)	100	100
2.5w	4-Cl,3-Me	-	-	-	-	-
2.5y	4-Cl,3-CF_3	-	-	-	-	-
2.5z	3-Br [c]	-	0 (0)	-	-	0 (0)
2.5aa	4-Br [c]	0 (1.3)	100	100	100	-
2.5bb	3-F [c]	0 (0.2)	0 (0.2)	0 (0)	0 (0)	0 (0.2)
2.5cc	4-F [c]	0 (0)	0 (0)	0 (13.4)	0 (0)	80 (0)
2.5dd	3,4-F_2	-	-	0 (-0.5)	-	0 (0)
2.5ee	3-OMe	0 (0)	-	0 (0)	-	0 (0)
2.5ff	4-OMe	0 (1.0)[d]	-	-	-	0 (0.4)
2.5gg	3,5-$(OMe)_2$	-	-	-	-	-
2.5hh	4-OH	0 (0.5)[d]	-	-	-	0 (0.2)
2.5ii	3-NO_2	-	-	-	-	0 (0)[d]
2.5jj	4-$N(Me)_2$	0 (0)	-	-	-	0 (0)
2.5kk	3-CF_3	0 (0)	100	100	100	100
2.5ll	4-CF_3	0 (0)	-	0 (0)	-	80 (2.0)
2.5mm	3,5-$(CF_3)_2$	0 (0)	-	0 (0)	-	0 (0)
2.5nn	3-SMe	0 (0.2)	-	0 (0)	-	0 (0)
2.5oo	4-SMe	0 (0.2)	-	0 (0.5)	-	0 (0.5)
2.5pp	4-SO_2Me	0 (0)[d]	-	-	-	0 (0.2)
2.5qq	4-CN	0 (0.5)[d]	-	-	-	0 (0)
2.5rr	4-CO_2H	0 (1.0)[d]	-	-	-	0 (0.2)

[a] See section 7-3.1(i) for experimental details. [b] Most groups consist of 4 to 6 animals. [c] Compounds which were retested, for previous testing see references [42,76]. [d] Denotes a group of two mice. [e] Toxic at 80 mg/kg.



Cmpd no.	$(X)_n$	Per cent cured and increase in mean survival (days) at dose, mg/kg [b]				
		40	50	60	70	140
2.5a	H	-	0 (0.3)	20 (0.5)	60 (0)	[e]
2.5c	3-Me	-	0 (0.6)	-	0 (0)	0 (-1.5)[d]
2.5d	4-Me	-	0 (0.3)	-	0 (0)	0 (0.5)[d]
2.5f	$3,4\text{-}(Me)_2$	-	20 (0.25)	60 (0.5)	100	-
2.5g	$3,5\text{-}(Me)_2$ [c]	100	60 (5.5)	0 (0.4)	-	-
2.5j	3-Et	-	-	-	0 (0.3)	-
2.5k	4-Et	-	0 (0.5)[d]	-	0 (-0.4)	-
2.5l	4-*n*-butyl	-	0 (0)	-	0 (0.5)	0 (0.5)[d]
2.5n	3-Cl [c]	-	0 (0.4)	50 (-0.5)	100	-
2.5o	4-Cl [c]	100	20 (-0.8)	-	0 (-1.0)	-
2.5r	$3,4\text{-}Cl_2$ [c]	-	0 (0)	-	0 (0)	100[d]
2.5s	$3,5\text{-}Cl_2$ [c]	100	-	-	60 (16.5)	-
2.5t	3-Cl,4-Me	-	-	-	0 (0)[d]	100
2.5u	3-Cl,5-Me	100	100	-	100	-
2.5w	4-Cl,3-Me	0 (0.25)	25 (-0.33)	100	80 (2.0)	50 (0)[d]
2.5y	$4\text{-Cl},3\text{-}CF_3$	-	-	-	0 (0.2)	0 (0)[d]
2.5z	3-Br [c]	0 (0)	80 (1.0)	80 (-1.0)	100	-
2.5aa	4-Br [c]	-	40 (9.0)	-	0 (7.4)	-
2.5bb	3-F [c]	0 (0.8)	20 (0)	-	0 (-1.0)	-
2.5cc	4-F [c]	80 (0)	20 (-0.5)	-	0 (-0.6)	-
2.5dd	$3,4\text{-}F_2$	-	-	-	0 (-1.0)	-
2.5ee	3-OMe	-	-	-	0 (0)	0 (0.5)
2.5ff	4-OMe	20 (1.0)	75 (2.0)	40 (0.7)	60 (-0.5)	-
2.5gg	$3,5\text{-}(OMe)_2$	-	0 (0)	20 (3.0)	100	100 [d]
2.5hh	4-OH	-	-	-	0 (0)[d]	0 (0)[d]
2.5ii	$3\text{-}NO_2$	-	-	0 (1.0)[d]	0 (-0.5)[d]	0 (-1.0)[d]
2.5jj	$4\text{-}N(Me)_2$	-	-	-	0 (0)	-
2.5kk	$3\text{-}CF_3$	100	100	40 (10)	0 (7.5)[d]	-
2.5ll	$4\text{-}CF_3$	-	100	100	100	-
2.5mm	$3,5\text{-}(CF_3)_2$	-	-	-	0 (0)	0 (0)[d]
2.5nn	3-SMe	-	-	-	0 (0)	0 (0)[d]
2.5oo	4-SMe	-	-	-	0 (0)	0 (1.5)[d]
2.5pp	$4\text{-}SO_2Me$	0 (-0.5)	0 (-0.8)	-	0 (-2.0)	-
2.5qq	4-CN	-	-	-	0 (0)	0 (0)[d]
2.5rr	$4\text{-}CO_2H$	-	-	-	0 (-0.2)	0 (-0.5)[d]

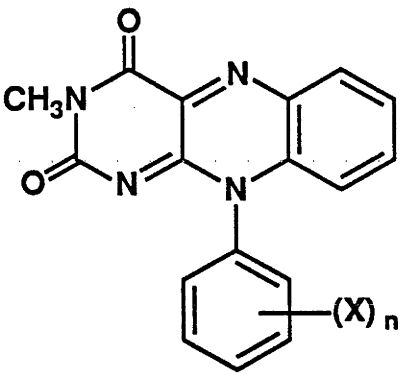
Table 2-6: Antimalarial activity of 3-methyl-10-(2'-substituted phenyl)flavins against *P. vinckei vinckei* in mice [a]



Cmpd no.	$(X)_n$	Per cent cured and increase in mean survival (days) at dose, mg/kg [b]							
		10	15	20	25	30	40	50	70
2.5b	2-Me	0 (-0.2)	-	0 (-1.0)	-	0 (-2.0)	-	-	-
2.5e	2,4-$(Me)_2$	0 (0.4)	-	-	-	20 (1.3)	40 (-1.7)	0 (-2.0)	0 (-2.0)
2.5h	2,6-$(Me)_2$	0 (0)	-	0 (-0.3)[c]	0 (-2.0)	-	-	-	0 (-2.0)[d]
2.5i	2-Et	0 (0)	-	0 (-2.0)	-	0 (-2.0)	-	-	-
2.5m	2-Cl [e]	-	-	-	-	0 (-0.3)	-	-	0 (-2.0)
2.5p	2,4-Cl_2 [f]	0 (1.7)	0 (0.5)	0 (0)	0 (-1.2)	0 (-1.0)	-	-	-
2.5q	2,5-Cl_2 [e]	-	-	-	-	0 (0.3)	-	-	0 (-0.6)[g]
2.5v	4-Cl,2-Me [f]	0 (0.5)	0 (1.2)	0 (1.2)	0 (-1.4)	0 (-2.0)	-	-	-
2.5x	4-Cl,2-F	0 (1.0)[d]	-	0 (4.0)[d,h]	-	0 (-2.0)[d]	-	-	-

[a] See section 7-3.1(i) for experimental details. [b] Most groups consist of 4 to 6 animals. [c] Activity detected by day two blood smear, average parasitemia 50 %. [d] Denotes a group of two mice. [e] Compounds which were retested, for previous testing see reference [42]. [f] This compound included for comparison; see reference [42]. [g] Activity detected by day two blood smear, average parasitemia 56 %. [h] Activity detected by day two blood smear, average parasitemia 27 %.

Table 2-7: **ED_{40} values of some active 3-methyl-10-(substituted phenyl)flavins [a]**



Cmpd no.	$(X)_n$	ED_{40} (mmoles/kg x10^{-3})	95 % confidence limits	
			lower	upper
2.5aa	4-Br	38.4	35.4	40.9
2.5o	4-Cl	38.8	35.5	41.6
2.5s	3,5-Cl_2	40.2	36.0	44.1
2.5kk	3-CF_3	79.3	73.1	86.1
2.5u	3-Cl,5-Me	85.7	80.2	92.6
2.5cc	4-F	103	95.0	112
2.5g	3,5-$(Me)_2$	105	94.2	118
2.5ll	4-CF_3	135	124	146
2.5hh	4-OMe	138	133	143
2.5z	3-Br	148	138	162
2.5w	4-Cl,3-Me	182	176	195
2.5f	3,4-$(Me)_2$	210	205	216
2.5gg	3,5-$(OMe)_2$	219	210	229
2.5n	3-Cl	229	221	242
2.5a	H	248	235	290
2.5k	4-Et	281	271	292
2.5t	3-Cl,4-Me	456	363	582

[a] See section 2-4.3 for details.

2-4.4 Discussion

(i) Structure activity relationships (SAR)

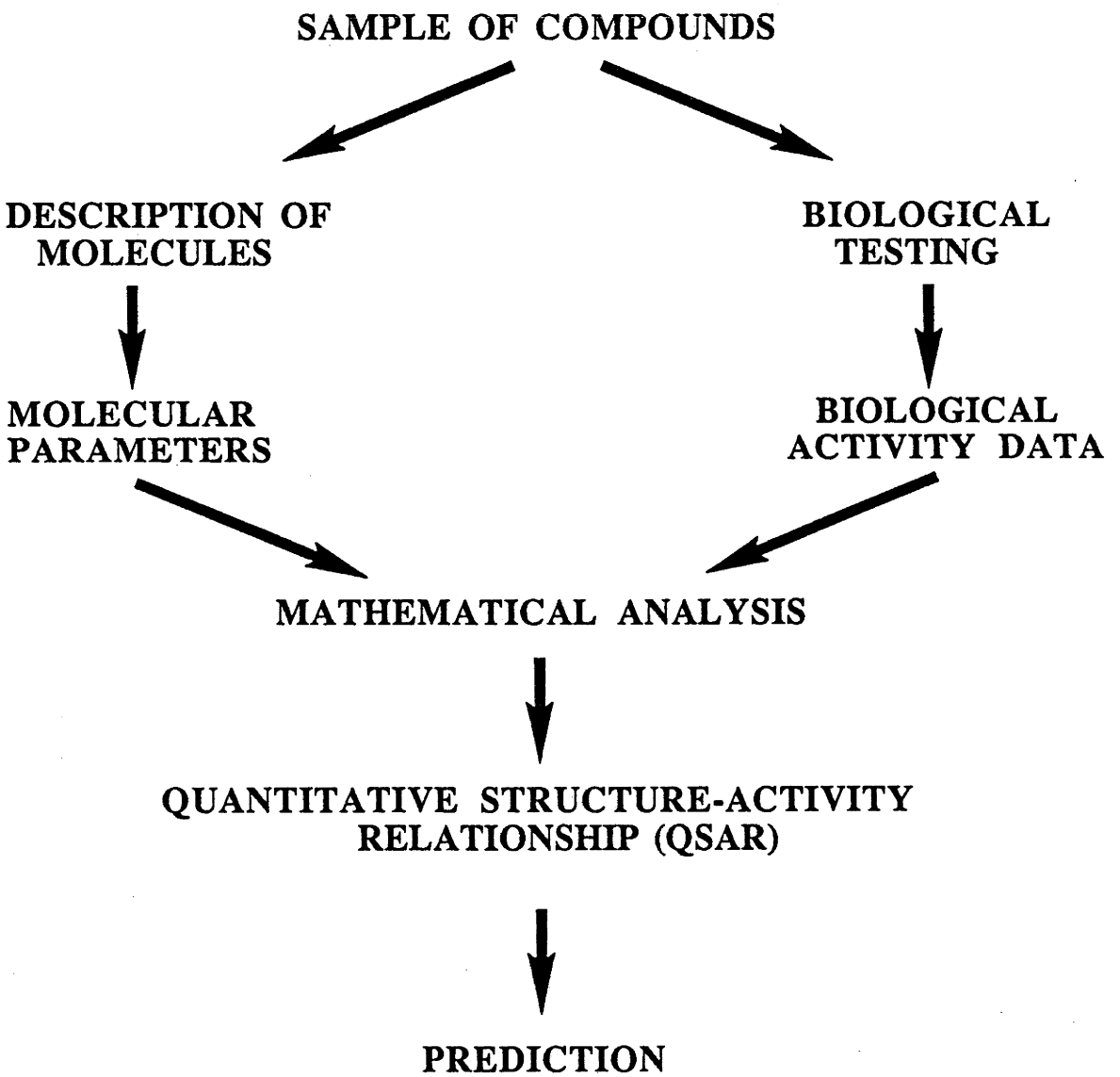
On enlarging the series of 3-methyl-10-(substituted phenyl)flavins first investigated by Cowden *et al.* [42,76], ten new active compounds were found though none were more active than the previously noted 4'-Br (**2.5aa**), 4'-Cl (**2.5o**) and 3',5'-Cl_2 (**2.5s**) substituted compounds. The two new compounds which showed the next best activity were the 3'-CF_3 (**2.5kk**) and 3'-Cl, 5'-CH_3 (**2.5u**) substituted flavins. The relative order of activity of the active compounds is shown in Table 2-7. The action of these compounds as blood schizontocides is clear by the suppression of parasitemia on day two after treatment.

When activity was detected in a flavin with a single substituent it was noted that the 4'-substituted substance was more active than its 3'-substituted positional isomer, the only exception to this was the 3-methyl-10-(3'-trifluoromethylphenyl)flavin which was found to be more active than its 4'-substituted analogue. Of the disubstituted compounds those that were 3',5'-disubstituted and active, were always found to have higher potency than their 3',4'-disubstituted isomers. This is exemplified by the substituted flavins 3',5'-Cl_2 (**2.5s**), 3',5'-Me_2 (**2.5g**) and 3'-Cl, 5'-Me (**2.5u**) as seen in Table 2-5.

All the flavins in the subgroup comprising 2'-substituted compounds (activity shown in Table 2-6) showed toxicity in the dose range tested and were always more toxic than any other positional isomer tested. A number of these compounds had activity, the most obvious one being the 2',4'-Me_2 compound (**2.5e**), which showed activity at 30 and 40 mg/kg. Of the other 2'-substituted phenyl compounds activity could only be detected by examination of the day two parasite counts, since toxicity masked activity as gauged by percentage cured or extension in lifespan. The three other active 2'-substituted phenyl compounds included the 2',5'-Cl_2 (**2.5q**), 2',6'-Me_2 (**2.5h**) and 2'-F, 4'-Cl (**2.5x**) substituted derivatives.

It has been shown that large groups in the 2'-positions of similar 10-phenyl flavins restrict the rotation of the phenyl group around the C(1') - N(10) bond because of steric hindrance [77]. It was thought that steric hindrance by the groups in the 2' position

Figure 2-6 Schematic diagram of the principles of quantitative structure-activity analysis



and the resulting loss of free rotation of the 10-phenyl group may have been the common feature responsible for the high toxicity of this subgroup. To investigate this possibility the 2' position was substituted with the small fluoro group in the 2'-F, 4'-Cl compound **2.5x**. However, while this compound did show activity it still remained toxic indicating that the relatively high toxicity characteristic of this subgroup is apparently not dependent upon the steric bulk of the 2'-substituent. Because of the high toxicity and obvious low therapeutic index of these flavins work was terminated in this area after testing five new compounds.

(ii) Quantitative structure-activity analysis (QSAA)

A systematic method was required for selecting substituents to be introduced into the 10-phenyl ring that would lead to compounds with higher antimalarial activity. An approach dealing with this problem is quantitative structure-activity analysis (QSAA). This technique assumes that congeners of a series are all acting in the biosystem by the same mechanism to produce biological activity and that the activity is related to the chemical structure of the compound. Fig 2-6 schematically illustrates the principles of this method [78]. The aim of this technique is to obtain a quantitative structure-activity relationship (QSAR) from the biological and molecular descriptive data obtained from a sample of compounds. The QSAR reveals the molecular parameters (descriptors) of importance to the biological response and how they are quantitatively related. This allows the prediction of activity of as yet unsynthesised compounds.

In the present case it is the substituents on the 10-phenyl ring that are varied and the biological activity is their *in vivo* antimalarial activity. Variation of the substituents on benzene rings is a common method used to change biological activity and has been utilized extensively, in combination with QSAA, in the study of compounds with antimalarial [79], antiallergic [80], antileishmanial [81] and antiviral activity [82] to cite but a few examples. The advantage, among others, of altering substituents on a benzene ring is that there exists a large literature data base of molecular descriptors available for such substituents [83-87]. This obviates the need for experimentally determining the

Table 2-8: Physicochemical parameters used in QSAA of the 3-methyl-10-(3',4',5'-substituted phenyl)flavins

Cmpd no.	$(X)_n$ [b]	Physicochemical parameters [a]						
		$\Sigma\pi$ [c]	$\Sigma\pi$ [d]	$\Sigma\pi^-$ [d]	$\Sigma\sigma$	ΣF	ΣR	Σ MR
2.5a	H	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2.5c	3-Me	0.56	0.52	0.50	-0.07	-0.05	-0.05	4.70
2.5d	4-Me	0.56	0.60	0.48	-0.17	-0.05	-0.14	4.70
2.5f	3,4-$(Me)_2$	1.12	1.12	0.98	-0.24	-0.10	-0.19	9.40
2.5g	3,5-$(Me)_2$	1.12	1.04	1.00	-0.14	-0.10	-0.10	9.40
2.5j	3-Et	1.02	0.99	0.94	-0.07	-0.06	-0.04	9.40
2.5k	4-Et	1.02	1.10	0.98	-0.15	-0.07	-0.11	9.40
2.5l	4-n-butyl	2.13	2.10	1.98	-0.16	-0.06	-0.13	18.7
2.5n	3-Cl	0.71	0.77	1.04	0.37	0.68	-0.06	4.8
2.5o	4-Cl	0.71	0.73	0.93	0.23	0.69	-0.16	4.8
2.5r	3,4-Cl_2	1.42	1.50	1.97	0.60	1.37	-0.22	9.6
2.5s	3,5-Cl_2	1.42	1.44	2.08	0.74	1.36	-0.12	9.6
2.5t	3-Cl,4-Me	1.27	1.37	1.52	0.06	0.63	-0.20	9.5
2.5u	3-Cl,5-Me	1.27	1.29	1.54	0.30	0.63	-0.11	9.5
2.5w	4-Cl,3-Me	1.27	1.25	1.43	0.16	0.64	-0.21	9.5
2.5y	4-Cl,3-CF_3	1.59	1.83	2.42	0.66	1.31	-0.09	8.8
2.5z	3-Br	0.86	0.96	1.17	0.39	0.71	-0.06	7.6
2.5aa	4-Br	0.86	1.19	1.13	0.23	0.73	-0.18	7.6
2.5bb	3-F	0.14	0.22	0.47	0.34	0.69	-0.12	-0.04
2.5cc	4-F	0.14	0.15	0.31	0.06	0.71	-0.34	-0.04
2.5dd	3,4-F_2	0.28	0.37	0.78	0.40	1.40	-0.46	-0.08
2.5ee	3-OMe	-0.02	0.12	0.12	0.12	0.41	-0.17	6.5
2.5ff	4-OMe	-0.02	-0.03	-0.12	-0.27	0.41	-0.50	6.5
2.5gg	3,5-$(OMe)_2$	-0.04	0.24	0.24	0.24	0.82	-0.34	13.0
2.5hh	4-OH	-0.67	-0.61	-0.87	-0.37	0.49	-0.64	1.5
2.5ii	3-NO_2	-0.28	0.11	0.54	0.71	1.09	0.05	6.0
2.5jj	4-$N(Me)_2$	0.18	-0.08	-0.69	-0.83	0.03	-0.85	14.4
2.5kk	3-CF_3	0.88	1.10	1.49	0.43	0.62	0.07	4.0
2.5ll	4-CF_3	0.88	1.04	1.05	0.54	0.63	0.19	4.0
2.5mm	3,5-$(CF_3)_2$	1.76	2.20	2.98	0.86	1.24	0.14	8.0
2.5nn	3-SMe	0.61	0.64	0.55	0.15	0.33	-0.07	13.0
2.5oo	4-SMe	0.61	0.87	0.32	0.00	0.33	-0.19	13.0
2.5pp	4-SO_2Me	-1.63	-1.20	-1.02	0.72	0.90	0.22	12.5
2.5qq	4-CN	-0.56	-0.33	0.14	0.66	0.85	0.18	5.2
2.5rr	4-CO_2H	-4.36	[e]	[e]	0.00	[e]	[e]	[e]

[a] Physicochemical parameters defined in section 2-4.4(ii). [b] Indicates substitution of the 10-phenyl of the flavins. [c] Obtained from reference [88]. [d] Obtained from reference [86]. [e] Not avialable.

molecular parameters. It is therefore possible to attempt to relate changes in biological activity to changes in the molecular descriptors of the different substituents used.

The molecular descriptors are defined in terms of physicochemical properties which usually consist of the three major subgroups of lipophilic, electronic and steric parameters. In this study no mode of action was previously known and thus there was no guide suggesting which of these physicochemical properties might have been most important to antimalarial activity, so parameters related to all three properties were used in the following analysis. The 3-methyl-10-(substituted phenyl)flavins and their physicochemical parameters considered are shown in Table 2-8.

To define the relative lipophilicity of the substituents, π values were used (π values from model systems are defined by the equation

$$\pi_X = \log P_X - \log P_H$$

where P_X is the partition coefficient of a X-substituted derivative and P_H that of the parent compound). A positive π value indicates that relative to hydrogen the substituent is lipophilic, while a negative value indicates it hydrophilic *vis-a-vis* hydrogen. In Table 2-8 the $\Sigma\pi$ values are the sum of the π values of substituents in the 3', 4' and 5' positions. The three different $\Sigma\pi$ values presented are based on: (1) π values obtained from Martin [88]; (2) π values obtained from Norrington *et al.* [86] in which π is positionally dependent; and (3) π^- values also obtained from Norrington *et al.* which are based on a model system containing an electron donating side chain on the benzene ring. These three represent alternative measurements of the same property. The importance of the π parameter lies in its established correlation in binding to biological macromolecules and transport through biological systems.

The electronic properties of a compound were represented by the sum of the Hammett constants (σ) of the 3', 4' and 5' substituents on the 10-phenyl ring. The σ constant is comprised of both inductive and mesomeric effects and therefore can also be represented by *F* and *R* values which are positionally weighted parameters indicating the inductive and mesomeric effects respectively, [86]. The $\Sigma\sigma$, and ΣF and ΣR values represent two alternate measures of the electronic properties of the substituted flavins.

The values used to obtain $\Sigma\sigma$ were obtained from Martin [88] and those for ΣF and ΣR were obtained from Norrington *et al.* [86].

The steric properties of the substituents were represented by the sum of their molar refractivity values (Σ MR). MR values are usually obtained experimentally by the Lorentz-Lorenz equation;

$$MR = \left(\frac{n^2-1}{n^2-2} \right) \times V$$

where V is molar volume and n is the refractive index. The MR values of common atoms and groups of atoms have been calculated using model systems. Since for liquid organic compounds n does not vary much, MR is really a "corrected" form of molar volume and as such represents a measure of the bulk of a substituent [83,85]. As MR increases so does the bulk of the substituents, the MR values used to obtain Σ MR were taken from Norrington *et al.* [86].

The importance of the electronic and steric properties of a drug relate to their intrinsic importance in the interactions, both physically and chemically, in the biosystem.

It was decided to use the methodology of QSAA as no obvious trend was observed between activity and any single one of the above parameters. It was thought that QSAA might indicate a relationship between activity and a combination of these parameters.

The first step in QSAA is choosing substituents to be used for synthesising a sample group of compounds. This group is referred to as the "training set", upon whose physicochemical and biological data the QSAR is to be based. The aim of this selection is to obtain a preliminary series in which all the physicochemical and structural properties that may be governing biological activity are varied systematically and independently from each other over a sufficiently large range. Different techniques for designing training series have been discussed by Craig [89], Wooldridge [90], Wootton *et al.* [91], Hansch *et al.* [92], and Dove *et al.* [93].

In the present study the training series was based on the expansion of an already

existing group of compounds made by Cowden *et al.* [42]. The selection of new congeners was made on the basis of the following:

1. With the aid of the TMIC (two-dimensional mapping of intraclass correlation matrices) method of Dove *et al.* [93], which allows the selection of single substituents that have high data variance and low collinearities of the major physicochemical parameters, by simple inspection of a two dimensional map of 35 possible single substituents;
2. For the majority of the disubstituted compounds, selection of substituents and position was made to determine the effect of various substitution patterns or, in an effort to increase activity, by combining single substituents which had shown activity; and
3. The selection processes above in 1. and 2. were subject to the synthetic feasibility of preparing potential congeners (see section 2-2).

The toxicity of and the difficulty in obtaining reliable physicochemical parameters for the 2'-substituted phenyl flavins meant that they were excluded from the following attempts at QSAA.

Initially an attempt was made to obtain a QSAR using the popular "Hansch" methodology. The Hansch method uses least squares multiple linear regression to correlate biological activity data to molecular parameters [94]. A requirement of the Hansch approach is that the biological data be in the form of a continuous quantity, usually the log molar dose of a drug required to produce a standard biological response. Of the training series made and tested shown in Table 2-5 such values were only obtainable for the subgroup of compounds in Table 2-7 in the form of ED_{40} values. For 16 of the flavins in the training series ED_{40} determination was not possible usually because they were inactive in the dose range tested.

The physicochemical data used in the multiple regression analysis is shown in Table 2-8. As well as these parameters four indicator variables, indicating whether the flavins were 3'-, 4'-, 3',4'- or 3',5'- substituted, were included as the substitution pattern appeared to be a factor of importance from the SAR analysis.

In the hands of a statistical analyst [e] a linear regression model was fitted;

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[e] The author is grateful to Mr R. Cunningham, statistical consultant, Statistics Department, The Australian National University, for the statistical analysis.

Table 2-9: Biological data used in QSAA and predicted ED_{40} values

Compound no.	$(X)_n$ [a]	Activity [b]	ED_{40} [c] (mmoles/kg x 10^{-3})	Predicted ED_{40} [d] (mmoles/kg x 10^{-3})
2.5a	H	1	248	204
2.5c	3-Me	0	-	201
2.5d	4-Me	0	-	201
2.5f	3,4-$(Me)_2$	1	210	221
2.5g	3,5-$(Me)_2$	1	105	221
2.5j	3-Et	0	-	214
2.5k	4-Et	1	281	216
2.5l	4-*n*-butyl	0	-	214
2.5n	3-Cl	1	229	113
2.5o	4-Cl	1	38.8	112
2.5r	3,4-Cl_2	1	-	62.7
2.5s	3,5-Cl_2	1	40.2	62.7
2.5t	3-Cl,4-Me	1	456	118
2.5u	3-Cl,5-Me	1	85.7	118
2.5w	4-Cl,3-Me	1	182	118
2.5y	4-Cl,3-CF_3	0	-	65.9
2.5z	3-Br	1	148	110
2.5aa	4-Br	1	38.4	109
2.5bb	3-F	1	-	112
2.5cc	4-F	1	103	110
2.5dd	3,4-F_2	0	-	60.8
2.5ee	3-OMe	0	-	142
2.5ff	4-OMe	1	138	142
2.5gg	3,5-$(OMe)_2$	1	219	100
2.5hh	4-OH	0	-	133
2.5ii	3-NO_2	0	-	79.6
2.5jj	4-$N(Me)_2$	0	-	198
2.5kk	3-CF_3	1	79.3	119
2.5ll	4-CF_3	1	135	118
2.5mm	3,5-$(CF_3)_2$	0	-	69.9
2.5nn	3-SMe	0	-	153
2.5oo	4-SMe	0	-	153
2.5pp	4-SO_2Me	0	-	93.5
2.5qq	4-CN	0	-	97.3
2.5rr	4-CO_2H	0	-	-

[a] Indicates substitution of the 10-phenyl of the flavins. [b] Activity is indicated by 1, inactivity by 0; based on the data in Table 2-5. [c] ED_{40} values taken from Table 2-7. [d] ED_{40} values predicted using equation 2-1 and parameters from Table 2-8.

computations were executed using the statistical package Genstat 5. The analysis involved first using all the independent variables (i.e. the physicochemical parameters and the four indicator variables) in a full model followed by removing the variables individually. Using the *F* distribution statistic (variance ratio) compared to the appropriate *F* value (according to degrees of freedom) it was possible to determine the significance of each independent variable as it was removed. The best model which could be found using this method is shown in equation 2-1.

$$\ln (ED_{40}) = -1.592 - 0.863\ \Sigma F \qquad \textbf{Equation 2-1}$$

$$(0.277)\quad (0.426) \qquad \text{estimated std errors}$$

$$n = 17 \qquad R^2_{adj} = 16.2 \qquad s = 0.725$$

This equation indicates that the greater the inductive electron withdrawing effect (large positive ΣF values) of the substituents in the 3', 4' and 5' positions the higher the activity. However, the *t* statistic (2.02) is barely significant (considered significant if greater than 2.1), thus this was a poor model for predicting activity. This is highlighted by the inactivity of the 3'-NO_2, 3',5'-$(CF_3)_2$, 4'-SO_2CH_3 and 4'-CN substituted flavins, which were not included in this analysis, but by equation 2-1 were predicted to have good activity (shown in Table 2-9).

This method, because of the type of statistical analysis used was limited to the biological data that was presented as ED_{40} values. This left out a large proportion of the training set, therefore limiting the physicochemical "space" covered in the model as well as reducing the number of compounds that could be included in the regression. The failure to use the entire training set could be responsible for the poor model produced.

To overcome this data gap an alternative statistical method was used that allowed inclusion of the entire series. This method, of logistic regression [95], allowed the biological response to be entered as binary data, in this case either as active or inactive. This method is similar to previously used methods of discriminant analysis [96,97] in that it allows the use of qualitative biological responses.

In this study logistic regression analysis was used to determine if the independent variables considered in the Hansch approach effect the probability of a compound being active or not. Activity is defined by detection of antimalarial activity (Table 2-5) and is

indicated by 1 in the activity column of Table 2-9; 0 indicates the compound to be inactive.

In a stepwise analysis of a full model similar to that done in the linear regression analysis, it was found that none of the terms were significant using the Chi-square test as a measure of significance and no equation could be found that was superior to the null hypothesis (the random probability of a compound being active).

Ariëns [98] has listed and discussed eight factors that could be involved in the apparent lack of a structure-action relationship. These include:

1. A lack of a relationship between the physicochemical parameters used and those of importance in the bioreaction that produces activity.
2. It is a metabolite which is responsible for activity and not the administered drug itself.
3. The action of a drug can be the result of a sequence of complicated events often involving pharmacokinetic processes. Each event may be related differently to the physicochemical properties of the drugs. If the physicochemical property requirements between events relate to each other in a contradictory or incompatible fashion then drug action as a whole may not be easily related to physicochemical properties. This is because, in essence, they are not of an additive nature amongst the different processes.
4. The possibility of a mixture of isomeric compounds which have different effects biologically being administered as a single test compound. Obviously such occurrences would severely compromise the nature of the biological data.
5. It is assumed that all members of a series are working by the same mechanism of action and in *in vivo* models that they have similar pharmacokinetic mechanisms. If this is not the case a clear cut QSAR could not be expected.
6. The route of administration and the pharmaceutical preparation of compounds can often have large influences on the potency of drugs, for example, the particle size and crystal structure of a drug may influence its potency. The disparity that may be occasioned by these differences may obscure a structure-activity relationship.

7. Drugs often have more than one biological action which may coincide or overlap to a considerable degree. Toxic side effects can be especially important in the *in vivo* model. Thus drugs which are multipotent have the ability to complicate the determination of a QSAR.

8. The diversity in drug action between species is well known, due in a large number of cases to pharmacokinetic differences amongst species. Such differences must be taken into account in formulating a QSAR.

With this in mind, it seems that the most likely reason for not obtaining a satisfactory QSAR for the flavins was the *in vivo* nature of the biological response. Thus, as noted *in vivo* results give valuable information about toxicity and compounds with favourable pharmacokinetics but in the determination of a QSAR this extra information may obscure the relationship. The possible factors that may be responsible in the present case include points 2, 3, 5, 6 and 7 from above. The importance of the complicated pharmacokinetic processes in this series is shown by the differences observed in the SAR of the *in vitro* and *in vivo* testing of these compounds (discussed in section 2-5.4) and the differences in the dose-response curves amongst the active flavins demonstrated in Fig 2-5.

The other major possibility of why no QSAR could to be obtained was that the descriptor parameters used were not those of importance to biological activity (point 1 above). This is always a possibility in QSAA in systems where there is no mechanistic model available on which to base the choice of molecular descriptors. In this flavin QSAA the selection of parameters was based on those that have most frequently been shown to be important in determining biological activity. The additional inclusion of the many other possible molecular parameters available [83,99] was not considered as these other parameters are usually less well defined and often have a high degree of collinearity with those parameters already tested. Besides this, it has been shown there is a limit to the number of explanatory variables, relative to sample size, which may be used in QSAA before the occurrence of chance correlations [100]. It is necessary to increase the sample size before increasing the number of variables screened in order to avoid obtaining correlations of doubtful significance.

In conclusion, a large number of compounds with different substituents, in which

the physicochemical properties were altered over a wide range, were made and tested. Since none were found to be more active than those in the original set prepared by Cowden *et al.* [42] and no useful predictive equation could be obtained using the methods outlined above, it was felt that further work on phenyl substitution to enhance potency was not warranted.

The information about the *in vivo* effects of substituents on the 10-phenyl ring was utilized to optimize activity when other structural changes were subsequently made in the following chapters.

2-5 *In vitro* antimalarial testing

2-5.1 Introduction

With the advent of a continuous culture method for *P. falciparum* in human erythrocytes, made possible by the work of Trager and Jensen [101] in 1976, it became possible to routinely test compounds against the principal human malarial parasite in a model free of the majority of the undesirable pharmacokinetic effects characteristic of *in vivo* models. Desjardins *et al.* [102] utilized the methods of continuous culture to develop a rapid, semiautomated, microdilution technique to measure the antimalarial activity of compounds against the asexual intraerythrocytic forms of *P. falciparum*. Their technique involved the growing of cultures in 96-well microtiter plates and the addition of a radiolabelled substrate which would be incorporated into the nucleic acid of the parasites. The 96-well plate allowed for large scale testing of compounds as each microwell is capable of being treated with different drugs and concentrations. In this particular method parasite development, after an incubation period, is quickly and accurately measured by determining the level of ^{3}H-hypoxanthine incorporation into the parasite using liquid scintillation counting.

The basic method of Desjardins *et al.* has been used successfully by numerous workers to, amongst other things, detect activity of potential antimalarials, identify the stage specificity of existing drugs and to investigate synergy of new and old antimalarials [102-105]. A brief review by Trager [106] relates the many applications of cultured

P. falciparum in both basic and applied malaria research.

In this investigation the Desjardins technique, with slight modifications, was used to confirm the activity of the flavins against the human parasite, look for cross resistance between the flavins and the established antimalarials chloroquine and pyrimethamine, and to carry out limited structure activity relationship studies in a situation free of many of the problems related to pharmacokinetics.

2-5.2 Materials and methods

In this work two variations of the Desjardins *in vitro* radioisotopic technique were used to determine the activity of ten flavins and three established antimalarials against *P. falciparum*. The first method (method 1) was conducted in collaboration with Dr G. A. Butcher [f] in which activity was determined against the chloroquine-sensitive FC-27 strain. The second method (method 2) involved testing against both the chloroquine-sensitive FC-27 and chloroquine-resistant K-1 strains. (The K-1 strain is also resistant to other established antimalarial drugs, see Table 2-10.) This was kindly carried out by Dr K. A. Rockett [g]. In both methods incubation times were 48 hours to allow one complete cycle of blood schizogony.

The experimental details of both methods are contained in section 7-3.1(ii).

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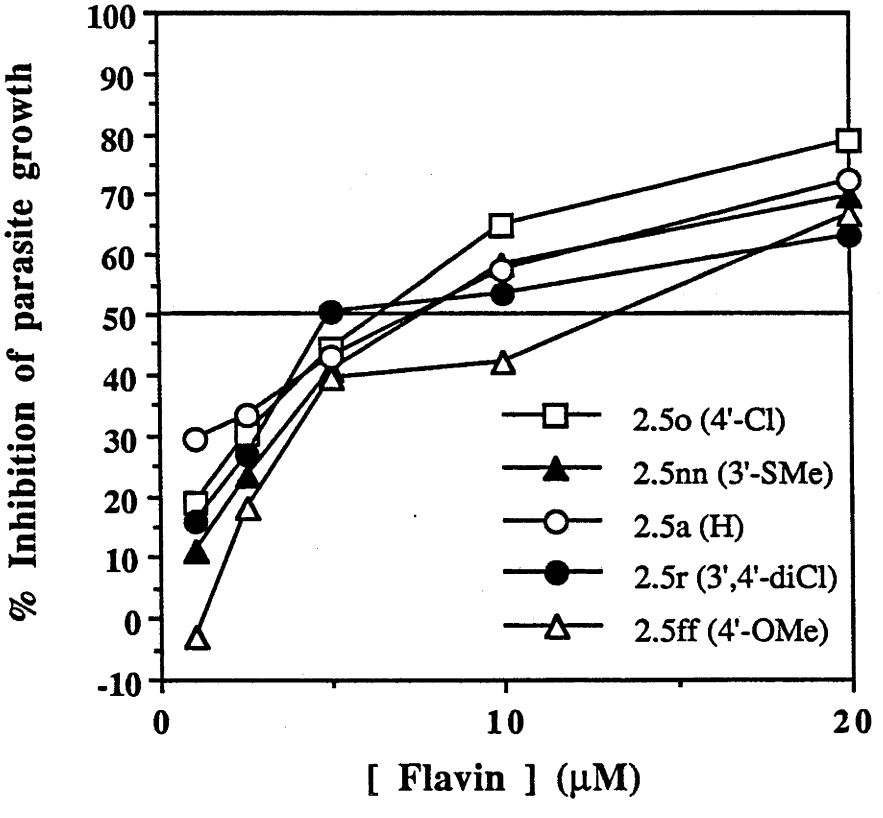
2-5.3 Results

Method 1

The results of the *P. falciparum* (FC-27) assay are presented in Fig 2-7, in which the percentage inhibition of parasite growth is measured by the inhibition of the uptake of tritiated hypoxanthine in drug-treated parasite cultures compared to drug-free control cultures.

Figure 2-7 Inhibition of cultured *P. falciparum* (FC-27 chloroquine-sensitive) by 3-methyl-10-(substituted phenyl)flavins



Each datum point is the mean for the sample tested in triplicate in a single experiment. Substituents on the 10-phenyl ring are shown in brackets. The point used to compare activity is the concentration that inhibits the uptake of ^{3}H-hypoxanthine by 50%.

Table 2-10: ***In vitro*** **antimalarial activity of chloroquine, pyrimethamine, quinine and flavins against 2 isolates of** ***P. falciparum*** **(FC-27 and K-1)**

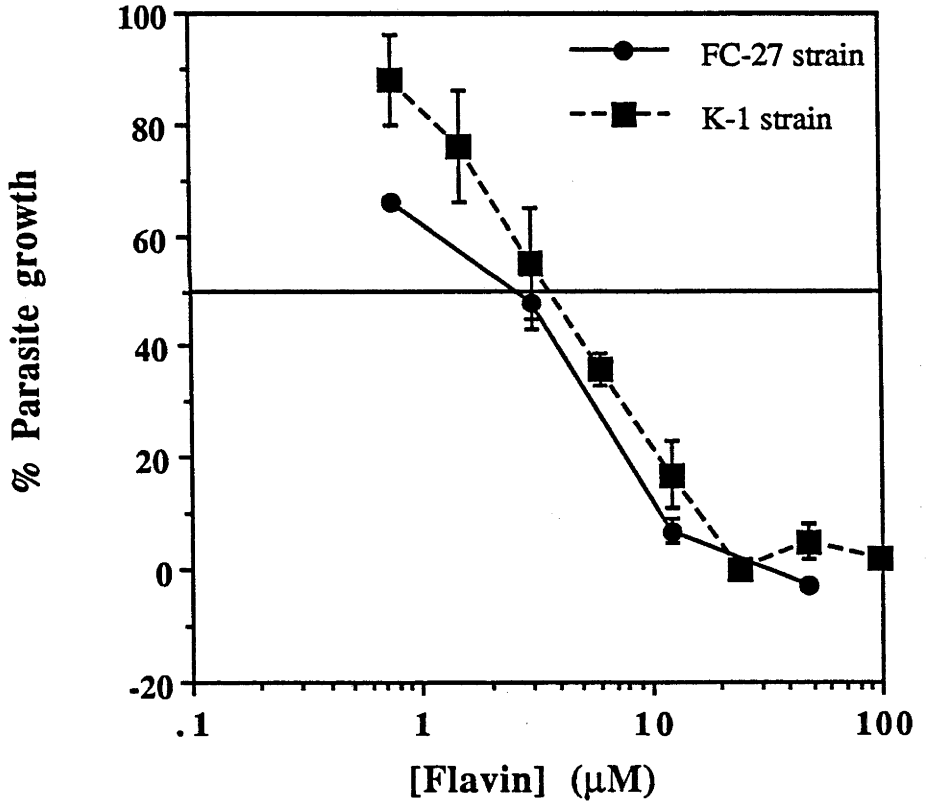
Compound [a]	IC_{50}[b,c] (μM)		Factor of resistance in K-1[d]
	FC-27	K-1	
Chloroquine	0.04	2.5	63
Pyrimethamine	0.34	>100	>294
Quinine	7.4×10^{-2}	0.56	8
2.5kk (3'-CF_3)	3.0	3.6	1.2
2.5s (3',5'-Cl_2)	2.3	4.8	2.1
2.5g (3',5'-Me_2)	5.6	13.6	2.4
2.5qq (4'-CN)	4.2	[e]	-
2.5rr (4'-CO_2H)	24.7	[e]	-

[a] Substitution of the 3-methyl-10-(substituted phenyl)flavins is indicated inside brackets. [b] IC_{50} is the concentration required to inhibit the uptake of ^3H-hypoxanthine by the culture by 50%. [c] These results were obtained from a single experiment done in triplicate. [d] This value was obtained by dividing the K-1 IC_{50} by the FC-27 IC_{50}. [e] Not done.

Method 2

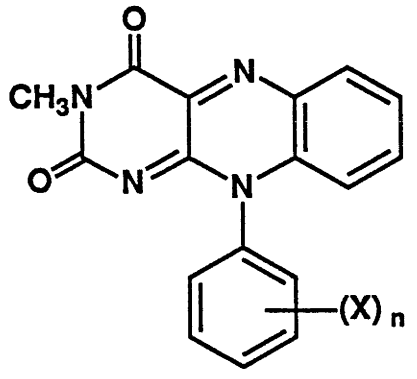
Results of a typical assay for 3-methyl-10-(3'-trifluoromethylphenyl)flavin against both the FC-27 strain and K-1 multidrug-resistant strain are shown in Fig 2-8. The IC_{50} values (concentration of the compound causing 50% inhibition of ^{3}H-hypoxanthine incorporation) of the flavins and other antimalarials are given in Table 2-10.

Figure 2-8 Inhibition of cultured *P. falciparum* strains FC-27 (chloroquine-sensitive) and K-1 (chloroquine-resistant) by 3-methyl-10-(3'-trifluoromethylphenyl)flavin



Each datum point is the mean ± 1 Std Dev for the sample tested in triplicate in a single experiment. The point used to compare activity is the dose that inhibits the uptake of ^{3}H-hypoxanthine by 50%. The concentration is plotted on a log scale.

Table 2-11: 3-Methyl-10-(substituted phenyl)flavins selected on the basis of *in vivo* activity and physicochemical parameters



Compound no.	$(X)_n$	*In vivo* activity [a]	$\Sigma\sigma$ [b]	$\Sigma\pi$ [b]	Σ MR [b]
2.5o	4-Cl	38.8	0.23	0.71	4.8
2.5nn	3-SMe	not active	0.15	0.61	13.0
2.5a	H	248	0.00	0.00	0.0
2.5ff	4-OMe	138	-0.27	-0.02	6.5
2.5r	3,4-Cl	active [c]	0.60	1.42	9.6

[a] Activity is reported as ED_{40} values (mmoles/kg x 10^{-3}) from Table 2-7 or as active or not active according to results from Table 2-5. [b] Physicochemical parameters are the same as those in Table 2-8. [c] Activity was detected at a dose of 140 mg/kg.

2-5.4 Discussion of results

Using method 1 a subgroup of five of the forty compounds in this series was selected for *in vitro* testing against *P. falciparum* (FC-27). The five compounds selected comprised a group that varied in *in vivo* activity and in their physicochemical properties (based on their 10-phenyl ring substituents). Table 2-11 shows the compounds selected, their *in vivo* activity and the physicochemical properties of the substituents. Figure 2-7 shows that all these compounds were active *in vitro* confirming similar findings of Cowden *et al.* [41] and Becker *et al.* [107] who also noted good *in vitro* activity for members of this series. By interpolation from this graph, the five flavins have IC_{50} values ranging from approximately 5 to 13 μ M.

This narrow range of potency from a series of compounds which vary widely in the nature of their substituents, makes it seem likely that variation of substituents on the 10-phenyl ring of the flavin has little effect in terms of direct anti-parasitic action. The significance of this observation becomes apparent when compared to the *in vivo* results. The fact that the order of the *in vivo* activities of these compounds was not reflected in the *in vitro* activities strongly suggests that alteration of the substituents on the 10-phenyl ring is primarily important in terms of *in vivo* events such as drug transport, distribution and excretion after injection. This difference in activity between culture and *in vivo* testing is especially highlighted in the case of 3-methyl-10-(3'-methylthiophenyl)flavin (**2.5nn**) which was inactive *in vivo* but showed nearly equivalent *in vitro* activity to the highly (*in vivo*) active 10-(4'-chlorophenyl)-3-methylflavin (**2.5o**). This indicates the *in vivo* results are not direct indicators of mechanistic activity.

Using method 2 a different group of five 3-methyl-10-(substituted phenyl)flavins were tested against *P. falciparum*. All of these flavins were tested against the FC-27 strain and three of the five were tested against the drug resistant K-1 strain.

The 10-(4'-carboxyphenyl)-3-methylflavin (**2.5rr**) is unique among the series of 3-methyl-10-(substituted phenyl)flavins in that at physiological pH it is likely to form a carboxylic acid anion. This compound was originally synthesised in order to take advantage of the reported increase in permeability to anions of malaria-infected

erythrocyte cell membranes [108], however, it failed to show any activity in the *in vivo* testing. This compound was selected for *in vitro* testing to determine if its anion forming properties had any detectable benefit in this more direct testing system. The IC_{50} of this compound showed it to be the least efficacious of the flavins examined, thus indicating that anion formation in this case had no beneficial effect.

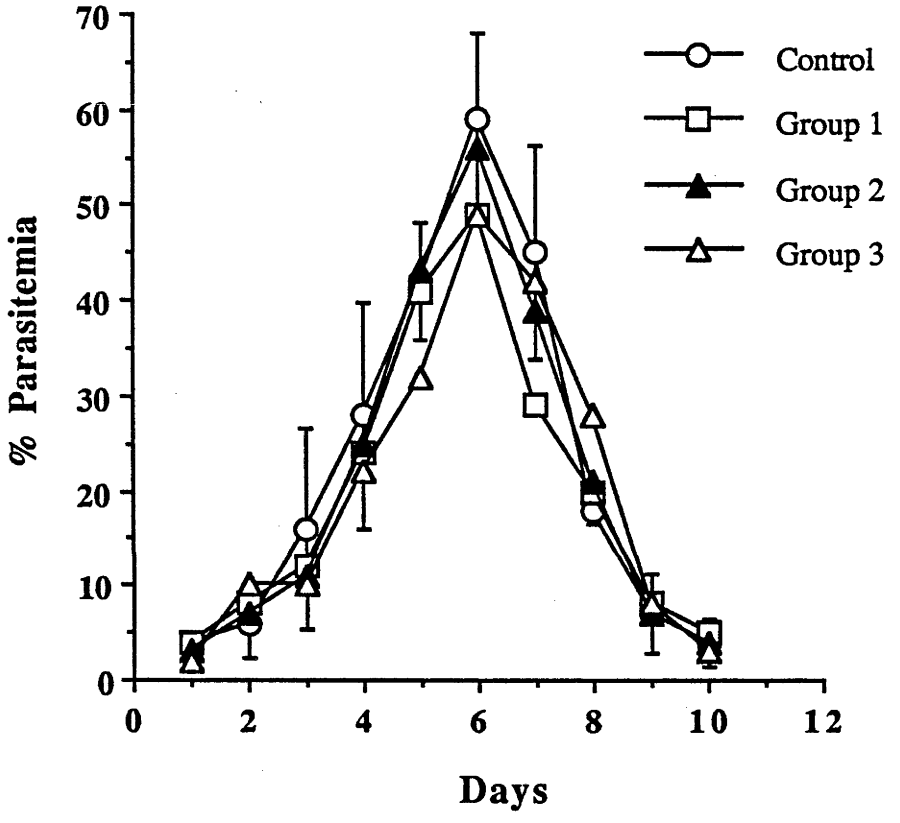
The IC_{50} values of the other four flavins examined against the FC-27 strain ranged from 2.3 to 5.6 μ M. These values are not directly comparable to the results obtained using method 1 because of differences in experimental procedure. Nonetheless, the potency and narrow range of activity are similar to those observed in method 1 and confirm the conclusions that: (i) variation of the 10-phenyl substituents usually has only small effects on i*n vitro* activity; and (ii) *in vitro* activity does not directly correlate with *in vivo* activity.

In a comparison of the drug sensitivity of the two *P. falciparum* strains FC-27 and K-1, the K-1 strain was found to be resistant to the currently used antimalarial drugs pyrimethamine and quinine by factors of >294 and 8, respectively, as well as to chloroquine by a factor of 63. These findings are consistent with previous reports [105,109] and established that the K-1 strain used in this study was indeed multidrug-resistant.

The results of testing the three flavins (**2.5kk, 2.5s, 2.5g**) against both the drug-sensitive and drug-resistant strains in Table 2-10 show that the flavins are consistently slightly less active against K-1 than FC-27 by a factor of 1.2 to 2.4. These small differences in activity between strains, when compared to the large differences in the chloroquine and pyrimethamine sensitivities indicate there is no substantial cross resistance between these agents. This finding is important since it shows that these flavins, which have good *in vivo* activity, are of potential use in cases of chloroquine- and pyrimethamine-resistant malaria. This finding also indicates that the flavins' mode of action is most likely dissimilar to that of chloroquine and pyrimethamine.

In addition to the above findings the *in vitro* assay also provides an estimate of the plasma drug levels that need to be achieved for *in vivo* inhibition of the parasite. Studies on plasma levels of active and inactive flavins could be used to confirm that variation in

Figure 2-9 **The ineffectiveness of flavins 2.5s (3',5'-Cl_2) and 2.5o (4'-Cl) against *B. microti* infection in mice**



For all groups treatment began on day 1. Group 1 with a daily dose of 15 mg/kg of compound **2.5s** for 5 days. Group 2 with a daily dose of 5 mg/kg of compound **2.5s** for 5 days. Group 3 with a daily dose of 10 mg/kg of compound **2.5o** for 4 days. The control received a daily injection of vehicle only for 5 days. Each datum point is the mean of the daily parasitemia of a group of 4-6 mice. For clarity only the control group has error bars representing ± 1 Std Dev.

in vivo activity of the 3-methyl-10-(substituted phenyl)flavins is primarily a function of pharmacokinetics. Such a study could also yield information concerning the possibility that antimalarial activity might be due to an active metabolite. Though there is no evidence for this supposition, the apparent lack of correlation between *in vivo* and *in vitro* antimalarial activity and the failure to obtain a QSAR could be explained in these terms [110]. It should be noted, however, that the observed activity of these compounds both *in vitro* and *in vivo* would tend to indicate that this is not the case.

In this study only blood schizontocidal activity was studied. The development of new culturing techniques for the exoerythrocytic and gametocyte stages of the human malaria parasite in the 1980s [111-114] means that drug testing assays against these stages could soon become commonly available. If this happens it would be interesting to determine the activity of the flavins against these other parasite stages.

2-6 Other test systems

2-6.1 *In vivo* antibabesial testing

Babesiosis is a tick-borne disease of wild and domestic animals, its major economic importance is in the cattle industry. It is caused by an intraerythrocytic protozoan parasite of the genus *Babesia*. Occasionally human infection occurs, of these the majority of cases have been caused by *Babesia microti*, a parasite of rodents [115].

The morphological similarities between *B. microti* and malaria parasites and the overlap of effective drugs for both diseases [115] lead to the decision to screen two of the more effective antimalarial flavins for antibabesial activity.

This screening, using *B. microti* in mice [116] involved the daily intraperitoneal injection of 10-(3',5'-dichlorophenyl)-3-methylflavin (**2.5s**) at 5 and 15 mg/kg and 10-(4'-chlorophenyl)-3-methylflavin (**2.5o**) at 10 mg/kg for 4-5 days after the disease first became patent. The result of this screen is seen in Fig 2-9 where the average daily percentage parasitemia of the treated groups are compared to a control group throughout the course of the disease. No significant suppression of parasitemia (the slight depression of the maximum parasitemia on day 6 in the treated groups was found not to

be significant by Student's *t* test) and the resemblance of the curves of the treated groups to that of the control group indicated that these compounds had no activity against *B. microti* in mice over the dose range tested. The experimental details of this procedure are presented in section 7-3.2.

2-6.2 *Giardia intestinalis*

The protozoan parasite *Giardia intestinalis* is found world-wide and is an important cause of chronic and sometimes serious gastrointestinal disease especially in children. It has been shown that *G. intestinalis* is susceptible to 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and 1-(2-chloroethyl)-3-(2-hydroxyethyl)-1-nitrosourea (HECNU) [117]. Both of these compounds are inhibitors of glutathione reductase (GR) and have been shown to have antimalarial activity [118-120]. Since the flavins are likewise antimalarials and inhibitors of GR (see Chapter 6) the two compounds **2.5o** and **2.5g** have been submitted for screening against *G. intestinalis* [h]. The screening method to be used is that of Boreham, Phillips and Shepherd [121]. Results of this screen are pending.

2-6.3 *In vitro* anti-HIV and anticancer testing

The National Cancer Institute [i] has recently begun a world-wide screening program to discover new antiviral agents effective against the human immunodeficiency virus (HIV). The screening method used is described by Weislow *et al.* [122]. The following compounds were evaluated for *in vitro* anti-HIV activity and classified as inactive; **2.5b** (2'-Me), **2.5d** (4'-Me), **2.5e** (2',4'-Me_2), **2.5g** (3',5'-Me_2), **2.5i** (2'-Et), **2.5l** (4'-*n*-butyl), **2.5n** (3'-Cl), **2.5q** (2',5'-Cl_2), **2.5y** (4'-Cl, 3'-CF_3), **2.5z** (3'-Br), **2.5aa** (4'-Br), **2.5bb** (3'-F), **2.5cc** (4'-F), **2.5dd** (3',4'-F_2), **2.5ee** (3'-OMe),

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[h] Testing is to be kindly carried out by Dr P. F. L. Boreham, Queensland Instituted of Medical Research, Bramston Terrace, Herston, Brisbane, Queensland, 4006.
[i] Dr V. L. Narayanan, Drug Synthesis and Chemistry Branch, Executive Plaza North, Suite 811, Bethesda, Maryland, 20892, USA.

2.5hh (4'-OH), **2.5mm** (3',5'-$(CF_3)_2$) and **2.5nn** (3'-SMe). These compounds were also submitted to the National Cancer Institute for an *in vitro* pre-screen consisting of human cell lines representing major tumour types such as lung, colon and melanoma. The results of this anticancer screen are pending.

CHAPTER 3

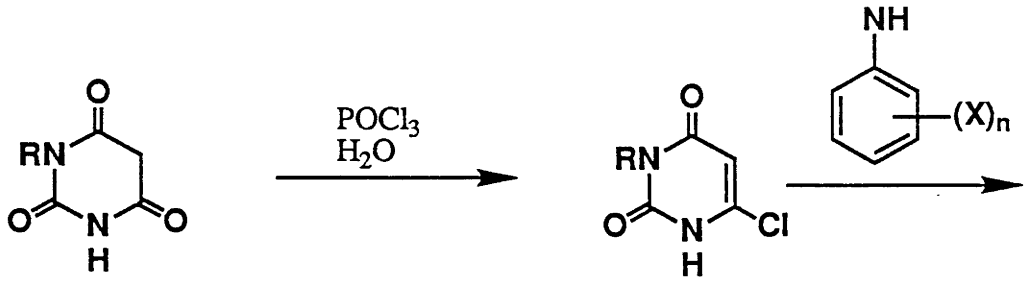
CHAPTER 3 Syntheses and antimalarial activity of some 3-substituted 10-(substituted phenyl)flavins

3-1 Introduction

In this chapter changes made at the 3-**N** position and to substituents on the 10-phenyl group of the flavin are outlined. The variation at the 3 position was undertaken primarily to effect changes in the overall lipophilicity of the flavins. Three subgroups were produced including a series of 3-ethyl-10-(substituted phenyl)flavins (**3.4a-l**), 3-substituted 10-phenylflavins (**3.7a, 2.5a, 3.4p-r**) and 3-substituted 10-(4'-chlorophenyl)flavins (**3.7b, 2.5o, 3.4m-o**). These compounds were tested for *in vivo* antimalarial activity where gains in both activity and lower toxicity, compared to their 3-methyl analogues (Chapter 2), were noted in a number of compounds. The 3-substituted 10-(4'-chlorophenyl)flavin series were tested *in vitro* against *P. falciparum* and found to have little variation in potency throughout the series.

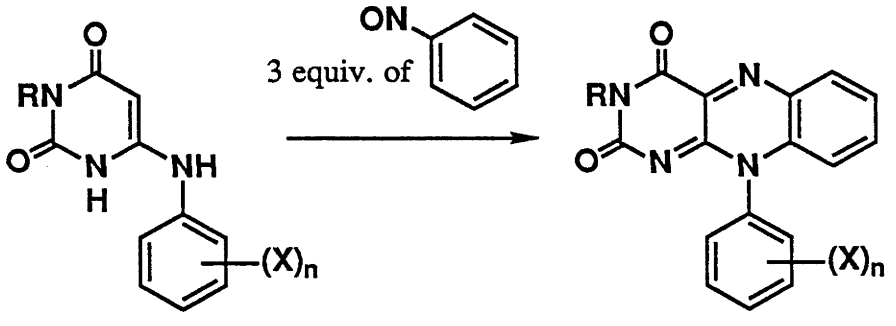
The synthesis, chemistry (electrophilic substitution), mass spectra and ^{1}H n.m.r. spectral data, along with results of antimalarial, anti-HIV and anticoccidial testing of these flavins are discussed in the following sections.

Scheme 3-1



3.1 3.2

3.1 and 3.2	R
a	Ethyl
b	*n*-Propyl
c	Phenyl
d	Benzyl



3.3 3.4

3.3 and 3.4	R	$(X)_n$	3.3 and 3.4	R	$(X)_n$
a	Ethyl	H	m	*n*-Propyl	4-Cl
b	Ethyl	4-Cl	n	Phenyl	4-Cl
c	Ethyl	4-Br	o	Benzyl	4-Cl
d	Ethyl	3-F	p	Phenyl	H
e	Ethyl	4-F			
f	Ethyl	3,5-Cl_2			
g	Ethyl	3,5-Me_2			
h	Ethyl	3-CF_3			
i	Ethyl	3-CN			
j	Ethyl	4-CN			
k	Ethyl	3-SMe			
l	Ethyl	4-SMe			

3-2 Syntheses

The two main routes used to produce the 3-substituted 10-(substituted phenyl)flavins were, firstly a modification of the main reaction pathway used to produce the flavins in Chapter 2 (shown in Scheme 3-1) and secondly, N-alkylation of the 10-phenyl flavins formed from the reaction of N-phenyl-1,2-benzenediamines (**3.6**) with alloxan (Scheme 3-2).

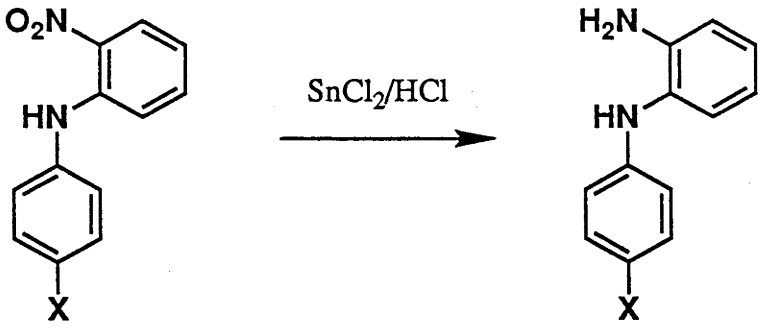
The advantage of the latter method is that it allows the introduction of the 3-N substituent at the last step in the reaction sequence. This method does, however, have limitations in that the 3-phenyl derivative cannot be produced in this way (because of the inactivity of phenyl halides towards nucleophilic displacement) and the frequent difficulty encountered in producing the N-phenyl-1,2-benzenediamine starting materials. Therefore, the former method is still advantageous when the aim of the synthesis is a concurrent alteration of substituents in both the 3-N position and the 10-phenyl ring or when a phenyl group is required at the 3 position.

A common method for the production of N-substituted barbituric acids, the starting material for Scheme 3-1, could not be found. The condensation of the monosubstituted ureas with malonic acid using acetic anhydride as a condensing agent was used to produce the N-ethyl and N-propyl barbituric acids [123].

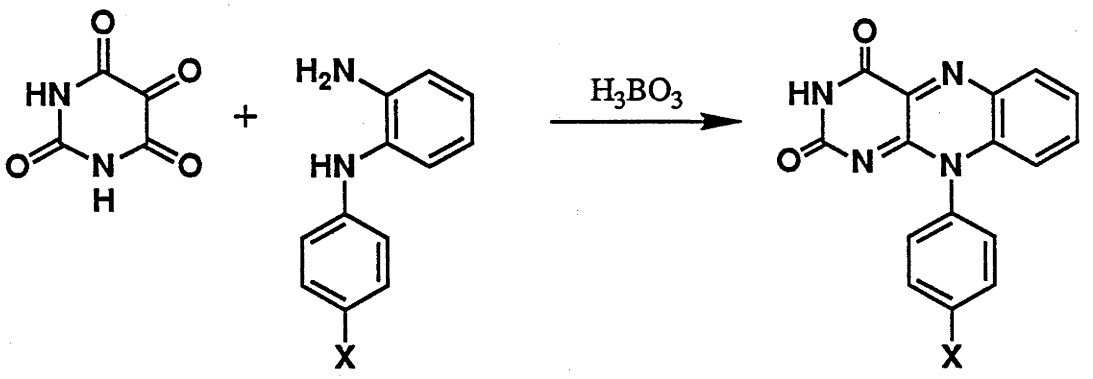
Pure N-phenylbarbituric acid was produced in 40% yield by the literature method of MacBeth *et al.* [124] using N-phenylurea, malonic acid and the condensing agent phosphorus oxychloride. Brückmann and Isaacs [123] reported a 58% crude yield of N-phenylbarbituric acid using sodium methoxide as the condensing reagent which they reported to be a superior method to that of using acetic anhydride.

Only low yields of N-benzylbarbituric acid were obtained using acetic anhydride, as the condensing agent, due to the formation of large amounts of a by-product, probably 5-acetyl-3-benzylbarbituric acid [63]. It was possible to convert this by-product to the desired N-benzylbarbituric acid by acid hydrolysis though only in low yields. An attempt to produce N-benzylbarbituric acid using phosphorous oxychloride as a condensing agent failed and starting material was recovered. A further attempt using the more reactive

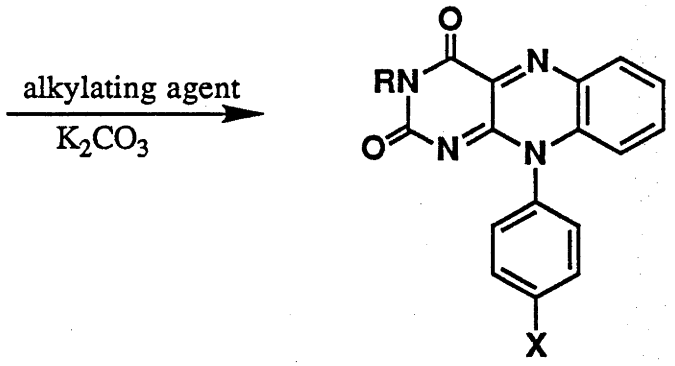
Scheme 3-2



3.5 3.6



3.6 3.7



3.4 or 2.5

3.5,3.6,3.7	X
a	H
b	4-Cl
c	4-$N(CH_3)_2$

	R	X
2.5a	Me	H
2.5jj	Me	$N(Me)_2$
3.4q	*n*-Propyl	H
3.4r	Benzyl	H

malonyl dichloride (instead of malonic acid) and N-benzylurea under similar conditions as those used by Whiteley [125] to make 1,3-diphenylbarbituric acid produced an intractable mixture. N-Benzylbarbituric acid was finally produced most satisfactorily in a high yielding clean reaction of N-benzylurea, diethyl malonate and sodium ethoxide.

Of the N-substituted ureas used to make the N-substituted barbituric acids (**3.1**) the ethyl and phenyl ureas are commercially available. The propyl and benzyl ureas were made by a modification of Wohler's famous synthesis of urea. An aqueous solution of sodium cyanate (isocyanic acid) was heated with the appropriate amine hydrochloride.

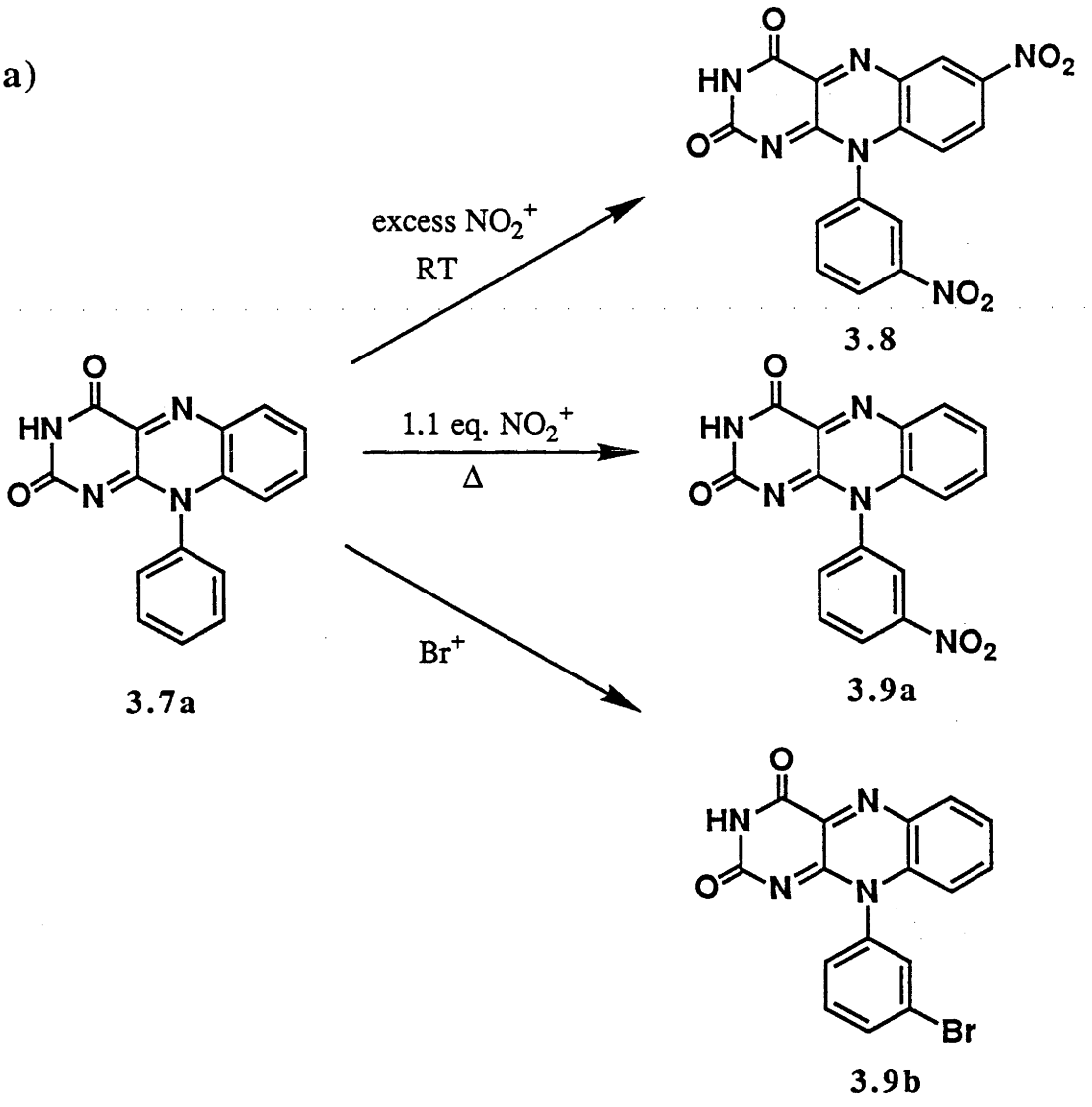
Monochlorination (position 6) of the barbituric acids (**3.1a-d**) was achieved in all cases with phosphorous oxychloride and water in yields of 22 to 55% after the method of Gauri [66]. The nucleophilic substitution of the 3-substituted 6-chlorouracils (**3.2a-d**) by the appropriate aniline produced 3-substituted 6-(substituted anilino)uracils (**3.3a-p**) which were reacted with nitrosobenzene to give the 3-substituted-10-(substituted phenyl)flavins (**3.4a-p**). These preceding three steps were carried out essentially in the same fashion as discussed in section 2-2.4.

Cowden *et al.* [42] produced 6-(4'-chloroanilino)uracil by the transamination of 6-aminouracil with excess 4-chloroaniline under harsh reaction conditions. A similar reaction, based on the method of Goldner *et al.* [68], using 3-trifluoromethylaniline hydrochloride and 6-aminouracil gave 6-(3'-trifluoromethylanilino)uracil (**3.3s**) in good yield; furthermore the reaction conditions required were less harsh. When this product was treated with nitrosobenzene in the usual way it formed 10-(3'-trifluoromethyl-phenyl)flavin (**3.4s**).

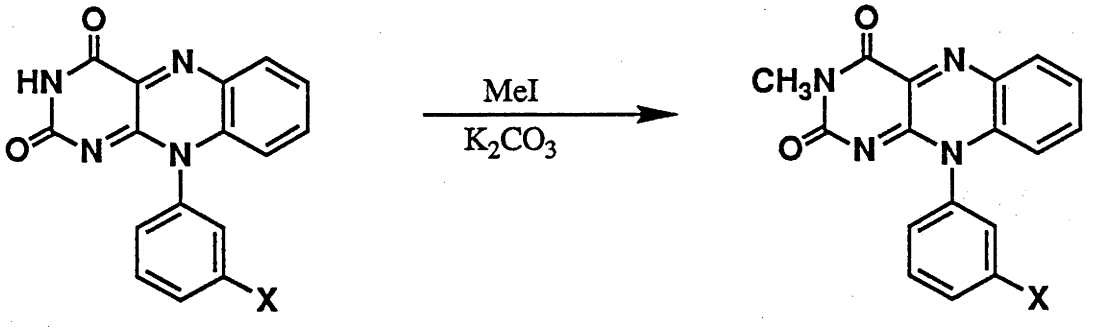
The production of the 2-nitro-N-phenylbenzenamines (**3.5b,c**) used as starting materials (Scheme 3-2) resulted from the action of two equivalents of 2-chloro-1-nitrobenzene on 1 equivalent of the appropriate aniline. Harsh reaction conditions were required as the 2-chloro is only activated towards nucleophilic substitution by the single nitro group. The reaction conditions required the reactants to be heated at 160-180° for 7 to 10 hours with 4 equivalents of sodium acetate [126]. The two 2-nitro-N-(4'-substituted phenyl)benzenamines (**3.5b,c**) produced under these conditions were obtained in low yields. In the case of N,N-dimethyl-N'-(2'-nitrophenyl)-1,4-

Scheme 3-3

(a)



(b)



3.9a or **3.9b**

2.5z (X=Br)

2.5ii (X=NO_2)

benzenediamine, the reaction was done under nitrogen to retard the degradation of the reactive benzenediamine compounds. Fortunately, the necessity to synthesise 2-nitro-N-phenylbenzenamine was obviated by its recent commercial availability [a]. The 2-nitrobenzenamines (**3.5a-c**) were easily reduced to the corresponding 1,2-benzenediamines (**3.6a-c**) with acidic stannous chloride. The condensation of these 1,2-benzenediamines (**3.6a-c**) with alloxan in the presence of boric acid offered a facile method of producing 3-unsubstituted flavins (**3.7**). Some of these flavins were then easily alkylated by methyl iodide, propyl iodide and/or benzyl bromide. Alkylations were carried out using a modification of the methylation procedure reported by Shinkai *et al.* [77]. Thus the flavin, potassium carbonate and alkylating agent were heated at 60° for 30 minutes. All alkylations of flavins worked well giving moderate to good yields of products.

3-3 Electrophilic reactions

The easy synthetic access to 10-phenylflavin (**3.7a**) (because of the commercial availability of 2-nitro-N-phenylbenzenamine (**3.5a**)) allowed a study of the electrophilic substitution of the parent structure to be undertaken.

Nitration of the 10-methyl and 3,10-dimethylflavin by stirring at room temperature for three days in a mixture of fuming nitric acid (d-1.5) and concentrated sulfuric acid (1:2 v/v) was reported by Knappe [127] to yield the 7-nitro derivatives. Under the same conditions (Scheme 3-3a) 10-phenylflavin gave 7-nitro-10-(3'-nitrophenyl)flavin (**3.8**), although, only a low yield was obtained as repeated recrystallizations were required to separate it from a small amount of contaminating 10-(3'-nitrophenyl)flavin. Mononitration (Scheme 3-3a) was achieved by heating 10-phenylflavin and 1.1 equivalents of fuming nitric acid (d-1.5) in concentrated sulfuric acid at 130° for 1.5 hours to obtain 10-(3'-nitrophenyl)flavin (**3.9a**) in 34% yield.

McCormick [128] achieved the bromination and chlorination of 7,8,10-trimethylflavin (lumiflavin) in the 9-position by treatment with N-halogenosuccinimide in

[a] From the Aldrich Chemical Company, 1988-89 Catalog.

trichloroacetic acid in the presence of catalytic amounts of benzoyl peroxide.

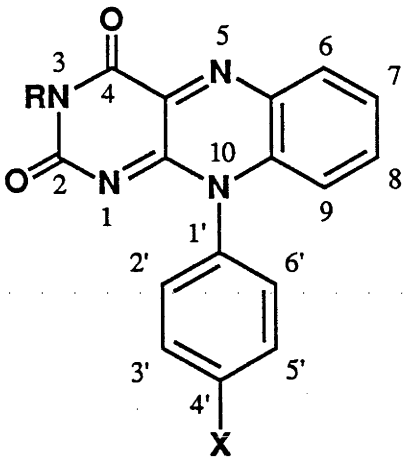
The 10-(3'-bromophenyl)flavin (**3.9b**) was produced by using an acidic solution of bromine and silver sulfate which favours the formation of the highly reactive bromine cation electrophile [129]. In the bromination one equivalent each of 10-phenylflavin, bromine and silver sulfate in 90% sulfuric acid were shaken for 16 hours to yield 25% of the product **3.9b**. An attempt to dibrominate under the same reaction conditions using two equivalents of bromine failed and only resulted in the formation of compound **3.9b**. A further attempt to form the dibrominated compound by brominating 10-(3'-bromophenyl)flavin and extending the reaction time to five days resulted in decomposition of the flavin.

The identity of the nitro **3.9a** and dinitro **3.8** products above was confirmed by ^{1}H n.m.r. spectroscopy and decoupling experiments discussed in section 3-4.1. The identity of 10-(3'-bromophenyl)flavin was confirmed by forming the 3-N-methyl derivative (Scheme 3-3b) which was found to be identical by melting point, mass spectra and ^{1}H n.m.r. spectra to an authentic sample of 10-(3'-bromophenyl)-3-methylflavin (sample provided by Dr W. B. Cowden).

The results of the above electrophilic reactions indicate that the 3' position of the 10-phenyl ring is more reactive towards electrophilic substitution than the 7 position of the flavin. The *meta* orientation of this substitution indicates that the flavin ring system is electron withdrawing towards the 10-phenyl ring. This is not unexpected because under the highly acidic conditions of the above reactions the protonation of the flavin at the 1-N position and localization of a positive charge in the flavin ring system would occur [130].

A general review of the reactivities of the flavins is given by Berezovskii *et al.* [131].

Table 3-1: **^{1}H n.m.r. spectral data (δ)[a] for 3-substituted 10-(4'-chlorophenyl and phenyl)flavins**



Cmpd no.	R	X	H 6 [b]	H 7 [c]	H 9 [b]
3.7a	H	H	8.19	e	6.74
2.5a	Me	H	8.24	e	6.78
3.4a	Et	H	8.22	e	6.78
3.4q [d]	Pr	H	8.23	e	6.78
3.4p [f]	Ph	H	8.36	e	6.92
3.4r [d,f]	$PhCH_2$	H	8.29	e	6.86
3.7b [d]	H	Cl	8.19	e	6.84
2.5o	Me	Cl	8.24	7.64	6.78
3.4b	Et	Cl	8.23	7.63	6.84
3.4m [f,g]	Pr	Cl	8.34	e	6.92
3.4n [f]	Ph	Cl	8.38	e	6.96
3.4o [d]	$PhCH_2$	Cl	8.25	e	6.88

[a] Chemical shifts reported as parts per million in CD_3SOCD_3 obtained using a Varian XL 200 spectrometer unless stated otherwise. [b] Peaks appear as doublets with integration of 1H, $J_{6,7}$ and $J_{8,9}$ ~ 8.0 Hz. [c] Peaks appear as triplets with integration of 1H, $J_{7,8}$ is 8.0 Hz. [d] Obtained using a Joel FX 90 spectrometer. [e] Peak obscured by hydrogen atom peaks of the 10-phenyl ring and/or the 3-N substituent. [f] Recorded in $CDCl_3$. [g] Obtained using a Varian VXR 300 spectrometer.

3-4 Physical properties

3-4.1 ^{1}H Nuclear magnetic resonance spectra

The ^{1}H n.m.r. spectral data of the two series of 3-substituted 10-(4'-chlorophenyl and phenyl)flavins are presented in Tables 3-1 and 3-2.

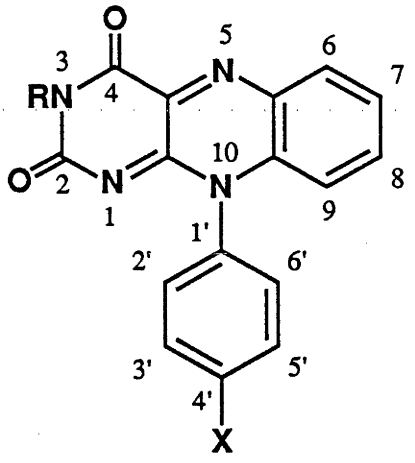
The 3-**N** substituents give the expected shifts and splitting patterns with the downfield influence of the **N**-3 nitrogen reflected in the chemical shifts of the signals of the adjacent methylene hydrogen atoms (Table 3-2). The remaining spectral features for the compounds are entirely consistent with the assignments made in section 2-3.2(i). In both the 4'-Cl substituted and unsubstituted 10-phenyl compounds the peaks due to H 6, H 7, H 8, H 9 and the 10-phenyl hydrogen atoms match (allowing for different solvent systems) that of the 3-**N** methyl analogues already discussed in Chapter 2.

The ^{1}H n.m.r. spectrum of 10-(3'-nitrophenyl)flavin (obtained in CD_3SOCD_3 using a Varian XL 200 spectrometer) consisted of a doublet (8.0 Hz, 1H) at 6.86 ppm, a multiplet (2H) at 7.60-7.78 ppm, a multiplet (2H) at 7.92-8.08 ppm, a doublet (8.0 Hz, 1H) at 8.21 ppm, a singlet (1H) at 8.42 ppm and a doublet (8.0 Hz, 1H) at 8.53 ppm. These peaks are assigned to H 9, H 7 and 8, H 5' and 6', H 6, H 2', and H 4', respectively.

The ^{1}H n.m.r. spectrum of the 7-nitro-10-(3'-nitrophenyl)flavin (obtained in CD_3SOCD_3 using a Varian XL 200 spectrometer) consisted of a doublet (9.4 Hz, 1H) at 7.08 ppm, a multiplet (2H) at 7.92-8.13 ppm, a multiplet (2H) at 8.40-8.45 ppm, a doublet (8.0 Hz, 1H) at 8.56 ppm and a singlet (1H) at 8.96 ppm. These peaks were assigned to H 9, H 5' and 6', H 8 and 2', H 4', and H 6, respectively. This assignment is consistent with the above 10-(3'-nitrophenyl)flavin spectrum and the expected changes in the chemical shifts and coupling pattern that would occur with a 7-nitro substituent [132].

For both the mononitro **3.9a** and the dinitro **3.8** compounds, decoupling experiments in which the doublets and multiplets of the spectra were decoupled confirmed the above assignments.

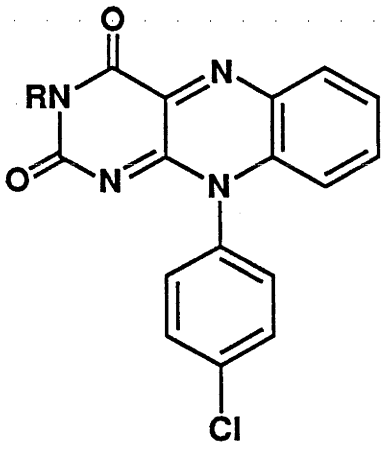
Table 3-2: **^{1}H n.m.r. spectral data (δ)[a] for 3-substituted 10-(4'-chlorophenyl and phenyl)flavins**



[a] Chemical shifts reported as parts per million in CD_3SOCD_3 obtained using a Varian XL 200 spectrometer unless stated otherwise. [b] Data are presented in the following form; chemical shift, multiplicity, coupling constants (where appropriate) and integration. The following abbreviations were used: s (singlet); d (doublet); t (triplet); q (quartet); sex (sextuplet); com (complex); and in cases where the coupling constants are the same in a spin system it is presented lastly. [c] For some compounds the hydrogen atom signals of the 3-N substituent or H 7 and H 8 are inseparable from signals from the 10-phenyl hydrogens. In these cases those signals which were not included in Table 3-1 or the adjacent column, are incorporated in this column and are apparent by integration. [d] Obtained using a Joel FX 90 spectrometer. [e] Recorded in $CDCl_3$. [f] Peaks expected in the aromatic region are obscured by the hydrogen atom peaks of 10-phenyl ring, H 7 or H 8. [g] Two symmetrical doublets seem to be present within this complex splitting pattern. [h] Obtained using a Varian VXR 300 spectrometer.

Cmpd no.	R	X	R [b]	10-Substituted phenyl [b,c]
3.7a	H	H	-	7.41 d 8.0 Hz 2H, 7.55-7.78 com 5H
2.5a	Me	H	3.25 s 3H	7.43 d 8.0 Hz 2H, 7.60-7.81 com 5H
3.4a	Et	H	1.13 t 3H, 3.89 q 2H 6.8 Hz	7.44 d 8.0 Hz 2H, 7.60-7.82 com 5H
3.4q [d]	Pr	H	0.88 t 3H, 1.57 sex 2H, 3.82 t 2H 7.4 Hz	7.39-7.68 com 7H
3.4p [e]	Ph	H	[f]	7.26-8.20 com 12H
3.4r [d,e]	$PhCH_2$	H	5.25 s 2H [f]	7.21-7.66 com 12H
3.7b [d]	H	Cl	-	7.42-7.85 com 6H
2.5o	Me	Cl	3.25 s 3H	7.48 d 2H, 7.81 d 3H 8.6 Hz
3.4b	Et	Cl	1.13 t 3H, 3.90 q 2H 6.8 Hz	7.28 d 8.6 Hz 2H, 7.73-7.85 com 3H [g]
3.4m [e,h]	Pr	Cl	0.98 t 3H, 1.71 sex 2H, 4.03 t 2H 6.0 Hz	7.28 d 8.6 Hz 2H, 7.62-7.68 com 4H [g]
3.4n [e]	Ph	Cl	[f]	7.27-7.68 com 11H
3.4o [d]	$PhCH_2$	Cl	5.08 s 2H [f]	7.32-7.86 com 11H [g]

Table 3-3: **Mass spectral data for 3-substituted 10-(4'-chlorophenyl)flavins**



Cmpd no.	R	Nominal mass m/z and intensities (relative % of base peak) M+2	M	M-1	281	253	246	239	218
3.7b	H	326 (12)	324 (24)	323 (96)	(11)	(35)	(10)	(5)	(17)
2.5o	Me	340 (13)	338 (31)	337 (100)	(21)	(31)	(13)	(8)	(9)
3.4b	Et	354 (34)	352 (100)	351 (61)	(39)	(39)	(16)	(12)	(11)
3.4m	Pr	368 (38)	366 (100)	365 (9)	(33)	(30)	(13)	(10)	(11)
3.4n	Ph	402 (22)	400 (42)	399 (20)	(100)	(31)	(18)	(11)	(8)
3.4o	$PhCH_2$	416 (37)	414 (85)	413 (2)	(100)	(22)	(11)	(10)	(8)

3-4.2 Mass spectra

The electron impact induced fragmentation of the 3-substituted 10-(4'-chlorophenyl)flavin series has been studied. From Table 3-3 it is seen that peaks at M+2, M, M-1, m/z 281, 253, 246, 239 and 218 appear throughout the series.

The M+2 peak is characteristic of chlorine containing molecules and results from a 33 % natural abundance of the ^{37}Cl isotope. The M-1 peaks are seen to varying degrees throughout the series but are most pronounced in the 3-unsubstituted, 3-methyl and 3-ethyl compounds.

A proposed fragmentation pattern for this series based on the fragmentation pattern of 3,7,8,10-tetramethylflavin described by Holzmann and coworkers [133] is shown in Fig 3-1. After the C-4a/C-4 bond cleavage of the molecular ion, the major fragmentation involves initial loss of RNCO depicted by a peak at 281. Subsequent losses of CO or Cl or NCO appear as peaks at 253, 246 and 239 respectively. The peak at 218 is probably formed by the further loss of CO from the 246 fragment. Exact mass measurements of the peaks at 281, 253, 246, 239 and 218 for 10-(4'-chlorophenyl)-3-methylflavin support the above proposition.

The direct cleavage of the R groups or fragments thereof from the molecular ion is seen in 10-(4'-chlorophenyl)-3-ethylflavin with peaks at m/z 337 (M-CH_3) and 323 (M-C_2H_5) and for 10-(4'-chlorophenyl)-3-propylflavin with peaks at m/z 351 (M-CH_3), 337 (M-C_2H_5) and 323 (M-C_3H_7).

Details of experimental methods are given in section 7-1.

Figure 3-1 **Generalized fragmentation pathway of 3-substituted 10-(4'-chlorophenyl)flavins**

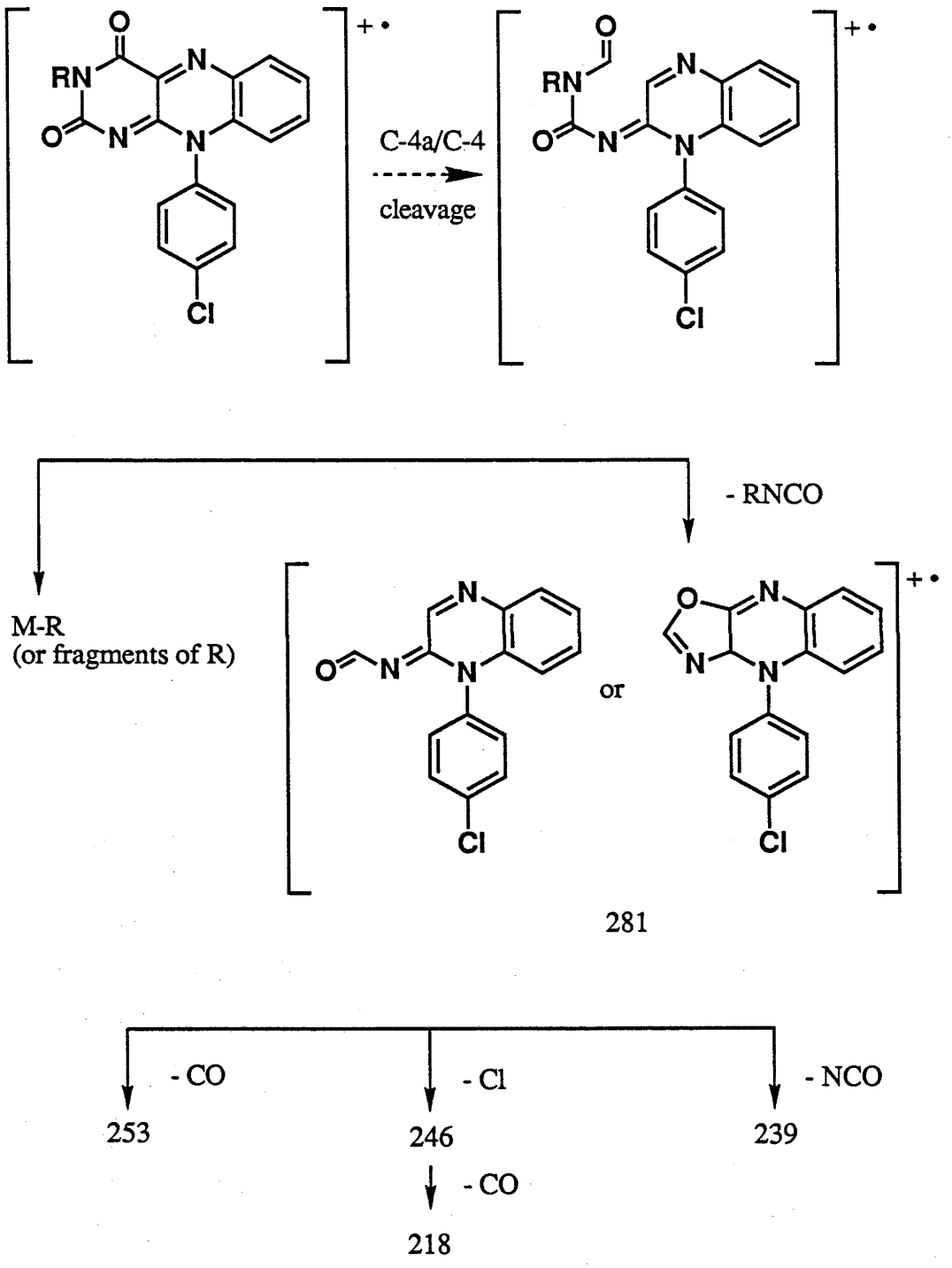
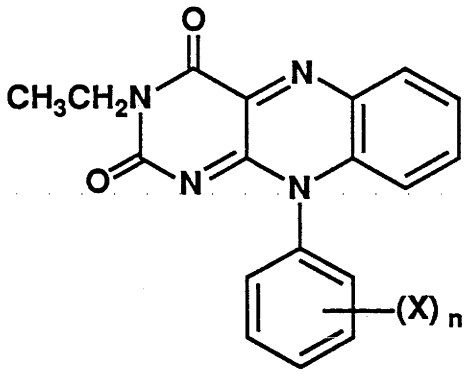


Table 3-4: **Antimalarial activity of 3-ethyl-10-(substituted phenyl)flavins against *P. vinckei vinckei* in mice** [a]



Cmpd no.	$(X)_n$	Per cent cured and increase in mean survival (days) at dose, mg/kg [b]				
		10	15	20	25	30
3.4a	H	0 (0.7)	-	0 (1.3)	-	100
3.4b	4-Cl	100	-	100	100	100
3.4c	4-Br	60 (2.0) [c]	80 (39)	100	80 (7)	75 (8.0)
3.4d	3-F	0 (0.4)	-	20 (4.3)	-	40 (2.3)
3.4e	4-F	0 (0.6)	-	50 (1.0)	-	100
3.4f	$3,5\text{-}Cl_2$	80 (9.0) [d]	20 (9.3)	0 (10)	0 (12.2)	0 (12.2)
3.4g	$3,5\text{-}Me_2$	0 (0.6)	0 (0.4)	0 (0.6)	0 (1.0)	0 (1.6) [e]
3.4h	$3\text{-}CF_3$	100	100	100	0 (-1.0)	-
3.4i	3-CN	0 (0)	-	0 (0)	-	0 (-0.25)
3.4j	4-CN	0 (0.4)	0 (1.2)	0 (0.6)	0 (0.8)	0 (0)
3.4k	3-SMe	0 (0)	-	0 (0.5)	-	0 (1.2)
3.4l	4-SMe	0 (0)	-	0 (0)	-	0 (0)

[a] See section 7-3.1(i) for experimental details. [b] All groups consist of 4 to 6 animals. [c] Average per cent day 2 parasitemia of 29 %. [d] Average per cent day 2 parasitemia of 12 %. [e] 100 Per cent cured at 70 mg/kg.

3-5 Antimalarial activity

3-5.1 Methods and results

The compounds prepared in this study were tested against *P. vinckei vinckei in vivo* as described in sections 2-4 and 7-3.1(i). For the 3-ethyl-10-(substituted phenyl)flavin series the dose range examined was 10 to 30 mg/kg. For the other members of the 3-substituted 10-(4'-chlorophenyl and 10-phenyl)flavin series the dose range examined was 10 to 70 mg/kg. The results of the *in vivo* testing are given in Tables 3-4 and 3-5. The 10-(3'-trifluoromethylphenyl)flavin (**3.4s**) tested was found to be inactive at 10, 20 and 30 mg/kg.

The 3-substituted 10-(4'-chlorophenyl)flavin series was tested *in vitro* for antimalarial activity against *P. falciparum* (FC-27). The method used was the same as in sections 2-5 and 7-3.1(ii) (method 1). The results of this screen are shown in Fig 3-2. Results for compound **3.4o**, at all concentrations, and compound **3.4m** at 20 μM could not be obtained due to their insolubility in the culture medium.

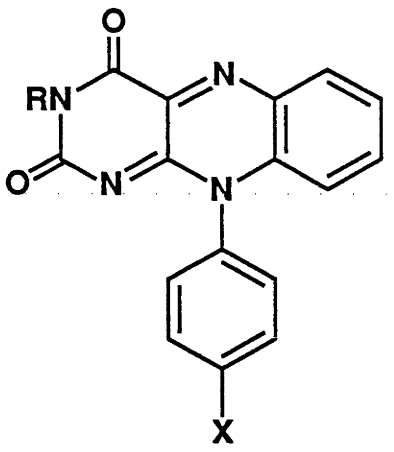
3-5.2 Discussion

The compounds described in this section are discussed in terms of three main subgroups. These are the 3-ethyl-10-(substituted phenyl)flavins (**3.4a-l**), the 3-substituted 10-phenylflavins (**3.7a**, **2.5a** and **3.4p-r**) and the 3-substituted 10-(4'-chlorophenyl)flavins (**3.7b**, **2.5o** and **3.4m-o**).

Table 3-4 shows the *in vivo* antimalarial activity of the 3-ethyl-10-(substituted phenyl)flavins in the dose range tested. In terms of activity this series reflects the findings of the 3-methyl-10-(substituted phenyl)flavin series discussed in Chapter 2 (except for compound **3.4i** (3'-CN) for which no 3-methyl analogue was made).

All the active compounds of this series, except compound **3.4g** (3',5'-Me_2), had higher schizontocidal activity than their corresponding 3-methyl analogues as gauged by parasite counts on day 2 after treatment. When the active flavins in this series were compared to their 3-methyl analogues it was found that:

Table 3-5: Antimalarial activity of 3-substituted 10-(4'-chlorophenyl and phenyl)flavins against *P. vinckei vinckei* in mice [a]



Cmpd no.	R	X	Per cent cured and increase in mean survival (days) at dose, mg/kg [b]				
			10	20	30	50	70
3.7a	H	H	-	-	0 (0.4)	-	0 (0)
2.5a [c]	Me	H	0 (0)	-	0 (0)	0 (0.3)	60 (0)
3.4a	Et	H	0 (0.7)	0 (1.3)	100	0 (-2.0)	0 (-2.0)
3.4q	Pr	H	20 (0)	20 (1.0)	75 (3.0)	100	100
3.4p	Ph	H	-	-	-	0 (-0.2)	0 (-0.6)
3.4r	$PhCH_2$	H	-	-	-	0 (0.4)	0 (2.8) [e]
3.7b	H	Cl	0 (0) [d]	-	0 (0)	-	0 (0)
2.5o [c]	Me	Cl	20 (0.25)	100	100	20 (-0.8)	0 (-1.0)
3.4b	Et	Cl	100	100	100	-	100 [d]
3.4m	Pr	Cl	0 (0.5) [d]	0 (0)	0 (0)	80 (1.0)	100
3.4n	Ph	Cl	0 (0)	0 (0)	0 (0)	-	0 (0)
3.4o	$PhCH_2$	Cl	0 (0)	0 (0)	0 (0)	-	0 (0)

[a] See section 7-3.1(i) for experimental details. [b] Most groups consist of 4 to 6 animals. [c] Data taken from Table 2-5. [d] Denotes a group of two mice. [e] A group of 2 mice failed to cure at 140 mg/kg.

1. Compounds **3.4a** (unsubstituted), **3.4b** (4'-Cl), **3.4d** (3'-F) and **3.4e** (4'-F) had higher potency (while not showing any toxicity in the dose range tested).
2. Compound **3.4h** (3'-CF_3) was more active but also more toxic.
3. For compound **3.4f** (3',5'-Cl_2) and to a lesser degree compound **3.4c** (4'-Br), it was noted that a number of mice and/or groups of mice with day two parasite counts low enough to indicate that a cure should be expected died after a mean extension of lifespan of 7 to 39 days. This curious discrepancy was probably due to the failure of these flavins to effect radical cures resulting in recrudescence and ultimately death from malaria.

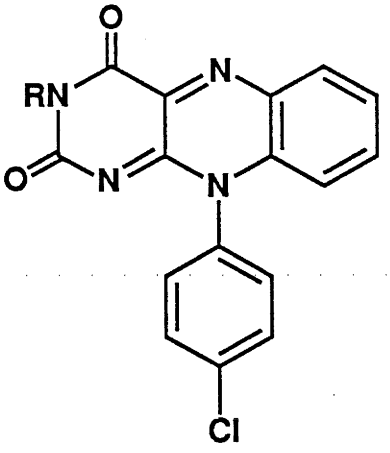
To investigate the effect of modifying substituents at the 3-N position two series of compounds were produced by maintaining the 10-substituent as either phenyl or 4'-chlorophenyl while the 3-N position was varied. The groups used as substituents as well as their associated π, σ and molar refractivity values are shown in Table 3-6.

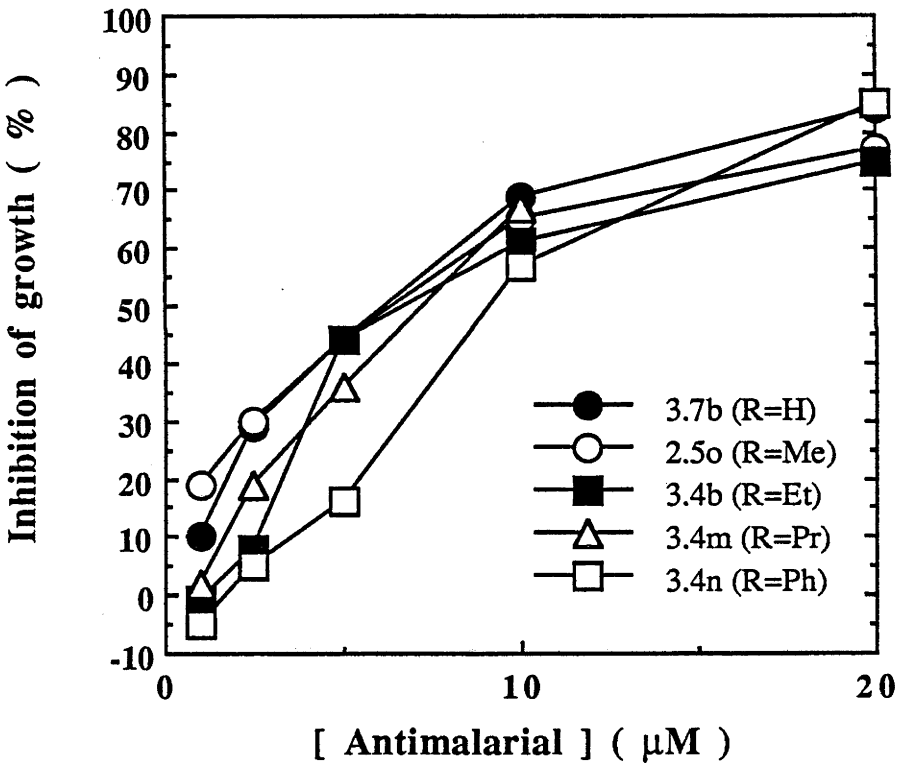
Table 3-6: Some physicochemical parameters of the various 3- and 10-phenyl substituents of the flavins

3-N substitutent	10-phenyl substitutent	Physicochemical parameters [a]		
		π (lipophilic) [b]	σ (electronic) [b]	MR (steric) [c]
H	-	0.00	0.49	1.68
Me	-	0.50	0.00	6.34
Et	-	1.00	-0.10	11.0
Pr	-	1.50	[d]	15.66
Ph	-	2.13	0.60	25.66
CH_2Ph	-	2.69	0.22	31.32
-	H [e]	0.00	0.00	1.68
-	Cl [e]	0.71	0.23	6.64

[a] For definition of parameters see section 2-4.4(ii). [b] Parameter values obtained from reference [134]. [c] Parameter values obtained from reference [89]. [d] Not available. [e] Aromatic substituent parameters.

Fig 3-2 **Growth inhibition of *P. falciparum in vitro* after 48 hr incubation with 3-substituted 10-(4'-chlorophenyl)flavins**





Details of this assay are given in sections 2-5 and 7-3.1(ii) (method 1).

In both series the 3-unsubstituted (as well as 10-(3'-trifluoromethylphenyl)flavin (**3.4s**)), 3-phenyl and 3-benzyl compounds failed to show any significant activity.

In the 10-(4'-chlorophenyl) series activity increased when the 3-substituent was changed from methyl to ethyl but decreased when changed to propyl. In the 10-phenylflavin series the activity increased when the 3-substituents were changed from methyl to ethyl and again from ethyl to propyl (Table 3-5). These results indicate that overall lipophilicity may be related to activity. Two reasons for this assertion are:

1. The *P. falciparum* inhibition data (Fig 3-2) on the 3-substituted 10-(4'-chlorophenyl)flavin series indicates that alteration at the 3-N position has little effect on the activity *in vitro*. From this it is reasonable to assume that the differences in *in vivo* activity among this series are due to the pharmacokinetic processes of distribution and elimination occurring *in vivo*. A parameter of major importance in these processes is lipophilicity; and
2. Using the combined π values of the 3- and 10-phenyl substituents as an estimate of overall lipophilicity, it is apparent that the most active compound from each series, that is 10-(4'-chlorophenyl)-3-ethylflavin ($\Sigma\pi = 1.71$) and 10-phenyl-3-propylflavin ($\Sigma\pi = 1.50$) have similar overall calculated lipophilic values.

10-(4'-Chlorophenyl)-3-ethylflavin was not only the most active member of both series (Table 3-5) but is also better tolerated at higher doses than its 3-methyl analogue.

In conclusion, some activity and toxicological advantages have been gained by alteration of substituents at the 3-N position in the above two series of compounds. These gains seem to be associated with increased lipophilicity, although, the benefit appears to be limited by either a natural lipophilic optimum or possibly the associated increase in bulk or changes in the electronic nature (albeit minor) occurring as the substituents at the 3 position increase in lipophilicity in these two series.

3-6 Other biological screens

Compounds **3.4a** and **3.4l** were evaluated for *in vitro* anti-HIV activity by the National Cancer Institute as described in section 2-6.3 and classified as inactive.

It has been reported by Graham *et al.* [135], and Ryley and Wilson [136] that some riboflavin antagonists have activity against poultry coccidiosis. With this in mind, 3-ethyl-10-(3'-trifluoromethylphenyl)flavin was tested [b] and found to have activity against the Coccidia *Eimeria vermiformis* in C57/BL6 mice. A reduction by 90% compared to controls of the number of oocysts expelled during the course of the disease was achieved by a daily oral dose of 25 mg/kg given for twelve days after infection. The experimental details of this coccidiosis screen are given in section 7-3.3.

[b] Testing was done by Mr C. Reiger and Dr K. Ovington of the Department of Zoology, The Australian National University, Canberra, ACT, 2601.

CHAPTER 4

CHAPTER 4 Syntheses and *in vivo* antimalarial activity of some 7,8,9-substituted 3-methyl-10-(substituted phenyl)flavins and a 6,8-diazaflavin

4-1 Introduction

An investigation into substituent effects in the ring-3 of 10-phenylflavins are described in this chapter. A series of compounds with dimethyl or chloro substituents in the 7, 8 or 9 positions of the 10-phenylflavins and a 10-phenyl-6,8-diazaflavin were prepared (shown in Schemes 4-1, 4-2 and 4-4). These compounds were tested for antimalarial activity *in vivo* and found to be mostly inactive.

The syntheses, some of the physical properties, the results of biological testing and the rationale for preparing these compounds are presented and discussed in the following sections.

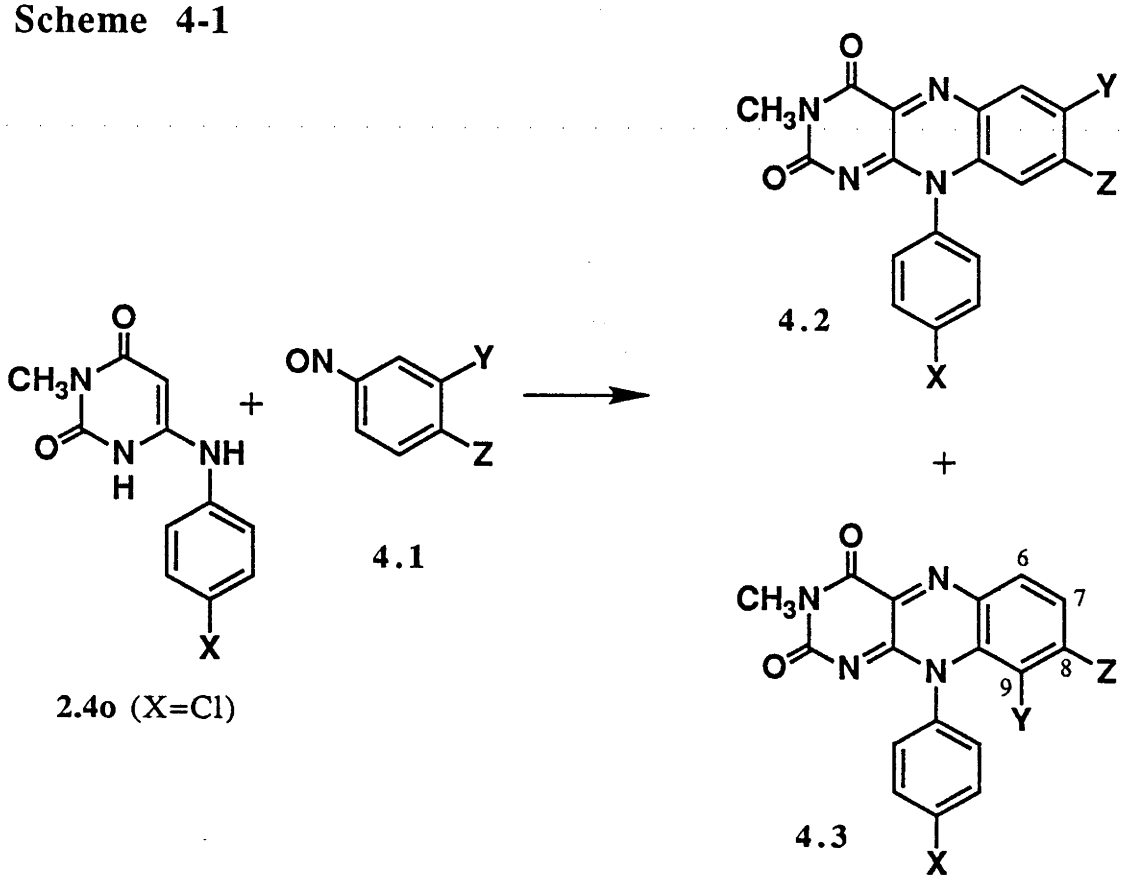
4-2 Syntheses

4-2.1 7,8,9-Substituted-10-(4'-chlorophenyl and phenyl)-3-methylflavins

The two routes considered for obtaining substituents on the ring-3 of the flavin are shown in Schemes 4-1 and 4-2 and were discussed generally in section 2-2. It is clear from these schemes that in both cases the formation of positional isomers is possible.

The starting materials for the first preparative programme were substituted nitrosobenzenes and 6-(4'-chloroanilino)-3-methyluracil (**2.4o**) (general synthesis discussed in section 2-2.4). The nitrosobenzenes for this reaction were prepared by oxidation of the appropriate anilines with Caro's acid (H_2SO_5) [137,138]. The formation of the 7,8,9-substituted-10-phenylflavins **4.2a-c** and **4.3a,b** involved the condensation of 6-(4'-chloroanilino)-3-methyluracil (**2.4o**) with 3 equivalents of the appropriately substituted nitrosobenzene in the presence of acetic anhydride, essentially according to the method of Yoneda *et al.* [60,61]. ^{1}H N.m.r. spectroscopy was used to detect the presence and proportion of the possible isomers.

Scheme 4-1



4.1	Y	Z
a	Me	Me
b	Cl	H
c	H	Cl

4.2	X	Y	Z
a	Cl	Me	Me
b	Cl	Cl	H
c	Cl	H	Cl

4.3	X	Y	Z
a	Cl	Me	Me
b	Cl	Cl	H

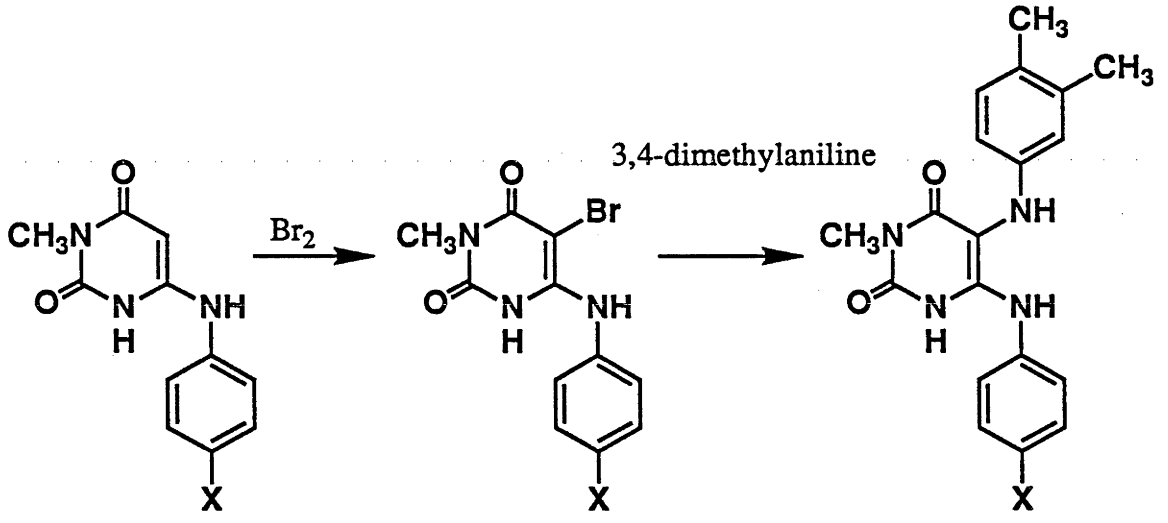
The condensation with 3-chloronitrosobenzene (**4.1b**) gave a mixture of approximately 1 to 2 of the 7- and 9-chloro isomers **4.2b** and **4.3b**, respectively. The 9-chloro isomer **4.3b** was isolated by fractional recrystallization from dimethylformamide. A 7-chloro isomer enriched mixture was recovered from the filtrate and recrystallized from glacial acetic acid to obtain the pure 7-chloro compound **4.2b**. By using the same reaction as described by Yoneda *et al.* [61], the condensation with the symmetrical 4-chloronitrosobenzene (**4.1c**) gave 8-chloro-10-(4'-chlorophenyl)-3-methylflavin (**4.2c**) in 37% yield.

The condensation of 3,4-dimethylnitrosobenzene (**4.1a**) gave a mixture of approximately one third of the 7,8-dimethyl isomer **4.2a** and two thirds of the 8,9-dimethyl isomer **4.3a**. Only the 8,9-dimethylflavin **4.3a** could be recovered from the isomer mixture by repeated recrystallizations with glacial acetic acid and dimethylformamide.

The above reaction scheme was successful in yielding the 7- (**4.2b**),8- (**4.2c**) and 9- (**4.3b**) monochlorinated and 8,9-dimethyl **4.3a** compounds but failed to make available a pure sample of the 7,8-dimethyl flavin **4.2a**. In the second synthetic scheme (Scheme 4-2) it was reported by Sako *et al.* [62] that ring closure of 6-anilino-5-(3',4'-dimethylanilino)-3-methyluracil (**4.5a**) gave only the 7,8-dimethyl isomer **4.2d**. Therefore, this reaction sequence was undertaken in an effort to take advantage of this reported propensity to form a single isomer and apply it to produce 10-(4'-chlorophenyl)-3,7,8-trimethylflavin (**4.2a**).

The 5-bromouracil (**4.4**) starting materials for the reaction were prepared by bromination of the 6-anilino-3-methyluracils **2.4a** and **2.4o**. Two possible sites for bromination are found on 6-anilinouracils, they are the 4' position of the anilino group and the 5 position in the uracil ring. (Bromination of the 2' and 6' positions of the anilino substituent is less likely on steric grounds.) In the bomination of 6-(4'-chloroanilino)-3-methyluracil only the 5-position is available but in the case of 6-anilino-3-methyluracil care had to be taken to keep the reaction mixture at -7° (in a salt-ethanol ice bath) during the addition of one equivalent of bromine to ensure monobromination of the slightly more active 5-position. Dibromination readily occurred at temperatures above this to give

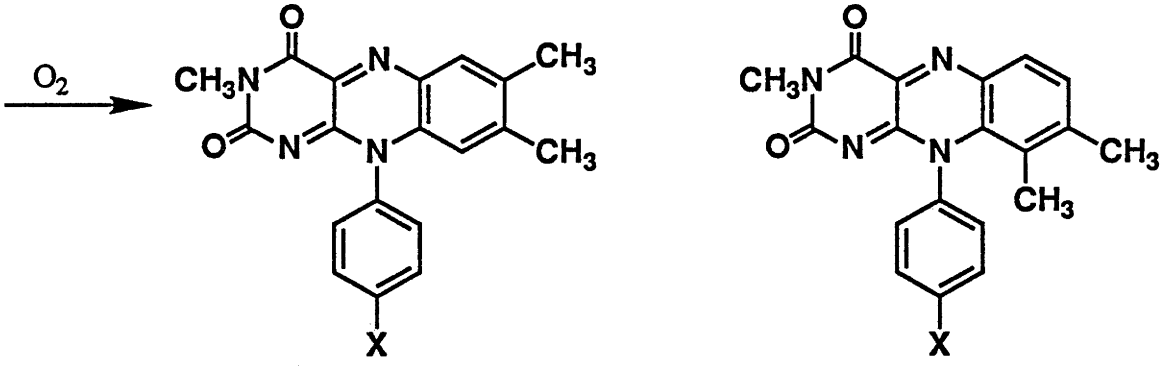
Scheme 4-2



2.4a (X=H)
2.4o (X=Cl)

4.4a (X=H)
4.4b (X=Cl)

4.5a (X=H)
4.5b (X=Cl)

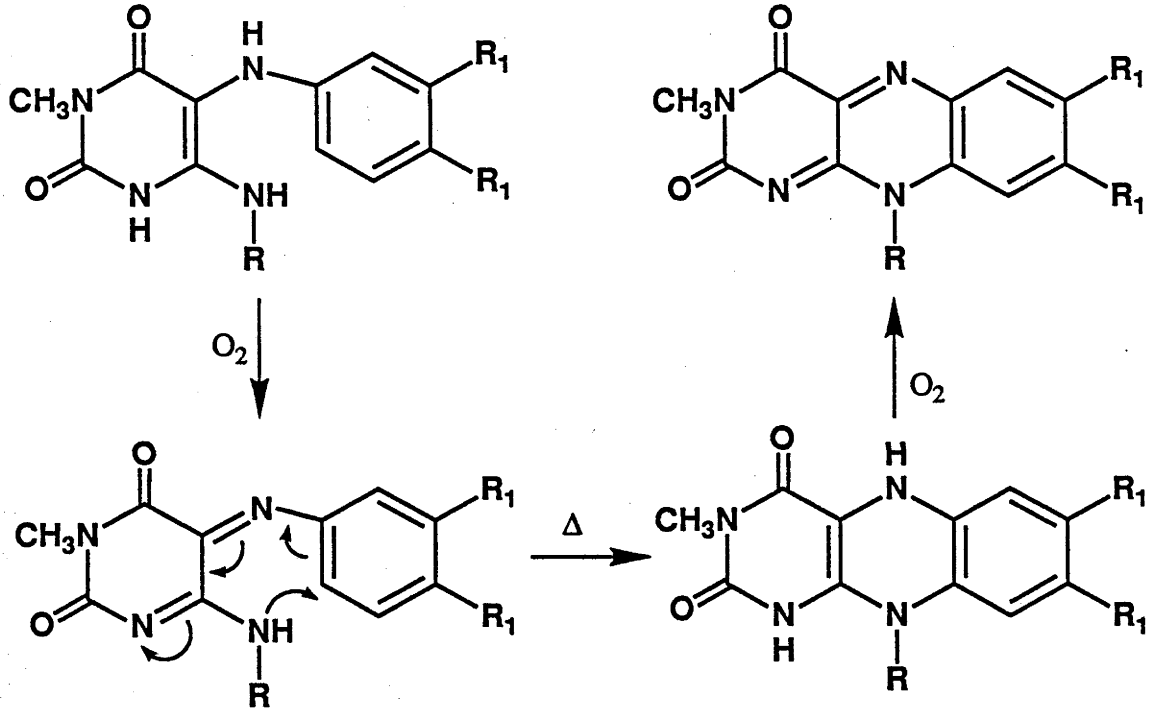


4.2a (X=Cl)
4.2d (X=H)

4.3a (X=Cl)

5-bromo-6-(4'-bromoanilino)-3-methyluracil (**4.4c**, X=Br). The 5-bromo compounds (**4.4a,b**) were treated with excess 3,4-dimethylaniline at room temperature for 17 hours. Nucleophilic substitution occurred smoothly under these conditions. The resulting 6-(substituted anilino)-5-(3',4'-dimethylanilino)-3-methyluracils (**4.5a,b**) were heated in an oxygen atmosphere at 120° for 2 hours in dimethylformamide to achieve cyclization. The mechanism proposed for this reaction by Sako *et al.* [62] is shown in Scheme 4-3.

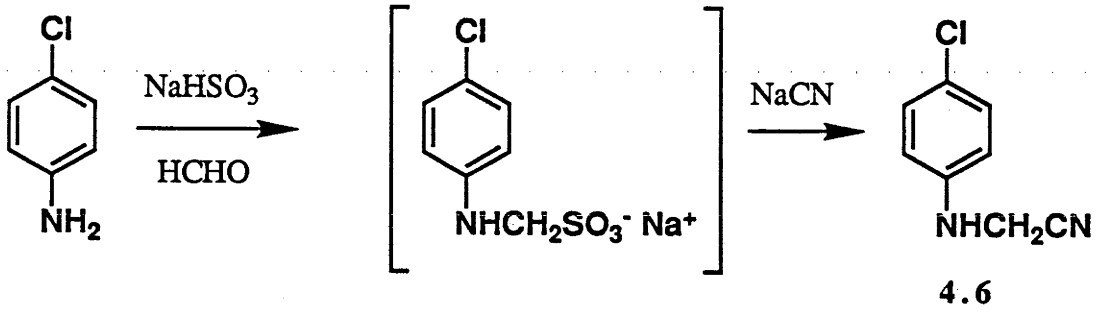
Scheme 4-3



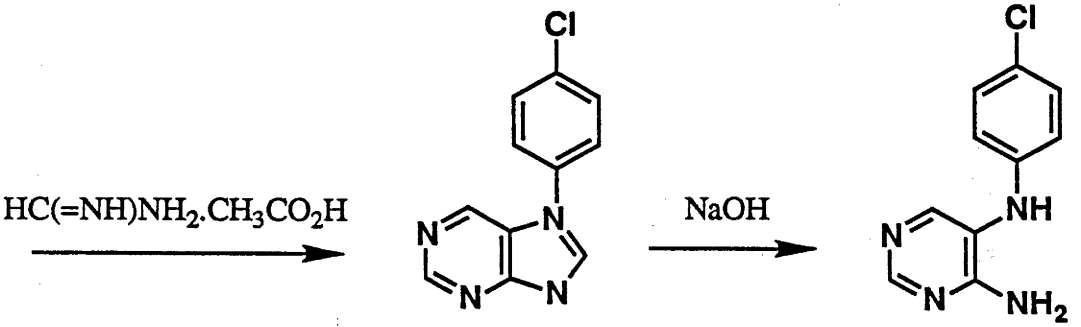
In this last synthetic step it was found that the 6-anilino-5-(3',4'-dimethylanilino)-3-methyluracil (**4.5a**) gave only the 7,8-dimethyl substituted flavin **4.2d** as reported [62]. However, under the same reaction conditions the 6-(4'-chloroanilino)-5-(3",4"-dimethylanilino)-3-methyluracil (**4.5b**) gave a majority of 7,8-dimethyl isomer **4.2a** but approximately 14% of the crude product consisted of the 8,9-dimethyl isomer **4.3a** (as assessed by ^{1}H n.m.r. spectroscopy). This indicated that Scheme 4-2 cannot always be relied upon to give exclusively 7,8-dimethyl substituted flavins as obviously the substituent effect of the 4'-chloro group promotes the formation of both possible isomers.

Scheme 4-4a,b

(a)

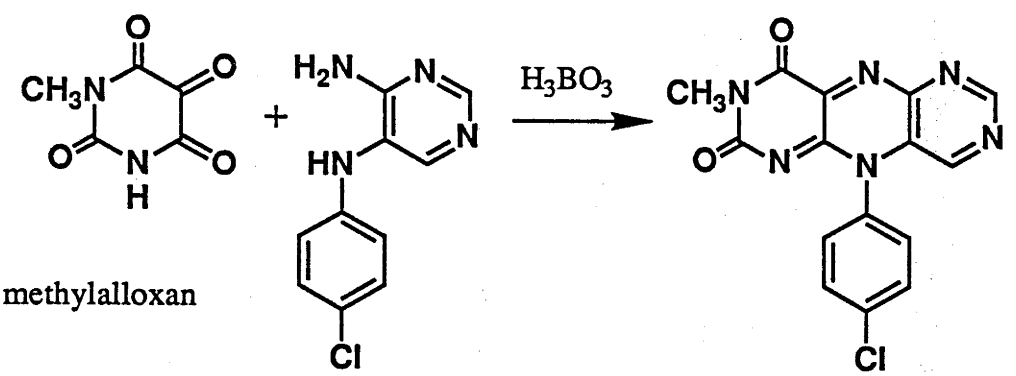


4.6



4.7 4.8

(b)



methylalloxan

4.8 4.9

The 7,8-dimethyl isomer (**4.2a**) was eventually obtained in its pure form by recrystallization of the above mixture from acetic acid and dimethylformamide.

4-2.2 10-(4'-Chlorophenyl)-3-methyl-6,8-diazaflavin

The synthetic pathway to the title compound is shown in Scheme 4-4. The last step (Scheme 4-4b) is a modification of the alloxan, N-substituted 1,2-benzenediamine flavin synthesis discussed in section 2-2.2(i). 3,10-Dimethyl-6,8-diazaflavin has been produced by Yano *et al.* [139,140] through condensation of N-methylalloxan and 4-amino-5-methylaminopyrimidine. Therefore, 4-amino-5-(4'-chloroanilino)pyrimidine (**4.8**) was needed in order to obtain the desired 4'-chlorophenyl substituent in the 10-position of the 6,8-diazaflavin **4.9**. The synthesis of this key intermediate used an analogous method to that used by Bredereck *et al.* [141] in their synthesis of 4-amino-5-anilinopyrimidine.

Synthesis of the starting material 4'-chloroanilinoacetonitrile (**4.6**) was first attempted using the Strecker condensation with 4-chloroaniline, formaldehyde and sodium cyanide as described by Marxer [142]. It was latter found that the so-called "Knoevenagel-Bucherer" modification [143], which involves the initial formation of $4\text{-}ClC_6H_4NHCH_2SO_3^-Na^+$ before addition of cyanide gave a better yield and was a simpler method for the production of 4'-chloroanilinoacetonitrile (**4.6**; Scheme 4-4a). The purine **4.7** was formed in a "one pot" reaction by refluxing 4'-chloroanilino-acetonitrile and 7.5 equivalents of formamidine acetate in 1-butanol. The volatile 7-aryl purine **4.7** was purified from reaction by-products by sublimation. The purine **4.7** was then readily hydrolysed with 0.5 M sodium hydroxide to give 4-amino-5-(4'-chloroanilino)pyrimidine (**4.8**) in excellent yield.

The N-methylalloxan required in the next step was formed by treatment of N-methylbarbituric acid with benzaldehyde to give 5-benzal-3-methylbarbituric acid, which following chromic acid oxidation gave N-methylalloxan in reasonable yield [144].

The 6,8-diazaflavin **4.9** was formed by heating a solution of the diaminopyrimidine **4.8**, N-methylalloxan and boric acid at 60° for 30 minutes

(Scheme 4-4b). The identity of the product was confirmed by ^{1}H n.m.r. spectroscopy, mass spectrometry and microanalysis.

The experimental details of the above syntheses (sections 4-2.1 and 4-2.2) are reported in section 7-2.

4-3 Physical properties

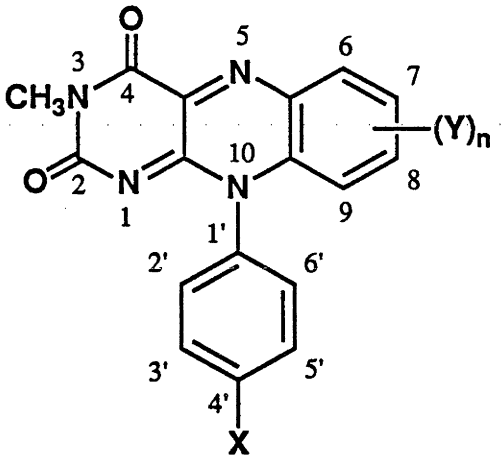
4-3.1 ^{1}H Nuclear magnetic resonance spectra

The ^{1}H n.m.r. data for this series of compounds is consistent with that obtained for the 3-methyl-10-(substituted phenyl)flavins reported in section 2-3.2(i). The substituent location in ring-3 of the flavins was detected by both the absence of the positionally corresponding hydrogen atom peak and the new coupling pattern in the peaks of adjacent hydrogens.

For example, the 8-chloro flavin (**4.2c**) when compared to its 7,8,9-unsubstituted analogue (**2.5o**) has an H 7 doublet instead of a triplet and a H 9 singlet instead of a doublet and no signal for a corresponding H 8. The rest of the 7,8,9-substituted flavins presented in Table 4-1 are similarly consistent.

The substituted chloro group has very little detectable effect on the shifts of the surrounding hydrogen atoms while the dimethyl substituents exerted a shielding effect of between 0.10-0.27 ppm on the two remaining hydrogen atoms in ring-3. The 9-Me hydrogen atoms in the 8,9-dimethyl isomer **4.3a** were assigned to the peak at 1.58 ppm because of the shielded nature at the 9 position and by comparison with the unassigned methyl peaks in the 7,8-dimethyl isomers **4.2a** and **4.2d.**

Table 4-1: **^{1}H n.m.r. spectral data (δ)[a] for 7,8,9-substituted-3-methyl-10-(substituted phenyl)flavins**



[a] Chemical shifts reported as parts per million in CD_3SOCD_3 using a Varian XL 200 spectrometer unless stated otherwise. Data are usually presented in the following form; chemical shift, multiplicity, coupling constants (where appropriate) and integration. The following abbreviations were used: s (singlet); d (doublet); t (triplet); com (complex); and in cases where the coupling constants are the same in a spin system it is presented lastly. [b] All peaks appear as singlets integrating for 3H. [c] All reported peaks integrate for 1H. [d] Spectral data taken from Tables 2-1 and 2-2 and included for comparison. [e] Peak obscured by hydrogen atom peaks of the 10-phenyl ring but detected by integration. [f] Spectrum recorded in $CDCl_3$ using a Joel FX 90 spectrometer.

Cmpd no.	$(Y)_n$	X	H 6 [c]	H 7 [c]	H 8 [c]	H 9 [c]
2.5o [d]	H	Cl	8.24 d 8.0 Hz	7.64 t 8.0 Hz	e	6.88 d 8.0 Hz
4.2b	7-Cl	Cl	8.39 s	-	e	6.89 d 8.6 Hz
4.2c	8-Cl	Cl	8.27 d 8.7 Hz	7.70 d 8.7 Hz	-	6.85 s
4.3b	9-Cl	Cl	8.23 d 7.6 Hz	e	7.89 d 7.6 Hz	-
4.2a	7,8-Me_2	Cl	8.03 s	-	-	6.66 s
4.3a	8,9-Me_2	Cl	7.97 d 8.4 Hz	7.54 d 8.4 Hz	-	-
4.2d [f]	7,8-Me_2	H	8.08 s	-	-	6.65 s

Cmpd no.	$(Y)_n$	X	NMe [b]	10-substituted phenyl	$(Y)_n$ [b]
2.5o [d]	H	Cl	3.25	7.48 d 2H, 7.81 d 3H 8.6 Hz	-
4.2b	7-Cl	Cl	3.25	7.47 d 2H, 7.81 d 3H 8.2 Hz	-
4.2c	8-Cl	Cl	3.25	7.48 d 2H, 7.81 d 2H 8.1 Hz	-
4.3b	9-Cl	Cl	3.24	7.44 d 2H, 7.66 d 3H 8.4 Hz	-
4.2a	7,8-Me_2	Cl	3.22	7.46 d 2H, 7.79 d 2H 8.5 Hz	2.31, 2.38
4.3a	8,9-Me_2	Cl	3.22	7.46 d 2H, 7.68 d 2H 8.8 Hz	1.58, 2.38
4.2d [f]	7,8-Me_2	H	3.47	7.27-7.66 com 5H	2.36, 2.42

The ^1H n.m.r. spectrum of the 10-(4'-chlorophenyl)-3-methyl-6,8-diazaflavin (**4.9**) (obtained in $CDCl_3$ on a Jeol FX 90 spectrometer) consisted of the following: a sharp singlet at 3.47 ppm assigned to the 3-N methyl group; a pair of symmetrical doublets ($J_{2',3'}$ = 8.6 Hz) at 7.34 and 7.70 ppm consistent with the 10-(4'-chlorophenyl) group; a singlet integrating for 1H at 8.64 ppm; and a singlet integrating for 1H at 9.35 ppm. The unassigned peaks due to H 7 and H 9 appear considerably downfield undoubtedly due to the deshielding effect of the adjacent nitrogen atoms N-6 and N-8.

4-3.2 Mass spectra

The mass spectra (electron impact) of the three chloro isomers were nearly identical and offered no clear way of distinguishing between them. The spectra showed a molecular ion peak at m/z 372 and a closely associated but more intense peak at M-1. Both these peaks had the associated +2 and +4 isotopic peaks characteristic of a compound containing two chlorine atoms. The expected ratio of these associated peaks was more pronounced for the M-1 peak. The spectra also showed prominent peaks at m/z 315 (M-57) and 287 (M-85). These two peaks are consistent with the decomposition of the molecular ion by loss of CH_3NCO (57) followed by further loss of CO (28). Such a fragmentation pattern is in accordance with that reported [133] for 3-methyl lumiflavin and that of the 3-substituted 10-(4'-chlorophenyl)flavins discussed in section 3-4.2.

The mass spectra (electron impact) of the 6,8-diaza compound (**4.9**) gave a peak corresponding to the molecular ion at m/z 340 which was associated with a more intense M-1 peak at m/z 339 both of these peaks had an associated +2 peak typical of monochlorinated compounds. This spectrum is similar to those above for the flavins as it has prominent peaks at m/z 283 (M-57) and m/z 255 (M-85). It is interesting to note that chemical ionization with ammonia, which is usually associated with the formation of a quasi-molecular ion of M+1 [145], gave a base peak of M+3 and a M+5 peak (^{37}Cl isotope peak) of approximately a third the intensity. This may indicate that the compound has become reduced during chemical ionization. Similar mass spectrometer reductions have been noted in the case of the benzoquinones [146].

4-4 Results and discussion of *in vivo* testing

The compounds prepared in this chapter were tested for antimalarial activity against *P. vinckei vinckei* in mice. The results of this screen are presented in Table 4-2, the lead compound 10-(4'-chlorophenyl)-3-methylflavin has been included for comparison. The percentage cured, mean extension in lifespan and parasitemia on day 2 were used to monitor activity. More details of this procedure are given in section 2-4.

Table 4-2: Antimalarial activity of 7,8,9-substituted-3-methyl-10-(substituted phenyl)flavins and 10-(4'-chlorophenyl)-3-methyl-6,8-diazaflavin against *P. vinckei vinckei* in mice [a]

Cmpd no.	ring-3 subst.	10-phenyl subst.	Per cent cured and increase in mean survival (days) at dose, mg/kg [b]			
			30	50	70	140
2.5o [c]	-	4-Cl	100	20 (-0.8)	0 (-1.0)	-
4.2b	7-Cl	4-Cl	0 (0)	0 (-0.6) [d]	0 (-2.0)	-
4.2c	8-Cl	4-Cl	0 (0)	-	0 (0.4)	0 (-1.0)
4.3b	9-Cl	4-Cl	-	-	0 (0.5) [e]	0 (0)
4.2a	7,8-Me_2	4-Cl	-	-	0 (0.6)	0 (0) [e]
4.3a	8,9-Me_2	4-Cl	-	-	0 (0)	0 (0)
4.2d	7,8-Me_2	H	-	-	0 (0)	0 (-0.8)
4.9	6,8-diaza	4-Cl	0 (0)	0 (0)	0 (-1.0) [e]	-

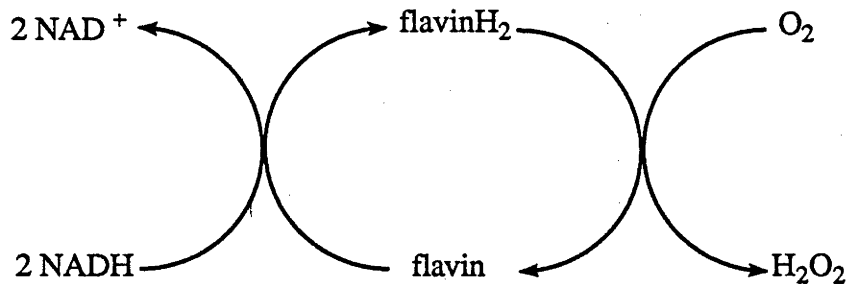
[a] See section 7-3.1(i) for experimental details. [b] Most groups consist of 4 to 6 animals. [c] Data taken from Table 2-5 and included for comparison. [d] Activity detected with day two blood smears. [e] Denotes a group of two mice.

The rationale for introducing groups into ring-3 of the lead flavin compound are twofold.

Firstly, the introduction of 7,8-dimethyl substituents would produce a molecule that more closely resembles the natural flavin analogue riboflavin. If these compounds were inhibiting riboflavin metabolism a closer structural resemblance might be expected to improve their antagonism. Thus, the analogues produced and tested were the 10-phenyl and 10-(4'-chlorophenyl)-3,7,8-trimethylflavins as well as the incidentally produced 10-(4'-chlorophenyl)-3,8,9-trimethylflavin. All of these flavins proved to be inactive over the same dose range in which their 7,8,9-unsubstituted analogues were active. Interestingly, their toxicity relative to their active analogue counterparts was reduced.

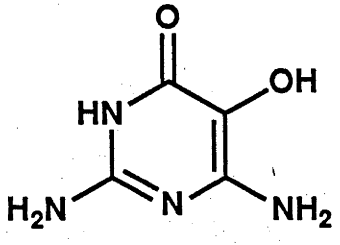
Flavins in many flavoproteins act as oxidizing agents for many biochemical reactions, some of these flavin enzymes include glucose oxidase, glycolate oxidase and amino acid oxidases. In all these cases, once the flavin is reduced it is reoxidized with molecular oxygen and hydrogen peroxide is produced. This ability for redox cycling is not exclusive to protein bound flavins. It has been shown that non-enzymatic oxidation of NADH by flavins occurs at moderate speeds in water at room temperature and that dihydroflavins are oxidized with molecular oxygen to form hydrogen peroxide [147]. This could create the possibility for a redox cycle as seen in Fig 4-1 to occur.

Fig 4-1 Flavin redox cycle

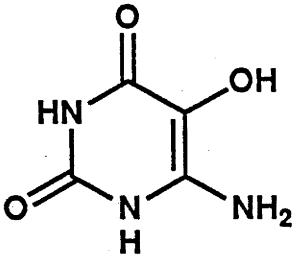


The malaria parasite is very susceptible to oxidative stress. The naturally occurring pyrimidine aglycones divicine and isouramil (shown in Fig 4-2) are known to undergo redox cycling in which hydrogen peroxide is formed. It is believed that these compounds function as antimalarials by exerting oxidant stress on the parasite [25].

Fig 4-2 Chemical structure of divicine and isouramil



divicine



isouramil

A possible mode of antimalarial action for the flavin analogues under investigation could therefore be oxidative damage to the parasite due to hydrogen peroxide produced by redox cycling of the flavin compounds. This hypothesis would seem to be supported by the observation of Divo *et al.* [148] that the antimalarial flavin analogue 8-methylamino-8-desmethyl riboflavin had enhanced activity *in vitro* in a high per cent oxygen atmosphere. Though this hypothesis was not investigated as such it did suggest the production of the 7-, 8- and 9-chloroflavin isomers (**4.2b, 4.2c, 4.3b**) and the 6,8-diazaflavin (**4.9**) might be worthwhile. This is because introducing electron withdrawing groups into the flavin ring system increases the oxidizing activity of the flavin [140]. Thus, in light of the above hypothesis this might have been a way of increasing oxidative stress on the parasite and therefore increasing the biological activity of the flavins.

Of the three positional chloro isomers synthesised only the 7-chloro compound (**4.2b**) showed activity. Even here, however, the activity was at a lower level than the lead compound (**2.5o**). The 7-chloro flavin which produced an average 80% suppression in parasitemia of the surviving mice on day two after treatment, was toxic at the dose where activity was observed. The 8- and 9- chloro substituted flavins were not active in the dose range tested but were less toxic than 10-(4'-chlorophenyl)-3-

methylflavin.

The 6,8-diaza flavin compounds produced by Yano *et al.* have not only been found to be the most active flavin mimics known for oxidation of thiols and nitroalkanes but also redox cycle in the present of oxygen [139,140]. The 6,8-diaza flavin (**4.9**) produced in this study should therefore have high oxidative activity and avoid any problems which might be associated with any increased steric hindrance that occurs when substituents are introduced into the ring-3 to increase oxidative activity (such as in the chloro series above). However, the 10-(4'-chlorophenyl)-3-methyl-6,8-diazaflavin was found to be inactive though less toxic in comparison to the lead compound.

Although no attempt was made to investigate the above two hypotheses it is clear from the paucity of activity in this series that it can be concluded on a purely SAR basis that substitution in ring-3 of the flavin is detrimental to *in vivo* activity.

4-5 Other biological testing

Compounds **4.2a-c** and **4.3a,b** were evaluated for *in vitro* anti-HIV activity by the National Cancer Institute as described in section 2-6.3 and classified as inactive.

CHAPTER 5

CHAPTER 5 Syntheses and antimalarial activity of some 6,7,8-substituted 3-methylpteridine-2,4(3*H*,8*H*)-diones

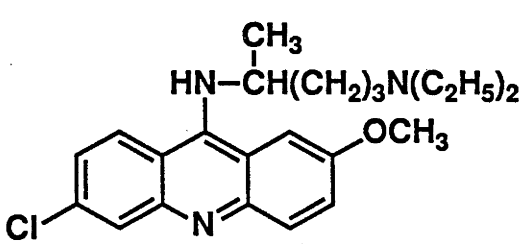
5-1 Introduction

In this chapter the synthesis of a series of 6,7,8-substituted 3-methylpteridine-2,4(3*H*,8*H*)-diones (Scheme 5-1) and their *in vitro* and *in vivo* antimalarial activity are described. This work was undertaken in order to examine the role of the benzenoid ring (ring-3) of the flavins in antimalarial activity. An interesting historical precedent paralleling this was the removal of the 7-methoxy containing benzenoid ring of the antimalarial acridine, quinacrine, to give the superior antimalarial chloroquine (see Fig 5-1)[15a]. The pteridinediones were also of interest as it has been reported [15b] that agents with the pteridine ring structure such as 2,4-diamino-6,7-diphenylpteridine shown in Fig 5-2 have antimalarial activity (these agents probably act as antifolates).

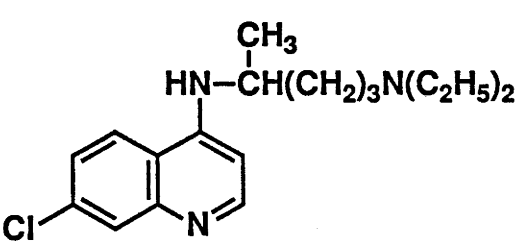
The substituents 4'-chloro and 3',5'-dimethyl, in the 8-phenyl ring of the pteridinediones (**5.3a-f**, Scheme 5-1), were selected because of their previously demonstrated beneficial effect on antimalarial activity in the 3-methyl-10-(substituted phenyl)flavin series (see Table 2-5, pg46).

It was shown that ring-3 of the flavin series is necessary for *in vivo* activity. Additionally only the 6,7-unsubstituted pteridinediones (**5.3a, 5.3b**) were active in the *in vitro* screen.

Fig 5-1 Chemical structures of quinacrine and chloroquine

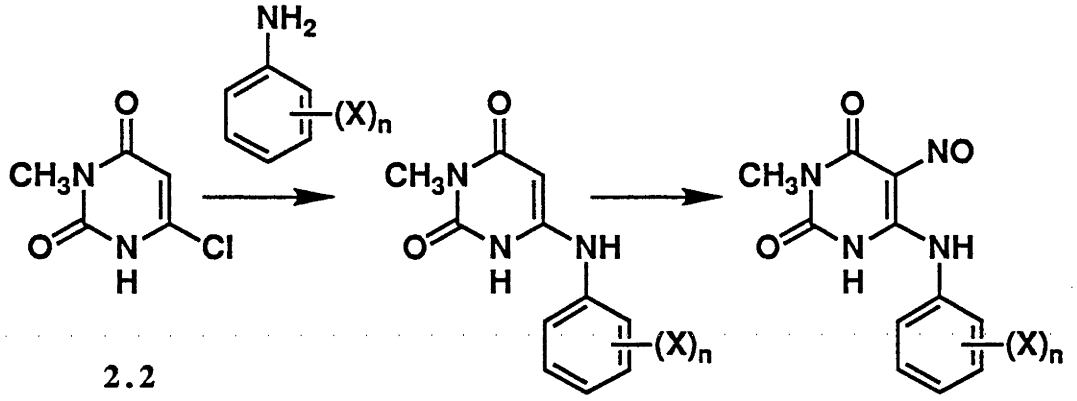


Quinacrine



Chloroquine

Scheme 5-1



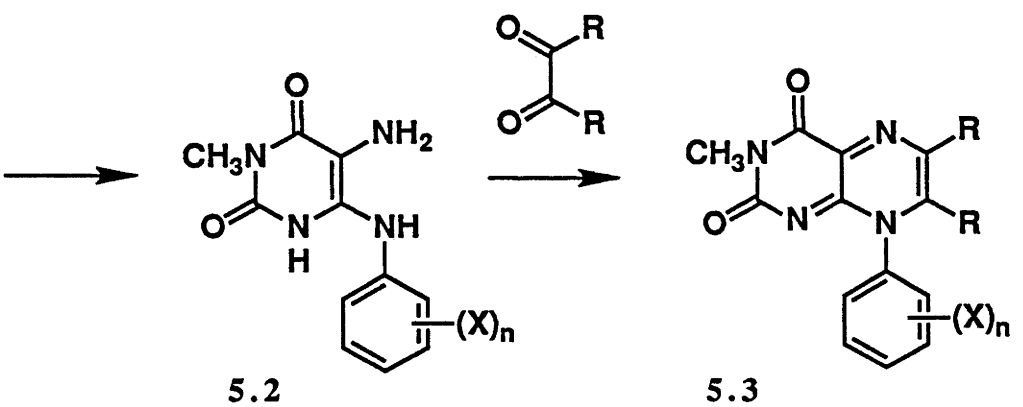
2.2

2.4o (X=Cl)

2.4g (X=3,5-Me_2)

5.1

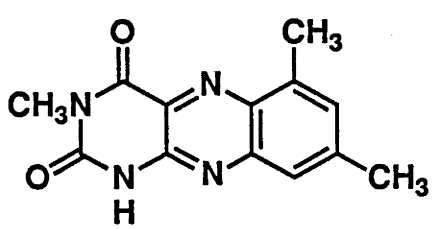
5.1 and 5.2	$(X)_n$
a	4-Cl
b	3,5-Me_2



5.2

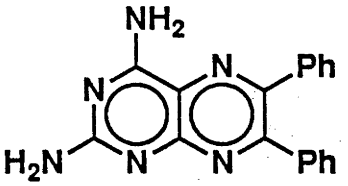
5.3

5.3	R	$(X)_n$
a	H	4-Cl
b	H	3,5-Me_2
c	Me	4-Cl
d	Me	3,5-Me_2
e	Ph	4-Cl
f	Ph	3,5-Me_2



5.4

Fig 5-2 2,4-Diamino-6,7-diphenylpteridine



5-2 Syntheses

The synthesis of the 6,7,8-substituted 3-methylpteridine-2,4(3*H*,8*H*)-diones is shown in Scheme 5-1. Nitrosation of the 6-(substituted anilino)-3-methyluracils (**2.4o, 2.4g**; general synthesis discussed in section 2-2.4) in trifluoroacetic acid readily gave the corresponding 6-(substituted anilino)-3-methyl-5-nitrosouracils (**5.1a, 5.1b**) as trifluoroacetic acid salts (not characterized) which were easily reduced with sodium dithionite to the corresponding 5-amino-6-(substituted anilino)-3-methyluracils (**5.2a, 5.2b**). In an attempt to purify 5-amino-6-(3',5'-dimethylanilino)-3-methyluracil (**5.2b**) by recrystallization it was found that an unexpected product formed. The ^{1}H n.m.r. and mass spectra of this compound indicated that it was 3,6,8-trimethylbenzo[*g*]pteridine-2,4(1*H*,3*H*)-dione (**5.4**). This is in accordance with reports that compounds of similar structure to the 5-nitroso (**5.1a, 5.1b**) and 5-amino (**5.2a, 5.2b**) uracils easily convert, *via* ring closure involving the 5-nitroso/amino group and C-2' of the anilino substituent, into the corresponding benzo[*g*]pteridinediones or their *N*-oxides [58,68,149]. It's structure was confirmed by preparing it in a similar manner to that used by Goldner *et al.* [68] to prepare similar compounds, by briefly heating 6-(3',5'-dimethylanilino)-3-methyl-5-nitrosouracil (**5.1b**) in acetic acid. Care was therefore taken not to allow the 5-nitroso (**5.1a, 5.1b**) and 5-amino (**5.2a, 5.2b**) uracils to be heated during their preparation. The final synthetic step in the preparation of the 8- or 6,7,8-substituted 3-methylpteridine-2,4(3*H*,8*H*)-diones (**5.3a-f**) involved the Gabriel and Colman condensation of the 5-amino compounds (**5.2a, 5.2b**) with α-dicarbonyl

reagents. This gave the 6,7,8-substituted 3-methylpteridine-2,4(3*H*,8*H*)-diones (**5.3a-f**) in reasonable yields.

Ram *et al.* [150] have made similar compounds using an alternate reaction sequence shown in Scheme 5-2; this scheme was deemed less convenient to that used because the 6-(substituted anilino)-3-methyluracils were already available from previous syntheses and the straightforward conditions used in going from the 5-nitroso group to the 5-amino group were preferable to the more laborious process used by Ram and colleagues in going from the 5-nitro to the 5-amino group.

Scheme 5-2

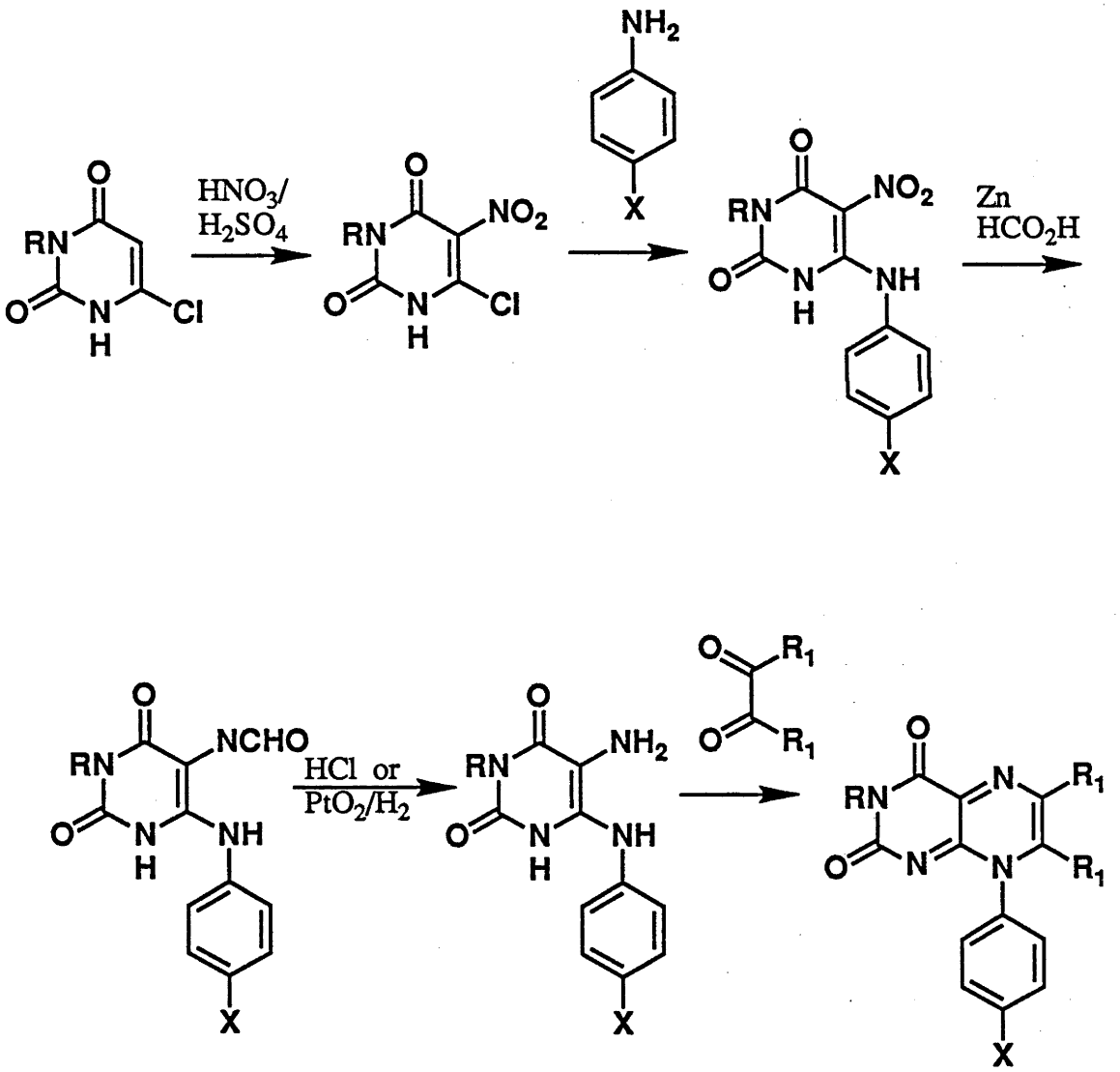
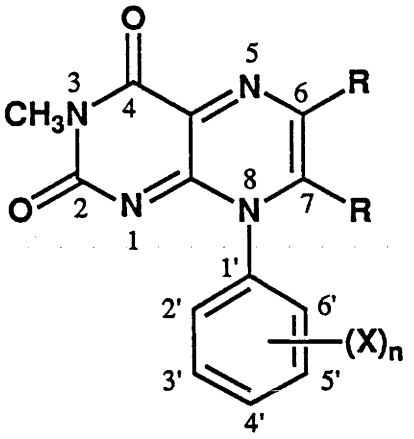


Table 5-1: ^{1}H N.m.r. spectral data (δ)[a] for the 6,7,8-substituted-3-methylpteridine-2,4(3*H*,8*H*)-diones



Cmpd no.	R	$(X)_n$	NMe [b]	$(X)_n$ [c]	R	8-Substituted phenyl
5.3a [d]	H	4-Cl	3.21	-	8.20 d 1H, 8.41 d 1H 4.0 Hz	7.60 d 2H, 7.72 d 2H 8.9 Hz
5.3b [d]	H	3,5-Me_2	3.21	2.35	8.17 d 1 H, 8.37 d 1H 4.0 Hz	7.15 s 2H, 7.24 s 1H
5.3c	Me	4-Cl	3.41	-	2.26 s 3H, 2.67 s 3H	7.19 d 2H, 7.60 d 2H 8.9 Hz
5.3d	Me	3,5-Me_2	3.45	2.39	2.26 s 3H, 2.67 s 3H	6.77 s 2H, 7.18 s 1H
5.3e	Ph	4-Cl	3.47	-	7.00-7.30 complex 14H	
5.3f	Ph	3,5-Me_2	3.50	2.19	6.68-7.30 complex 13H	

[a] Chemical shifts reported as parts per million in $CDCl_3$ using a Varian XL 200 spectrometer unless stated otherwise. Data are usually presented in the following form; chemical shift, multiplicity, coupling constants (where appropriate) and integration. The following abbreviations were used: s (singlet); d (doublet); and in cases where the coupling constants are the same in a spin system it is presented lastly. [b] All peaks appear as singlets integrating for 3H. [c] All reported peaks are singlets integrating for 6H. [d] This spectrum was recorded in CD_3SOCD_3.

5-3 ^{1}H Nuclear magnetic resonance spectra

The differences in solubility throughout the series of pteridine-2,4(3*H*,8*H*)-diones meant that dimethyl-d_6 sulfoxide was the solvent of choice to obtain the spectra of the 6,7-unsubstituted compounds while the spectra of the 6,7-dimethyl and 6,7-diphenyl pteridinediones were best obtained in deuterochloroform. Table 5-1 shows the peak assignments, multiplicity and coupling constants of this series.

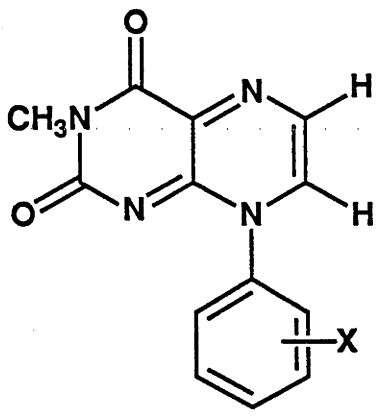
The signal due to the 3-N methyl group of this series appears as a singlet at 3.21 ppm in dimethyl-d_6 sulfoxide and between 3.41-3.50 ppm in deuterochloroform. This methyl peak appears downfield as expected due to the electron withdrawing effects of the neighbouring nitrogen and adjacent β-carbonyl groups.

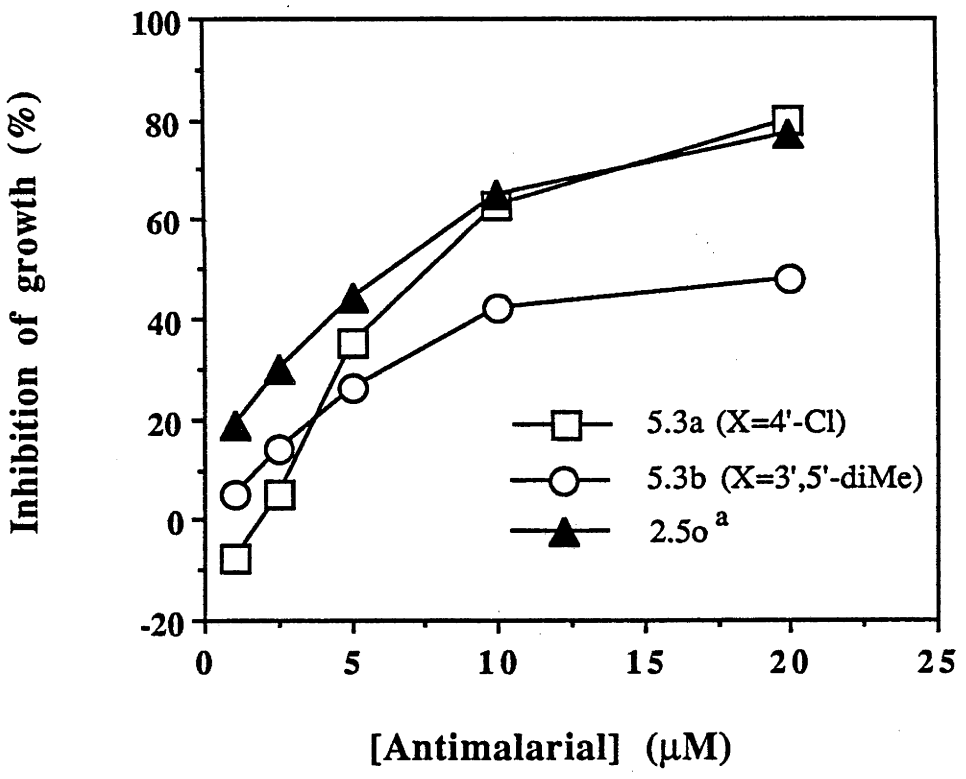
In the spectra of the 6,7-unsubstituted pteridinediones the 6 and 7 hydrogen atoms (not specifically assigned) appear as two doublets with a coupling constant of 4.0 Hz at 8.20 and 8.41 ppm for **5.3a**, and 8.17 and 8.37 ppm for **5.3b**. This spin system appears and can be interpreted as a first order AX coupling system ($\Delta\delta/J > 10$). The signals of these hydrogen atoms are downfield due to the π-deficient nature of the pteridine ring system.

The spectra of the 8-(substituted phenyl) hydrogen atoms for the 6,7-unsubstituted and 6,7-dimethyl pteridinediones are in accordance with their 4'-chloro or 3',5'-dimethyl substitution pattern. The spectra of the 4'-chloro substituted compounds have an AA'BB' quartet, characteristic of *para* disubstituted benzenes. The spectra of the 3',5'-dimethyl substituted compounds have two singlets, the more upfield peak integrates for two hydrogens indicating it results from H 2' and H 6' while the downfield peak integrates for one hydrogen H 4'. The signals (not specifically assigned) for the 6- and 7-methyl groups appear as singlets at 2.26 and 2.67 ppm for both **5.3c** and **5.3d.**

In the 6,7-diphenyl substituted compounds the aromatic resonances of the 6,7-diphenyl and the 8-substituted phenyl groups overlap to present complex spectra in the aromatic region. This disallows assignment of peaks or interpretation of splitting patterns, nevertheless, the integration and chemical shifts of the peaks in this region are consistent with the structures of these compounds.

Fig 5-3 Growth suppression of *P. falciparum* after 48 hr incubation with 3-methyl-8-(substituted phenyl)pteridine-2,4(3*H*,8*H*)-diones





[a] 10-(4'-Chlorophenyl)-3-methylflavin (**2.5o**) is included for comparison.

The unexpected 3,6,8-trimethylbenzo[*g*]pteridine-2,4(1*H*,3*H*)-dione (**5.4**) presents a simple spectrum which agrees with its predicted structure. The spectrum was obtained using dimethyl-d_6 sulfoxide (on a Varian XL 200 spectrometer) and consists of five singlets (integration shown in brackets) at 2.51 (3H), 2.70 (3H), 3.30 (3H) ,7.47 (1H) and 7.51 (1H) ppm. The two downfield peaks, though not specifically assigned, are due to the aromatic hydrogen atoms H 7 and H 9, the peak at 3.30 ppm was assigned to the N-methyl group, while the remaining peaks correspond to the methyl groups at positions 6 and 8 (not specifically assigned).

5-4 Biological activity, results and discussion

The two antimalarial screens used for this series of compounds were inhibition of the human parasite *P. falciparum in vitro* (method 1) and inhibition of lethal *P. vinckei vinckei* in mice. These screens have been discussed in sections 2-4 and 2-5 and the experimental details are in section 7-3.1.

The compounds which were unsubstituted in positions 6 and 7 (**5.3a, 5.3b**) showed activity in the *in vitro* screen over the same concentration range as the original lead compound 10-(4'-chlorophenyl)-3-methylflavin (**2.5o**) (Fig 5-3). The apparent lack of increased activity of compound **5.3b** at higher concentrations was due to its poor solubility in the testing medium. The other four pteridinediones (**5.3c-f**) containing 6,7-dimethyl or 6,7-diphenyl substituents failed to show any activity in the dose range tested. This suggests that the presence of bulky groups in both the 6 and 7 positions abolishes antimalarial activity in the 6,7,8-substituted 3-methylpteridine-2,4(3*H*,8*H*)-diones.

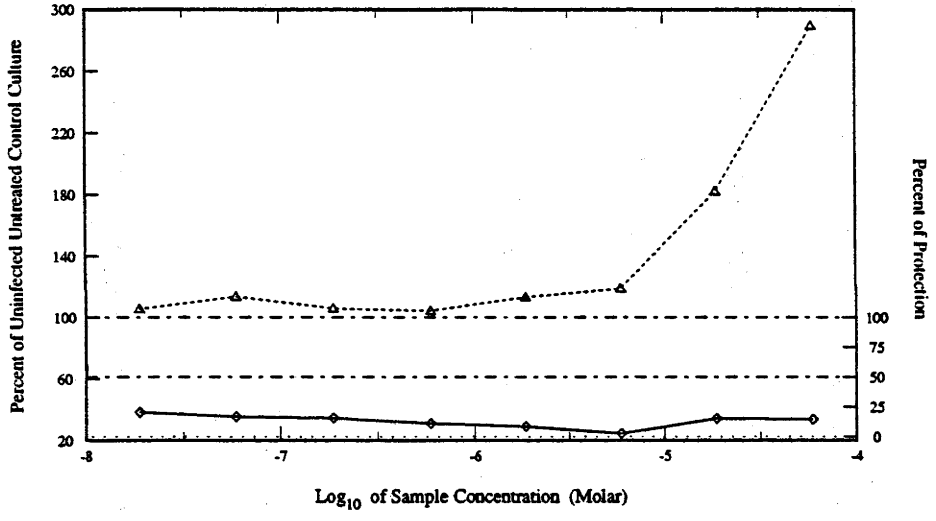
The complete series of pteridinediones (**5.3a-f**) and 3,6,8-trimethyl-benzo[*g*]pteridine-2,4(1*H*,3*H*)-dione (**5.4**) were tested *in vivo* and found to be inactive in the same dose range (10-70 mg/kg) over which the 10-(4'-chlorophenyl)-3-methylflavin had shown activity. In light of their *in vitro* activity compounds **5.3a** and **5.3b** were tested at the higher dose of 200 mg/kg in mice where still no activity was seen. This inactivity of the pteridinediones, when compared with the high activity of the 3-methyl-10-(substituted phenyl)flavins such as some of those in Chapter 2, could

Fig 5-5 National Cancer Institute anti-HIV testing sheet

National Cancer Institute
Developmental Therapeutics Program
In-Vitro Testing Results

NSC: 627996-N/ 1	Plate: 2972	Lab: 9N	Test Date: November 21, 1989	
Supplier Code: U50D	QNS:	MC:	Report Date: January 12, 1990	
COMI:			Cell Line: CEM-6	Molecular Weight:

IN VITRO ANTI-HIV DRUG SCREENING RESULTS

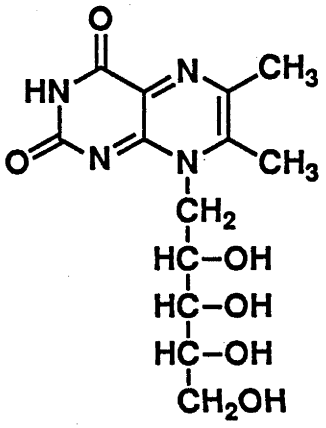


SUMMARY		DOSE	INFECTED RESPONSE	UNINFECTED RESPONSE
Index	Concentration	(Molar)	Percent of Control	Percent of Control
IC50 (Molar)	$>5.89 \times 10^{-5}$	1.87×10^{-8}	38.21	105.40
EC50 (Molar)		5.92×10^{-8}	35.40	113.55
TI50 (IC/EC)		1.87×10^{-7}	34.38	105.72
Conclusion		5.91×10^{-7}	31.01	104.31
INACTIVE		1.86×10^{-6}	28.97	113.23
		5.90×10^{-6}	24.43	118.87
		1.86×10^{-5}	34.22	182.15
		5.89×10^{-5}	33.59	289.58

possibly be attributed to pharmacokinetic effects brought about by the absence of the 3-benzenoid ring in the pteridine series. The results of the two types of antimalarial screens highlight the significance of this structure activity relationship.

As an aside, the 3,6,7-trimethyl-8-(substituted phenyl)pteridine-2,4(3*H*,8*H*)-diones are structural analogues of 6,7-dimethyl-8-(1'-D-ribityl) lumazine shown in Fig 5-4, which is the natural substrate of riboflavin synthase. As such this series represent an as yet untested group of potential riboflavin synthase inhibitors [151,152].

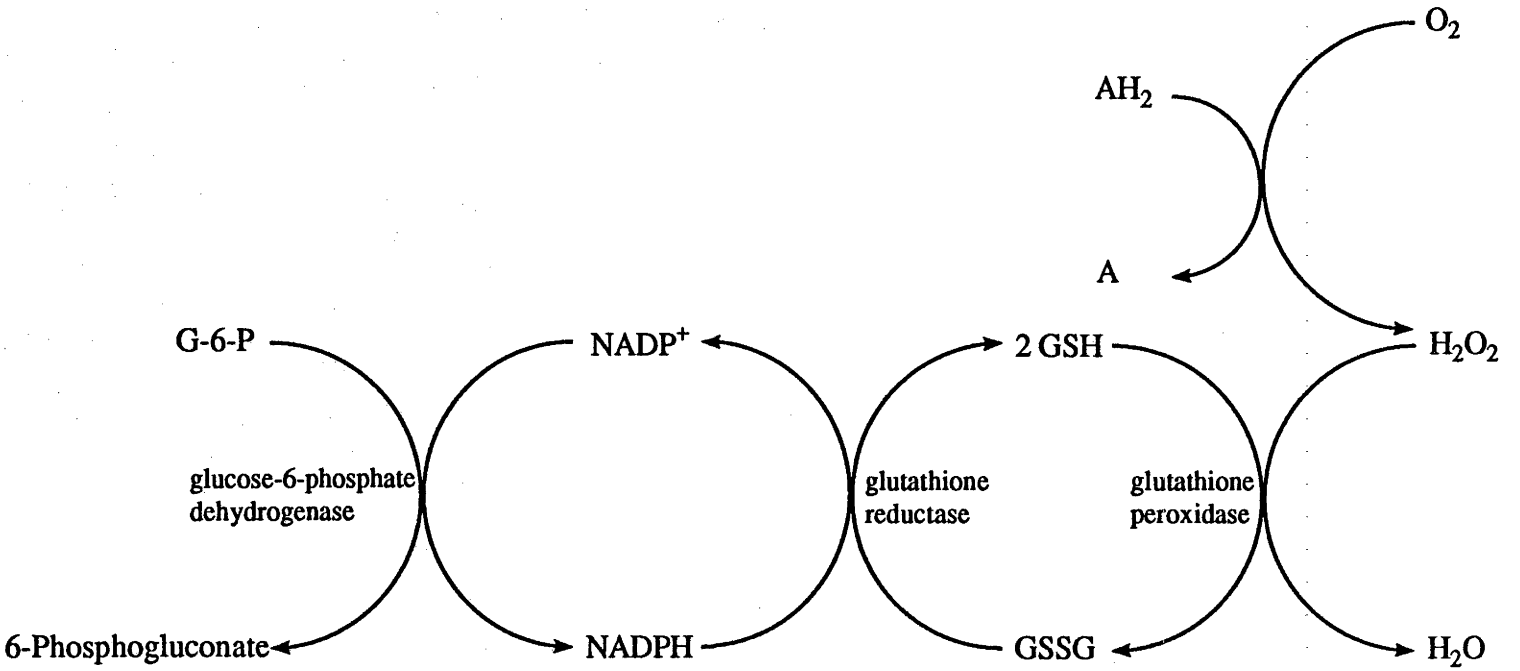
Fig 5-4 Chemical structure of 6,7-dimethyl-8-(1'-D-ribityl) lumazine



Compounds **5.3c** and **5.3e** were evaluated for *in vitro* anti-HIV activity by the National Cancer Institute as described in section 2-6.3 and classified as inactive. However, it is interesting to note that at higher doses compound **5.3e** caused the number of uninfected drug treated control T4 lymphocytes (CEM-6 cell line) to increase relative to the uninfected and untreated control. The results of this screen are shown in Fig 5-5.

CHAPTER 6

Fig 6-1 Glutathione cycle



CHAPTER 6 Antimalarial action of the flavins seems not to be due to the inhibition of glutathione reductase of host erythrocytes

6-1 Introduction

Reduced glutathione (GSH) plays an essential role in the anti-oxidant defence system of the red blood cell by the chemical reduction of hydrogen peroxide to water. Glutathione is maintained in its reduced form by the flavoenzyme, glutathione reductase (GR) which catalyses the reduction of oxidized glutathione (GSSG) at the expense of reduced nicotinamide adenine dinucleotide phosphate (NADPH) as shown in Fig 6-1.

The malaria parasite is susceptible to oxidant stress. It has been postulated that increased oxidant stress in the cell caused by increasing the production of reactive oxygen species and/or the suppression of normal anti-oxidant defense systems is an effective way of inhibiting *Plasmodium* species [24,25,107]. Two examples implicating suppression of anti-oxidant capacity as a major factor in preventing malaria are:

1. The high frequency of glucose-6-phosphate dehydrogenase (G-6-PD) deficiency in traditional malarial areas has been explained in terms of a positive selection pressure for this disorder because it confers protection against malaria [153]. This is thought to be the result of G-6-PD deficient individuals having a poor capacity to recycle glutathione. This impairment is a direct effect of their limited ability to produce the NADPH needed to reduce the GSSG to the important anti-oxidant GSH (Fig 6-1). The G-6-PD deficient erythrocyte is known to be a less viable environment for the intraerythrocytic malaria parasite when levels of oxidant stress are increased [153]; and
2. The inhibition of malaria both *in vivo* and *in vitro* by the well known glutathione reductase inhibitors 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and 1-(2-chloroethyl)-3-(2-hydroxyethyl)-1-nitrosourea (HECNU) reported by Schirmer *et al.* [118-120]. In their *in vitro P. falciparum* experiments they were able to correlate the level of glutathione reductase activity to parasite growth. It was also shown that HECNU had a curative effect in rats infected with *P. vinckei.*

When Thurnham and co-workers [33,34] reported that riboflavin deficiency suppressed *P. falciparum* infection in humans and *P. berghei* infection in rats they highlighted the necessity of riboflavin for GR activity, the possibility of GR activity being essential to the parasite and that GSH plays an important role in protecting the parasite from oxidant stress.

Some of the flavins of the present study including the lead compound 10-(4'-chlorophenyl)-3-methylflavin have been shown to be good inhibitors of GR [41,107]. The above information lead a number of workers [41,107] to suggest that the possible antimalarial mode of action of these compounds was by the inhibition of glutathione reductase.

To investigate this possibility, the structure activity relationship of a series of 3-substituted 10-(4'-chlorophenyl)flavins was examined (Table 6-1). These analogues exhibited considerable variation in their ability to inhibit human GR, which did not correlate with their inhibition of *P. falciparum* growth *in vitro*. These findings suggest that inhibition of human erythrocyte GR is probably not the primary mode of antimalarial action of this class of flavins.

GR inhibition assays of 3-methyl-10-phenylflavin analogues in which substituents on the 10-phenyl ring (Table 6-2) were varied were also conducted and revealed only small variations in activity amongst this series.

6-2 Materials and methods

6-2.1 Inhibitors

The preparation of the 3-substituted 10-(4'-chlorophenyl)flavins and the 3-methyl-10-(substituted phenyl)flavins used are described in sections 3-2 and 2-2.4.

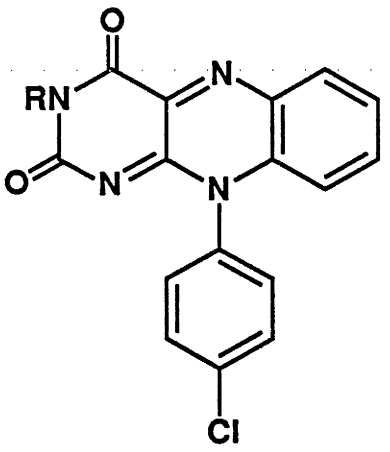
6-2.2 Glutathione reductase assay

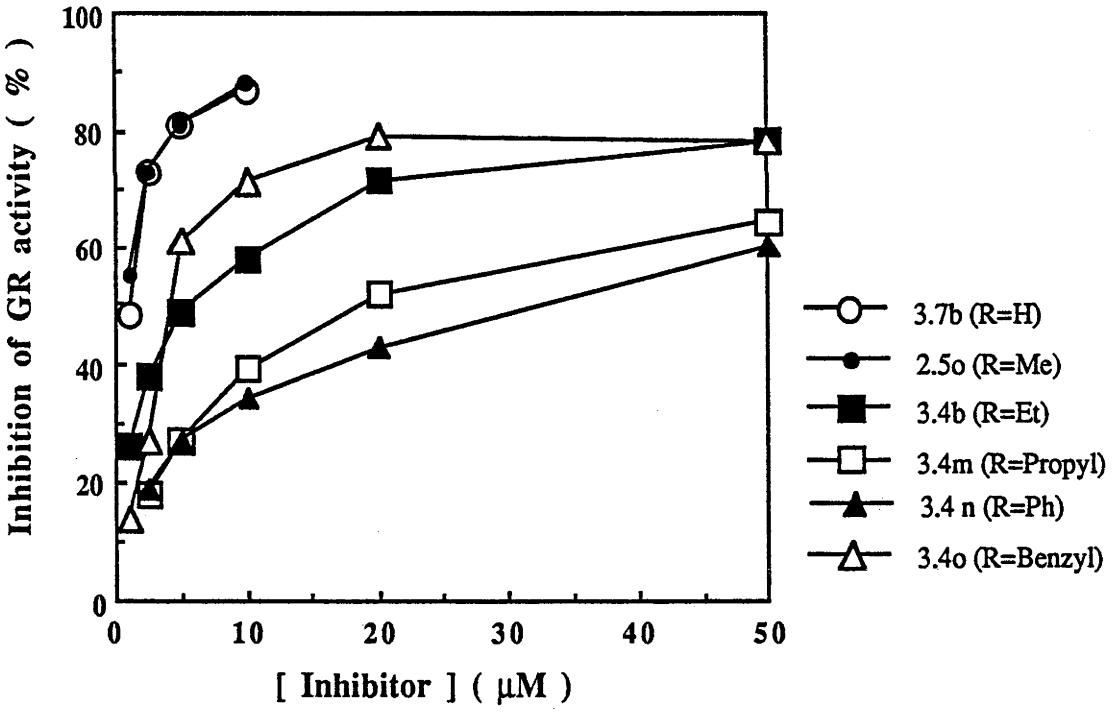
Enzyme activity was measured essentially by the method of Krohne-Ehrich *et al.* [154]. The GR reaction was monitored with a Varian DMS 100 UV/visible spectrophotometer at 25° using the decrease in absorbance at 340 nm that occurs when NADPH is oxidised to $NADP^+$. The assay mixture had a volume of 1 ml and a pH of 7.0. It contained 50 mM potassium phosphate, 200 mM KCl, 1 mM EDTA, 1 mM GSSG, 0.1 mM NADPH and 3 nM GR purified from human erythrocytes (a generous gift from Dr Heiner Schirmer, Heidelberg, FRG). The assay mixture had been incubated with various concentrations of the inhibitor for 2 minutes and the reaction was initiated by addition of GSSG. Inhibitor stock solutions (1 mM) of the flavin compounds in dimethyl sulfoxide were used. Reaction rates were obtained at various inhibitor concentrations; control samples contained dimethyl sulfoxide without inhibitor.

6-2.3 *In vitro* inhibition of *P. falciparum* growth

The inhibition of the growth of *P. falciparum* (FC-27, a Papua New Guinea strain maintained *in vitro* over several years) by the 3-substituted 10-(4'-chlorophenyl)flavins was determined by ^{3}H-hypoxanthine incorporation over 48 hours incubation as described in section 2-5 and 7-3.1(ii) (method 1).

Fig 6-2 Inhibition of human glutathione reductase by 3-substituted 10-(4'-chlorophenyl)flavins





6-3 Results

6-3.1 Flavin inhibition of glutathione reductase

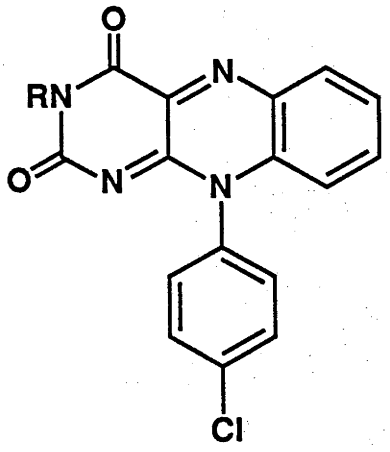
Concentration-inhibition curves for 3-substituted 10-(4'-chlorophenyl)flavins were determined (Fig 6-2). The values presented are the means of three experiments; the experimental values deviated from the mean by less than 7%. Double reciprocal plots of GR inhibition against flavin concentration gave straight lines ($r^2 > 0.97$), which allowed the calculation of IC_{50} values (Table 6-1). Based on the IC_{50} values the best inhibitor, 10-(4'-chlorophenyl)-3-methylflavin, (IC_{50} = 0.8 μM), was 57 times more active than the worst inhibitor, 10-(4'-chlorophenyl)-3-phenylflavin, (IC_{50} = 46.2 μM).

The 3-methyl-10-(substituted phenyl)flavins were tested in a single experiment at the concentrations of 1, 2.5, 5, 10 and 20 μM. Double reciprocal plots of the GR inhibition against flavin concentration gave straight lines (r^2 values given in Table 6-2). The IC_{50} values given in Table 6-2 range from 0.8 μM for 10-(4'-chlorophenyl)-3-methylflavin to 4.1 μM for 10-(3'-methoxyphenyl)-3-methylflavin.

6-3.2 Flavin inhibition of *P. falciparum* growth *in vitro*

Figure 6-3 shows inhibition of *P. falciparum* growth by the 3-substituted 10-(4'-chlorophenyl)flavins; it can be seen that all compounds inhibited to a similar extent. The IC_{50} of all the flavins lie in the narrow range of 6 to 9 μM. Results for the 3-benzyl compound **3.4o**, at all concentrations, and the 3-propyl derivative **3.4m** at 20 μM could not be obtained due to their insolubility in the testing medium.

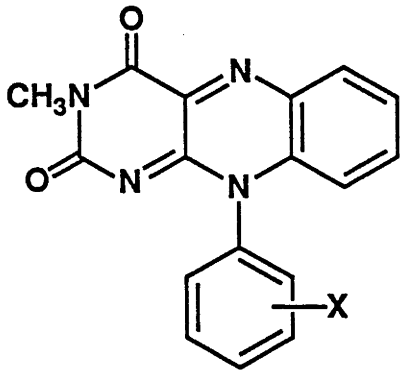
Table 6-1: Effects of 3-substituted 10-(4'-chlorophenyl)flavins on human erythrocyte glutathione reductase



Compound number	R	IC_{50} [a] (μM)
3.7b	H	1.1
2.5o	Me	0.8
3.4b	Et	4.3
3.4m	Pr	19.0
3.4n	Ph	46.2
3.4o	$PhCH_2$	5.7

[a] Concentration required to inhibit 50% of GR activity.

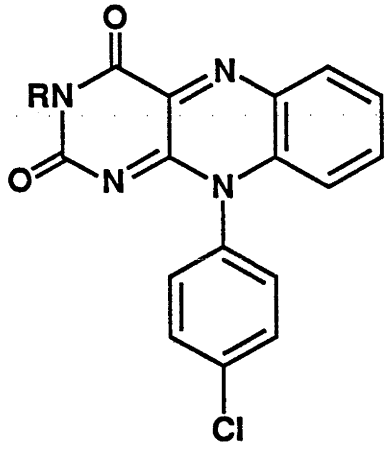
Table 6-2: Effects of 3-methyl-10-(substituted phenyl)flavins on human erythrocyte glutathione reductase

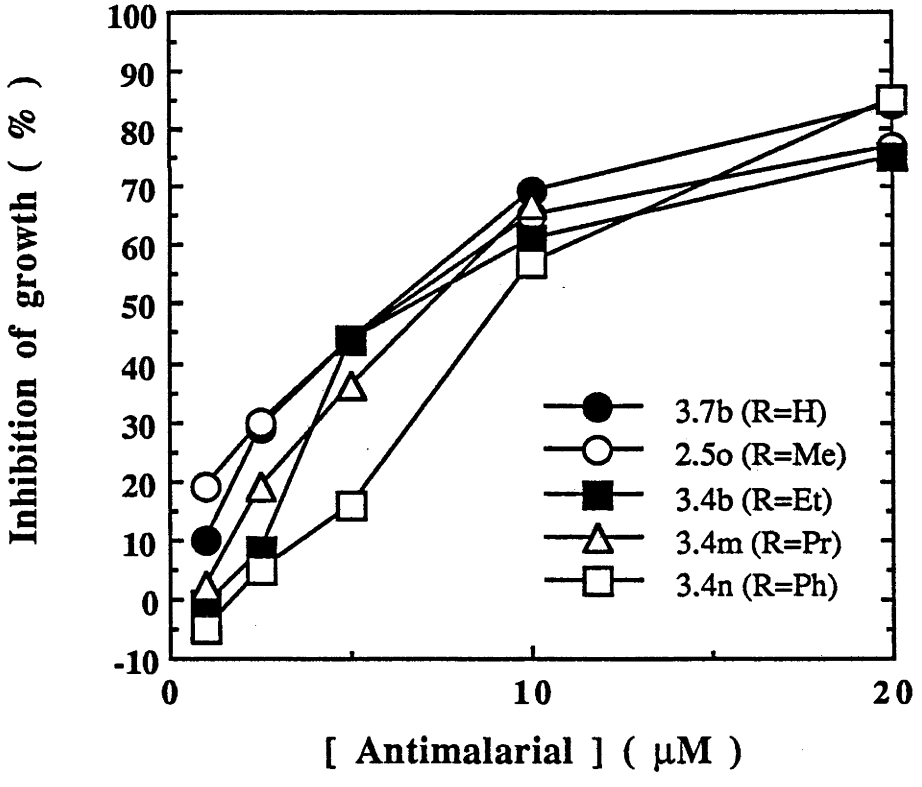


Compound no.	X	IC_{50} [a] (μM)	r^2
2.5o [b]	4-Cl	0.8	1.00
2.5h	2,6-$(Me)_2$	1.4	1.00
2.5n	3-Cl	1.5	0.99
2.5z	3-Br	1.5	1.00
2.5nn	3-SMe	1.9	0.99
2.5a	H	2.1	0.99
2.5j	3-Et	2.1	0.95
2.5kk	3-CF_3	2.2	1.00
2.5bb	3-F	2.5	0.99
2.5rr	4-CO_2H	2.6	1.00
2.5c	3-Me	3.3	0.99
2.5ee	3-OMe	4.1	0.97

[a] Concentration required to inhibit 50% of GR activity. [b] Data taken from Table 6-1.

Fig 6-3 **Growth inhibition of *P. falciparum in vitro* by 3-substituted 10-(4'-chlorophenyl)flavins**





6-4 Discussion

The two series, 3-methyl-10-(substituted phenyl)flavins and 3-substituted 10-(4'-chlorophenyl)flavins were tested for their ability to inhibit human GR and found to be active. The 3-substituted 10-(4'-chlorophenyl)flavins demonstrated considerably greater variation in potency than the 3-methyl-10-(substituted phenyl)flavins, therefore it was decided that this series would be used to delineate the role for inhibition of host erythrocytic GR in the antimalarial activity of the flavin compounds.

This series of flavin analogues with its wide range of lipophilic, electronic and steric properties when tested against human GR demonstrated a substantial change in effectiveness across the series. On the other hand, in the *P. falciparum* assay, the compounds proved to be essentially equipotent throughout the series. This lack of correlation, throughout the series, between the two test systems suggests that inhibition of host erythrocytic GR is probably not the principal mode of antimalarial action of these agents. The possibility that the these compounds are metabolized within the erythrocyte or parasite to a common active metabolite which inhibits GR cannot, of course, be excluded though there is no evidence to support this proposition. These results also indicate the importance of the substituent in the 3-N position of 10-phenylflavins in terms of host red cell GR inhibition, a factor apparently not crucial in their antimalarial action against *P. falciparum* in culture.

In an effort to explain the antimalarial activity of 10-(4'-chlorophenyl)-3-methylflavin (**2.5o**), a number of erythrocytic enzymes have previously been investigated as possible targets by Becker *et al.* [107]. The enzymes in which no significant inhibition by compound **2.5o** was noted include adenylate kinase, lactate dehydrogenase, pyruvate kinase, G-6-PD, hexokinase and adenylate kinase. They also showed that compound **2.5o** does not affect pyrimidine or purine nucleotide biosynthesis or metabolism of the parasite or the erythrocyte. In that report, GR presented itself as the most likely drug target, however, they found that addition of GSH to parasite cultures did not block the antimalarial action of 10-(4'-chlorophenyl)-3-methylflavin against *P. falciparum*, an observation consistent with the present conclusion that inhibition of

host erythrocytic GR is probably not the main site of antimalarial action for these drugs. It should be noted that compound **2.5o** has also been shown to be an inhibitor of the parasite's GR [107] and the flavins could conceivably be exerting their antimalarial activity *via* this route.

The finding by Becker *et al.* [107] that compound **2.5o** was not an inhibitor of its structural analogue, the cofactor flavin adenine dinucleotide (FAD), but that it competitively inhibited the binding of GSSG to GR, is of importance if further studies of flavins as GR inhibitors are to be undertaken.

In conclusion, the present work suggests that inhibition of human erythrocyte GR is probably not the primary mode of action of the 3-substituted 10-(4'-chlorophenyl)-flavin antimalarials. Additionally, substituents in the 3-N position of these compounds have a direct effect on their enzyme inhibitory activity, while the 10-phenyl substituted series showed only a small variation of 3.3 μM over a wide range of substituents indicating that the enzyme is relatively insensitive to changes at this site in the molecule.

CHAPTER 7

CHAPTER 7 Methods and materials

7-1 General

1. Melting points (mp) and decomposition points (dec) were taken with a Gallenkamp melting point apparatus and are uncorrected.
2. Analyses were performed by The Australian National University Analytical Services Unit, Canberra. All solids were dried for at least 3 hours under vacuum at appropriate temperatures prior to analysis.
3. ^{1}H Nuclear magnetic resonance spectra (^{1}H n.m.r.) were recorded on either a Jeol FX 90, Varian XL 200 or Varian VXR 300 fourier-transform spectrometer. Data are presented in the following order: chemical shift (ppm); multiplicity; coupling constant (J) in Hz; and assignment (where possible). The following abbreviations were adopted: s (singlet); d (doublet); t (triplet); q (quartet); quint (quintet) and m (multiplet).
4. ^{13}C Nuclear magnetic resonance techniques were performed at 299.95 MHz on a Varian VXR 300 instrument at 25°.
5. Low resolution mass spectra (MS) were recorded on an Incos data system attached to a VG-Micromass 7070F double focusing mass spectrometer using electron impact (EI) at 70 eV or chemical ionization (CI) with ammonia. High resolution mass measurements were made by peak matching using perfluorokerosene as a reference. Data are presented in the following order; m/z value, relative intensity as a percentage of the base peak. The mass spectra in the following section are EI unless stated otherwise.
6. Ultraviolet spectra were recorded on a Varian DMS 100 UV/visible spectrophotometer between 200-500 nm. Compounds were first dissolved in dimethyl sulfoxide and then diluted 1000 times with ethanol to a final concentration of 20 μM.

7. Analytical thin layer chromatography (t.l.c.) was performed on glass plates precoated with Merck Kieselgel 60 F_{254} of 0.25 mm thickness. Plates were visualized by both long and short wave ultraviolet light.
8. Starting materials, unless stated otherwise, were obtained commercially, usually from the Aldrich Chemical Company.

7-2 Synthetic experimental

Some of the 3-methyl-10-(substituted phenyl)flavins used in Chapter 2 were made by Dr W. B. Cowden. The description of the synthesis of those not reported in reference [42] has been included in this section.

The synthesis of some of the starting materials described below have been reported in the literature, however, conflicting results are often reported regarding reaction conditions, yields and characteristics for many of these. Thus, methods for their preparation are described herein.

N-Propylurea

Sodium cyanate (6.5 g, 0.1 mol) and *n*-propylamine hydrochloride (9.6 g, 0.1 mol) were dissolved in water (200 ml). The solution was brought to dryness on a steam-bath. The residue was recrystallized twice from the minimum amount of water to give white crystals which were dried over $CaCl_2$ to give N-propylurea (3.6 g, 36%), mp 107° (lit mp 110° [155]).

N-Benzylurea

A solution of sodium cyanate (6.5 g, 0.1 mol) in water (200 ml) was added to a solution of benzylamine (10.7 g, 0.1 mol) in 5 M hydrochloric acid (20 ml) and stirred for 10 min. An exothermic reaction occurred after which the mixture was heated for an hour on a steam bath. An oil formed. The mixture was diluted with water (400 ml) and stirred overnight. Long needle-like crystals formed and were filtered off. The crystals were dried over $CaCl_2$ to give N-benzylurea (90 g, 60%) mp 144-145° (lit mp 147-148° [155]).

1-Ethylbarbituric acid (3.1a)

A mixture of carefully dried malonic acid (40.0 g, 0.4 mol) and N-ethylurea (30.0 g, 0.34 mol) in acetic acid (90 ml) was heated to 60° and stirred until the solids dissolved. Acetic anhydride (65 ml, 0.69 mol) was then added dropwise over 90 min. The temperature was then raised to 90° for three hours before the solution was cooled and concentrated under vacuum to a thick red syrup. The syrup was crystallized by dilution with ethanol (200 ml). Recrystallization from 90% ethanol gave 1-ethylbarbituric acid (**3.1a**) (29 g, 55%) mp 119-122° (lit mp 119-120° [155]).

1-Benzylbarbituric acid (3.1d)

Method 1

N-Benzylurea (synthesis described) (51 g, 0.34 mol) was treated as for **3.1a** above. T.l.c. (ethanol) indicated the resulting crystals had a large amount of by-product present. Repeated recrystallizations from ethanol gave the title compound (**3.1d**) (0.7 g, 6%) mp 146-151°. (Found: C, 60.4; H, 4.6; N, 12.6. $C_{11}H_{10}N_2O_3$ requires C, 60.6; H, 4.6; N, 12.8%).

The filtrates from the above recrystallizations were combined and reduced in volume under vacuum and the resulting crystals filtered off. By t.l.c. (ethanol) the crystals consisted mainly of the by-product. The crude by-product gave an EIMS molecular ion peak at 260. The unpurified by-product (44.2 g) in 5 M hydrochloric acid (700 ml) was refluxed for 11 hrs. The solution was brought to dryness under vacuum and the residue recrystallized from ethanol to give the title compound (**3.1d**) (9.3 g, 13%) mp 147-151°. (Found: C, 60.5; H, 4.6; N, 12.9. $C_{11}H_{10}N_2O_3$ requires C, 60.6; H, 4.6; N, 12.8%).

Method 2

A mixture of phosphorous oxychloride (6 g, 40 mmol), malonic acid (2 g, 20 mmol) and N-benzylurea (synthesis described) (3 g, 20 mmol) in dry chloroform (20 ml) was heated under gentle reflux for 6 hrs. The chloroform was removed under vacuum, ice (2 g) was added and the mixture was allowed to stand for an hour before it

was adjusted to pH 7.5 with saturated sodium bicarbonate solution. The solid was filtered off and recrystallized from ethanol to give N-benzylurea (1.5 g). On acidification of the filtrate with concentrated hydrochloric acid no precipitate formed, t.l.c. (ethanol) indicated the absence of 1-benzylbarbituric acid.

Method 3

A mixture of N-benzylurea (synthesis described) (3 g, 20 mmol), malonyl dichloride [156] (2.8 g, 20 mmol) and dry toluene (50 ml) was refluxed for 21 hrs. The solution was reduced to dryness under vacuum and the residue recrystallized from 90% ethanol. T.l.c. (ethanol) revealed two spots with close R_f values, one of which correlated to the R_f of 1-benzylbarbituric acid. Repeated recrystallization from ethanol and 90% ethanol failed to isolate the pure 1-benzylbarbituric acid.

Method 4

Sodium metal (0.85 g, 37.5 mmol) was dissolved in dry ethanol (20 ml) before diethyl malonate (5.0 g, 31 mmol) and N-benzylurea (synthesis described) (4.7 g, 31 mmol) were added to the stirred solution. The reaction mixture was refluxed for 15 hrs. On cooling a precipitate formed. 2 M Hydrochloric acid (25 ml) was added to the reaction mixture with shaking. The solution was allowed to stand overnight before filtering off the precipitate, which after recrystallization from 90% ethanol gave 1-benzylbarbituric acid (**3.1d**) (4.7 g, 69%) mp 145-147° (lit mp 148-150° [157]).

3-Substituted 6-(substituted anilino)uracils (see Tables 7.1 and 7.2)

General method A

An intimate mixture of the appropriate 3-methyl (**2.2**) [63,66]; 3-ethyl (**3.1a**) [66]; 3-phenyl (**3.1c**) [124,66]; or 3-benzyl (**3.1d**) [66] -6-chlorouracil (10 mmol), the appropriate aniline (30 mmol) and acetic acid (0.5 ml) was heated in an oil bath at 180° for 25 min, cooled briefly and poured into ethanol (*ca* 50 ml) and stirred for 15 min. The solid was filtered off, washed with ether (80 ml), recrystallized from acetic acid or methanol, and dried under vacuum. The compounds produced, their melting points and yields are found in Table 7-1 and microanalyses are presented in Table 7-2.

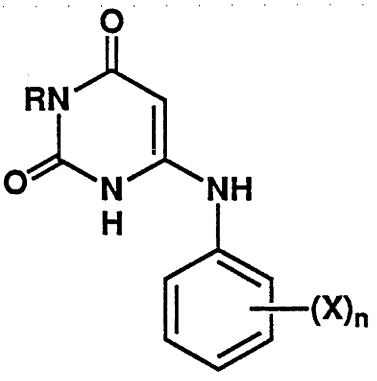
General method B

A mixture of the appropriate 3-methyl (**2.2**) [63,66]; 3-ethyl (**3.1a**) [66]; 3-*n*-propyl (**3.1b**) [123,158]; or 3-phenyl (**3.1c**) [66,124] -6-chlorouracil (10 mmol), the appropriate aniline (10 mmol; all except **2.3u** [70] and **2.3w** [69] were available from the Aldrich Chemical Company), N,N-diethylaniline (3 g, 3.2 ml, 20 mmol) and acetic acid (0.5 ml) was heated at 190° for 25 min, cooled briefly, poured into ethanol (*ca* 50 ml) and stirred until crystallization was complete. The solid was filtered off, washed with ether and recrystallized from either methanol or acetic acid and then dried under vacuum. The compounds produced, their melting points and yields are found in Table 7-1 and microanalyses are presented in Table 7-2.

Attempted condensation of 2,6-dimethylaniline and 6-chloro-3-methyluracil (2.2)

Using both general methods A and B above for 6-anilinouracil synthesis, the expected product was not formed as determined by t.l.c. (ethanol) and only starting materials were identified.

Table 7-1: Physical properties of 3-substituted 6-(substituted anilino)uracils produced by general methods A and B



[a] Literature mps are presented in brackets. [b] See reference [60]. [c] Synthesised by Dr W.B. Cowden. [d] See reference [159]. [e] See reference [67]. [f] See reference [160]. [g] See reference [161]. [h] See reference [68].

Cmpd no.	Substitutents R	$(X)_n$	Melting point (°C) [a]	% yield	Method
2.4a	Me	H	330 (336-338) [b]	91	B
2.4b	Me	2-Me	237-239	67	B
2.4c	Me	3-Me	273-274 (291) [b]	76	B
2.4d [c]	Me	4-Me	311-313 (325) [d]	70	A
2.4e	Me	2,4-Me_2	290-292	59	B
2.4f	Me	3,4-Me_2	309-312 (309) [e]	88	B
2.4g	Me	3,5-Me_2	288 (275-277) [f]	87	B
2.4i	Me	2-Et	223-226	22	B
2.4j [c]	Me	3-Et	246-248	71	A
2.4k	Me	4-Et	304-307	78	B
2.4l [c]	Me	4-*n*-butyl	268-270	62	A
2.4o	Me	4-Cl	345-346 (297) [b]	49	B
2.4t	Me	3-Cl,4-Me	299-300	88	B
2.4u	Me	3-Cl,5-Me	303-304	60	B
2.4w	Me	4-Cl,3-Me	334-335	76	B
2.4x	Me	4-Cl,2-F	335-336	42	B
2.4y [c]	Me	4-Cl,3-CF_3	283-285	75	A
2.4dd	Me	3,4-F_2	333-337	71	B
2.4ee [c]	Me	3-OMe	268-269 (276) [e]	69	A
2.4ff [c]	Me	4-OMe	311 (290-292) [g]	75	A
2.4gg	Me	3,5-$(OMe)_2$	298-299	88	B
2.4hh	Me	4-OH	330-333 (>330) [e]	82	B
2.4jj	Me	4-NMe_2	dec 320 (>330) [e]	92	A
2.4kk	Me	3-CF_3	257-259	56	B
2.4ll	Me	4-CF_3	300-302	42	B
2.4mm [c]	Me	3,5-$(CF_3)_2$	305-306	81	A
2.4nn	Me	3-SMe	257-259	87	B
2.4oo	Me	4-SMe	327-328	80	B
2.4qq	Me	4-CN	353 (357) [b]	30	B
3.3a	Et	H	328-329	91	B
3.3b	Et	4-Cl	267-268	65	A
3.3c	Et	4-Br	292-294	55	B
3.3d	Et	3-F	285-286	58	B
3.3e	Et	4-F	284-285	72	B
3.3f	Et	3,5-Cl_2	285-287	40	B
3.3g	Et	3,5-Me_2	253-254	45	A
				69	B
3.3h	Et	3-CF_3	250-251	41	B
3.3i	Et	3-CN	292-294	52	B
3.3j	Et	4-CN	318-320	37	B
3.3k	Et	3-SMe	213-216	41	B
3.3l	Et	4-SMe	316	71	B
3.3m	Propyl	4-Cl	263	46	B
3.3n	Phenyl	4-Cl	328-329	53	A
				47	B
3.3o	Benzyl	4-Cl	279-280	45	A
3.3p	Phenyl	H	300 (308-310) [h]	41	B

Table 7-2: Analytical data for the 3-substituted 6-(substituted anilino)uracils

Cmpd no.	Substitutents R	$(X)_n$	Formula	% calculated C	H	N	% found C	H	N
2.4a	Me	H	$C_{11}H_{11}N_3O_2$	60.8	5.1	19.3	60.8	5.4	19.6
2.4b	Me	2-Me	$C_{12}H_{13}N_3O_2$	62.3	5.7	18.2	62.4	5.6	18.2
2.4c	Me	3-Me	"	"	"	"	62.6	5.8	18.3
2.4d	Me	4-Me	"	"	"	"	62.3	5.8	18.2
2.4e	Me	2,4-Me_2	$C_{13}H_{15}N_3O_2$	63.7	6.2	17.1	63.9	6.3	17.2
2.4f	Me	3,4-Me_2	"	"	"	"	63.4	6.3	17.2
2.4g	Me	3,5-Me_2	"	"	"	"	63.7	6.3	17.2
2.4i	Me	2-Et	"	"	"	"	63.8	6.1	17.1
2.4j	Me	3-Et	"	"	"	"	63.7	6.2	16.9
2.4k	Me	4-Et	"	"	"	"	63.8	6.3	17.2
2.4l	Me	4-*n*-butyl	$C_{15}H_{19}N_3O_2$	65.9	7.0	15.4	66.1	7.1	15.4
2.4t	Me	3-Cl,4-Me	$C_{12}H_{12}ClN_3O_2$	54.3	4.6	15.8	54.6	4.8	16.1
2.4u	Me	3-Cl,5-Me	"	"	"	"	54.2	4.7	15.7
2.4w	Me	4-Cl,3-Me	"	"	"	"	54.0	4.5	15.8
2.4x	Me	4-Cl,2-F	$C_{11}H_9ClFN_3O_2$	49.0	3.4	15.6	48.9	3.2	15.7
2.4y	Me	4-Cl,3-CF_3	$C_{12}H_9ClF_3N_3O_2$	45.1	2.8	13.1	44.8	2.8	13.1
2.4dd	Me	3,4-F_2	$C_{11}H_9F_2N_3O_2$	52.2	3.6	16.6	52.5	3.8	16.6
2.4ee	Me	3-OMe	$C_{12}H_{13}N_3O_3$	58.3	5.3	17.0	58.5	5.3	17.1
2.4ff	Me	4-OMe	"	"	"	"	58.6	5.4	17.1
2.4gg	Me	3,5-$(OMe)_2$	$C_{12}H_{19}N_3O_4$	56.3	5.5	15.2	56.2	5.6	15.2
2.4hh	Me	4-OH	$C_{11}H_{11}N_3O_3$	56.7	4.8	18.0	57.0	4.7	18.1
2.4jj	Me	4-NMe_2	$C_{13}H_{16}N_4O_2$	60.0	6.2	21.5	59.8	6.5	21.5
2.4kk	Me	3-CF_3	$C_{12}H_{10}F_3N_3O_2$	50.5	3.5	14.7	50.3	3.3	14.5
2.4ll	Me	4-CF_3	"	"	"	"	50.6	3.5	14.4
2.4mm	Me	3,5-$(CF_3)_2$	$C_{13}H_9F_6N_3O_2$	44.2	2.6	11.9	44.4	2.3	11.8
2.4nn	Me	3-SMe	$C_{12}H_{13}N_3O_2S$	54.7	5.0	16.0	54.6	5.2	16.2
2.4oo	Me	4-SMe	"	"	"	"	55.0	5.1	16.1
2.4qq	Me	4-CN	$C_{12}H_{10}N_4O_2$	59.5	4.2	23.1	59.2	4.2	23.0
3.3a	Et	H	$C_{12}H_{13}N_3O_2$	62.3	5.7	18.2	62.3	5.8	18.2
3.3b	Et	4-Cl	$C_{12}H_{12}ClN_302$	54.3	4.2	15.8	54.0	4.5	15.7
3.3c	Et	4-Br	$C_{12}H_{12}BrN_3O_2$	46.5	3.9	13.6	46.1	3.8	13.4
3.3d	Et	3-F	$C_{12}H_{12}FN_3O_2$	57.8	4.9	16.9	58.1	5.0	17.1
3.3e	Et	4-F	"	"	"	"	58.2	4.9	17.0
3.3f	Et	3,5-Cl_2	$C_{12}H_{11}Cl_2N_3O_2$	48.0	3.7	14.0	48.3	3.7	13.9
3.3g	Et	3,5-Me_2	$C_{14}H_{17}N_3O_2$	64.9	6.6	16.2	65.1	6.8	16.3
3.3h	Et	3-CF_3	$C_{13}H_{12}F_3N_3O_2$	52.2	4.0	14.0	52.5	4.1	14.1
3.3i	Et	3-CN	$C_{13}H_{12}N_4O_2$	60.9	4.7	21.9	60.9	4.7	21.9
3.3j	Et	4-CN	"	"	"	"	60.6	5.0	21.8
3.3k	Et	3-SMe	$C_{13}H_{12}N_3O_2S$	56.3	5.5	15.2	56.5	5.7	15.2
3.3l	Et	4-SMe	"	"	"	"	56.6	5.5	15.1
3.3m	Propyl	4-Cl	$C_{13}H_{14}ClN_3O_2$	55.8	5.0	15.0	56.1	5.1	15.0
3.3n	Phenyl	4-Cl	$C_{16}H_{12}ClN_3O_2$	61.3	3.9	13.4	61.5	3.9	13.4
3.3o	Benzyl	4-Cl	$C_{17}H_{14}ClN_3O_2$	62.3	4.3	12.8	62.6	4.3	12.8
3.3p	Phenyl	H	$C_{16}H_{13}N_3O_2$	68.8	4.7	15.1	69.1	4.8	15.3

6-(3'-Trifluoromethylanilino)uracil (3.3s)

A mixture of 6-aminouracil (2.3 g, 18 mmol), 3-trifluoromethylaniline hydrochloride (4.1 g, 21 mmol), 3-trifluoromethylaniline (5.7 g, 36 mmol) and acetic acid (6 ml) were heated in an oil bath at 170° for 4 hrs. The melt was cooled and added to water (40 ml). The resulting solid was filtered off, washed with water and ethanol, and recrystallized from acetic acid to give the *title compound* (**3.3s**) (3.8 g, 78%) mp 325-327°. (Found: C, 48.8; H, 2.8; N, 15.5. $C_{11}H_8F_3N_3O_2$ requires C, 48.7; H, 3.0; N, 15.5%). ^{1}H n.m.r. (CD_3SOCD_3) δ 4.78, s, H 5; 7.50-7.58, m, H 2', 4', 5', 6'.

N-Phenyl-1,2-benzenediamine (3.6a)

To a solution of 2-nitro-N-phenylbenzenamine (4.3 g, 20 mmol) in acetic acid (100 ml) was added a solution of stannous chloride (22.6 g, 100 mmol) in concentrated hydrochloric acid (25 ml). The solution was warmed to 80° and stirred for 40 min. On cooling it was made strongly basic with 10 M sodium hydroxide solution, diluted with water (1 litre) and the residue was filtered off. The residue was washed with water (2 litres) and recrystallized from isopropanol to give the title compound (**3.6a**) (3.2 g, 87%) mp 72° (lit mp 76° [155]).

N-(4'-Chlorophenyl)-1,2-benzenediamine (3.6b)

N-(4'-Chlorophenyl)-2-nitrobenzenamine [126] (4.3 g, 20 mmol) was treated as for **3.6a** above. The product was recrystallized from a small amount of ethanol to give the title compound (**3.6b**) (3.5 g, 80%) mp 118° (lit mp 119° [126]).

10-Phenylflavin (3.7a)

The title compound was prepared by a similar method to that of Kraus *et al.* [162]. The crude product was recrystallized from acetic acid to give the title compound (**3.7a**) (2.6 g, 77%) dec 330° (lit mp 250° [162]). (Found: C, 65.9; H, 3.4; N, 18.9. $C_{16}H_{10}N_4O_2$ requires C, 66.2; H, 3.5; N, 19.3%.). ^{1}H n.m.r. data are presented in Tables 3-1 (pg 70o) and 3-2 (pg 71). m/z (rel. int.) 290 M (26), 289 (100), 246 (20), 219 (51), 218 (75).

10-(4'-Chlorophenyl)flavin (3.7b)

A mixture of N-(4'-chlorophenyl)-1,2-benzenediamine (**3.6b**) (2.5 g, 11.5 mmol), alloxan tetrahydrate (2.9 g, 13.7 mmol), boric acid (0.9 g, 14.6 mmol) and acetic acid (300 ml) was heated and stirred at 60° for 25 min. After crystallization was complete the solid was filtered off and recrystallized from acetic acid to give yellow crystals of the title compound (**3.7b**) (1.3 g, 36%) mp > 360° (lit mp >370° [42]). (Found: C, 59.5; H, 2.8; N, 17.4. $C_{16}H_9ClN_4O_2$ requires C, 59.2; H, 2.8; N, 17.3%). ^1H n.m.r. and mass spectral data are presented in Tables 3-1 (pg 70o) and 3-2 (pg 71), and Table 3-3 (pg 72o), respectively.

***N,N-Dimethyl-N'-(2'-nitrophenyl)-1,4-benzenediamine* (3.6c)**

A mixture of 2-chloronitrobenzene (25 g, 0.16 mol) and sodium acetate (25 g, 0.3 mol) was heated with stirring at 170-180° under a nitrogen atmosphere while N,N-dimethyl-1,4-benzenediamine (22.0 g, 0.16 mol) was added portionwise over 7 hrs. The resulting black tar was solubilized with a small volume of ethanol and made acidic with 1 M hydrochloric acid. The solution was steam distilled for 2.5 hrs to remove unreacted 2-chloronitrobenzene. The residue was made basic with aqueous ammonia and the resulting oil separated. The oil was washed with water (3 x 20 ml) and extracted several times with ether. The ether extracts were combined, dried (Na_2SO_4), filtered and the volatiles removed under reduced pressure. The resulting oily residue was dissolved in a small volume of hot ethanol, treated with charcoal, filtered, reduced in volume and chilled to give red crystals of the *title compound* (**3.6c**) (2.1 g, 5.1%). An analytical sample was recrystallized from isopropanol, mp 97-98°. (Found: C, 65.5; H, 5.9; N, 16.5. $C_{14}H_{15}N_3O_2$ requires C, 65.4; H, 5.9; N, 16.3%). m/z (rel. int.) 257 M (100), 223 (65), 209 (31), 167 (32), 136 (42).

10-(4'-Dimethylaminophenyl)flavin (3.7c)

N,N-Dimethyl-N'-(2'-nitrophenyl)-1,4-benzenediamine (**3.6c**) (1.2 g, 4.7 mmol) was added to a nitrogen saturated solution of stannous chloride (5.3 g, 23 mmol) in concentrated hydrochloric acid (10 ml) and acetic acid (50 ml) at 60° with stirring. A stream of nitrogen was passed through the solution continuously while stirring was continued for 1.3 hrs. The reaction was cooled, made basic with nitrogen saturated 10 M sodium hydroxide solution and diluted with nitrogen saturated water to dissolve the remaining tin salts. The resulting oil was extracted into chloroform, dried (Na_2SO_4), the chloroform removed under reduced pressure and the oil (1.0 g, 4.5 mmol) dissolved in acetic acid (10 ml). To this solution was added alloxan tetrahydrate (1.0 g, 4.5 mmol) and boric acid (0.3 g, 4.8 mmol) and the mixture heated at 60° for 1 hr under a nitrogen atmosphere. After evaporation to dryness the residue was washed with a small volume of water and ether, and recrystallized from methanol to give the *title compound* (**3.7c**) (0.4 g, 23%) dec 330°. (Found: C, 65.9; H, 3.4; N, 18.9. $C_{16}H_{10}N_4O_2$ requires C, 66.2; H, 3.5; N, 19.3%). ^{1}H n.m.r. (CD_3SOCD_3) δ 3.02, s, $N(CH_3)_2$; 6.93, d, 7.17, d, $J_{2',3'}$ 7.7 Hz, H 2', 3', 5', 6', 9; 7.53-7.82, m, H 7, 8; 8.16, d, $J_{6,7}$ 7.2 Hz, H 6. m/z (rel. int.) 334 (19), 333 M (95), 332 (71), 318 (17), 262 (54), 261 (100), 247 (25).

10-(3'-Nitrophenyl)flavin (3.9a)

To a solution of 10-phenylflavin [162] (1.5 g, 5 mmol) in concentrated sulfuric acid (10 ml) was added fuming nitric acid (d-1.5, 0.34 g, 5.5 mmol). The solution was heated to 130° for 1.5 hrs, allowed to cool, poured onto ice (100 g) and adjusted to pH 6 with concentrated ammonia solution. The precipitate was filtered off, washed with ethanol and recrystallized from methanol to give the *title compound* (**3.9a**) (0.6 g, 34%) dec 253°. (Found: C, 57.2; H, 2.7; N, 20.6. $C_{16}H_9N_5O_4$ requires C, 57.3; H, 2.7; N, 20.9%). ^{1}H n.m.r. (CD_3SOCD_3) δ 6.86, d, $J_{8,9}$ 8.0 Hz, H 9; 7.60-7.78, m, H 7, 8; 7.92-8.08, m, H 5', 6'; 8.21, d, $J_{6,7}$ 8.0 Hz, H 6; 8.42, s, H 2'; 8.53, d, $J_{4',5'}$ 8.0 Hz, H 4'. m/z (rel. int.) 335 M (34), 334 (100), 292 (16), 291 (22), 290 (21), 289 (76), 264 (19), 263 (30).

7-Nitro-10-(3'-nitrophenyl)flavin (3.8)

10-Phenylflavin [162] (1.5 g, 5 mmol) was added to a solution of fuming nitric acid (d-1.5, 12.5 ml) and concentrated sulfuric acid (25 ml) at 0°, and stirred at room temperature for 4 days. The solution was poured onto ice, adjusted to pH 3 with concentrated ammonia solution and filtered. The filtrate was allowed to stand for 4 days to ensure complete crystallization, the yellow solid was filtered off and recrystallized three times from ethanol to give the *title compound* (**3.8**) (0.2 g, 11%) dec 341°. (Found: C, 50.9; H, 1.9; N, 21.8. $C_{16}H_8N_6O_6$ requires C, 50.5; H, 2.1; N, 22.1%). ^{1}H n.m.r. (CD_3SOCD_3) δ 7.08, d, $J_{8,9}$ 9.4 Hz, H 9; 7.92-8.13, m, H 5', 6'; 8.40-8.45, m, H 8, 2'; 8.56, d, $J_{3',4'}$ 8.0 Hz, H 4'; 8.96, s, H 6. m/z (rel. int.) 380 M (11), 379 (37), 336 (11), 335 (24), 334 (70), 308 (11), 292 (15), 291 (22), 263 (28).

10-(3'-Bromophenyl)flavin (**3.9b**)

A mixture of 10-phenylflavin [162] (1 g, 3.3 mmol), concentrated sulfuric acid (10 ml), water (1 ml), silver sulfate (1.1 g, 3.3 mmol) and bromine (0.53 g, 3.3 mmol) was mechanically shaken for 16 hrs. The reaction mixture was poured onto ice (100 g) and filtered. The filtrate was adjusted to pH 6 with concentrated ammonia solution and the resulting precipitate was filtered off and recrystallized from acetic acid to give the *title compound* (**3.9b**) (0.3 g, 25%) dec 248°. (Found: C, 52.0; H, 2.3; N, 15.0. $C_{16}H_9BrN_4O_2$ requires C, 52.0; H, 2.5; N, 15.2%). ^{1}H n.m.r. (CD_3SOCD_3) δ 6.82, d, $J_{8,9}$ 6.8 Hz, H 9; 7.44-7.95, complex, H 2', 4', 5', 6', 7, 8; 8.21, d, $J_{6,7}$ 7.4 Hz, H 6.

Attempted dibromination of 10-phenylflavin

Method 1

10-Phenylflavin [162] (1 g, 3.3 mmol) was treated as for **3.9b** above except that twice the amount of bromine (1.06 g, 6.6 mmol) was used. The precipitate was recrystallized from acetic acid to give 10-(3'-bromophenyl)flavin (**3.9b**) (0.65 g, 51%) dec 248°. ^{1}H n.m.r. (CD_3SOCD_3) identical to that isolated above.

Method 2

10-(3'-Bromophenyl)flavin (**3.9b**) (1.2 g, 3.3 mmol) was treated as for **3.9b** above except that the reaction time was increased to 5 days. The black precipitate was taken up in acetic acid and filtered. T.l.c. (ethyl acetate) of the acetic acid solution showed no spots visible under long wavelength ultraviolet light. Attempts to isolate a product failed.

3-Substituted 10-(substituted phenyl)flavins (see Tables 7-3 and 7-4)

General method A - Condensation of 3-substituted 6-anilinouracils (**2.4, 3.3**) with nitrosobenzene.

The appropriate 3-substituted 6-(substituted anilino)uracil (**2.4, 3.3**) (10 mmol) and nitrosobenzene (3.2 g, 30 mmol) were refluxed in a mixture of acetic anhydride (16 ml) and acetic acid (6 ml) for 35 min. The volume of the reaction mixture was then reduced by approximately 50% under reduced pressure and ethanol (10 ml) added. After crystallization was complete the yellow solid was filtered off, washed with ethanol and ether, and recrystallized from methanol or acetic acid. The compounds produced, their melting points and yields are found in Table 7.3 and microanalyses are presented in Table 7-4. Some of the compounds' ^{1}H n.m.r. data are presented in Tables 2-1 (pg 32), 2-2 (pg 36), 3-1 (pg 70o) and 3-2 (pg 71). The UV and EIMS data of some of these compounds are presented in Tables 2-4 (pg 43) and 3-3 (pg 72o), respectively.

Attempted condensation of 6-(4'-dimethylaminoanilino)-3-methyluracil (2.4jj) with nitrosobenzene

6-(4'-Dimethylaminoanilino)-3-methyluracil (**2.4jj**) (2.6 g, 10 mmol) was treated according to general method A for flavin synthesis above. The t.l.c. (ethyl acetate) of the reaction mixture compared with an authentic sample of 10-(4'-dimethylaminophenyl)-3-methylflavin (**2.5jj**) revealed the flavin not to be present in the reaction mixture.

10-(4'-Carboxyphenyl)-3-methylflavin (**2.5rr**)

10-(4'-Cyanophenyl)-3-methylflavin (**2.5qq**) (0.5 g, 1.6 mmol) was refluxed in a mixture of acetic acid (100 ml) and 70% sulfuric acid (100 ml) for 2 hrs. The cooled mixture was poured onto ice (200 g) and the product precipitated with solid sodium carbonate. The solid was filtered off, washed with water and recrystallized from acetic acid to give the *title compound* (**2.5rr**) (0.19 g, 34%) mp >360°. (Found: C, 61.8; H, 3.7; N, 16.1. $C_{18}H_{12}N_4O_4$ requires C, 62.1; H, 3.5; N, 16.1%). UV and ^1H n.m.r. data are presented in Table 2-4 (pg 43), and Tables 2-1 (pg 32) and 2-2 (pg 36), respectively. m/z (rel. int.) 348 M (38), 347 (100), 291 (22), 290 (28), 263 (35), 262 (30).

3-Methyl-10-(4'-methylsulfonylphenyl)flavin (**2.5pp**)

3-Methyl-10-(4'-methylthiophenyl)flavin (**2.5oo**) (0.2 g, 0.57 mmol) was dissolved in acetic acid (50 ml) and cooled to 5°. To this solution was added 30% w/v hydrogen peroxide (0.26 g, 2.3 mmol), dropwise, with stirring over 1 hr. The mixture was allowed to come to room temperature and stirred for an additional 18 hrs. The acetic acid was evaporated off under a steam of nitrogen in an evaporating dish and the solid recrystallized from methanol to give the *title compound* (**2.5pp**) (0.1 g, 45%) mp 334-335°. (Found: C, 56.4; H, 3.8; N, 14.5. $C_{18}H_{14}N_4O_4S$ requires C, 56.5; H, 3.7; N, 14.5%). UV and ^1H n.m.r. data are presented in Table 2-4 (pg 43), and Tables 2-1 (pg 32) and 2-2 (pg 36), respectively. MSCI m/z (rel. int.) 383 M+1 (7), 369 (38), 368 (43), 367 (91), 366 (16), 365 (37), 354 (21), 353 (78), 352 (76), 351 (100).

3-Substituted 10-(substituted phenyl)flavins (see Tables 7-3 and 7-4)

General method B - Alkylation of 10-(substituted phenyl)flavins

To a solution of the appropriate 10-(substituted phenyl)flavin (**3.7a, 3.7c, 3.9a** or **3.9b**; 3.5 mmol) in dimethylformamide (250 ml) was added powdered potassium carbonate (4.7 g, 35 mmol) followed by the appropriate alkylating agent (methyl iodide, propyl iodide or benzyl bromide; 4.6 mmol). The mixture was heated and stirred at

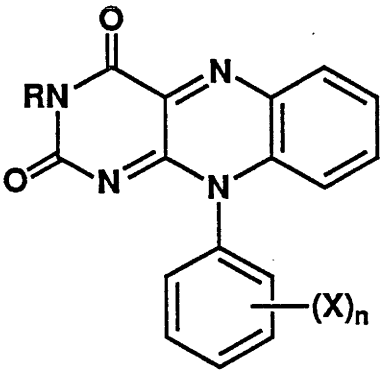
50-55° for 1 hr, cooled, filtered and the filtrate added to chloroform (400 ml). This was washed with water (5 x 200 ml), dried (Na_2SO_4), filtered and evaporated to dryness under reduced pressure. The solid residue was washed with ether, and recrystallized from methanol. The compounds produced, their melting points and yields are found in Table 7-3 and microanalyses are presented in Table7-4. Some of the compounds' ^1H n.m.r. data are presented in Tables 2-1 (pg 32), 2-2 (pg 36), 3-1 (pg 70o) and 3-2 (pg 71). The UV and EIMS data of some of these compounds are presented in Tables 2-4 (pg 43) and 3-3 (pg 72o), respectively.

The mass spectral data for **2.5jj** and **2.5z** are as follows:

10-(4'-dimethylaminophenyl)-3-methylflavin (**2.5jj**); m/z (rel. int.) 348 (21), 347 M (100), 346 (85), 332 (12), 290 (11), 262 (37), 261 (66); and

10-(3'-bromophenyl)-3-methylflavin (**2.5z**); m/z (rel. int.) 384 (27), 383 (88), 382 M (29), 381 (82), 327 (14), 326 (17), 325 (13), 324 (14).

Table 7-3: Physical properties of 3-substituted 10-(substituted phenyl)flavins produced by general methods A and B



[a] Literature mps are presented in brackets. [b] See reference [60]. [c] See reference [47]. [d] Synthesised by Dr W.B. Cowden. [e] See reference [77]. [f] See reference [42]. [g] See reference [161]. [h] See reference [163].

Cmpd no.	Substitutents R	$(X)_n$	Melting point (°C) [a]	% yield	Method
2.5a	Me	H	>360 (>360) [b]	56	B
2.5b	Me	2-Me	>360 (372) [c]	50	A
2.5c	Me	3-Me	341 (326) [b]	91	A
2.5d [d]	Me	4-Me	367-368	64	A
2.5e	Me	2,4-Me_2	333	56	A
2.5f	Me	3,4-Me_2	333-334 (347) [b]	47	A
2.5g	Me	3,5-Me_2	318-319	51	A
2.5i	Me	2-Et	270 (262-263) [e]	23	A
2.5j [d]	Me	3-Et	277-279	42	A
2.5k	Me	4-Et	318-319	51	A
2.5l [d]	Me	4-*n*-butyl	298-300	42	A
2.5t	Me	3-Cl,4-Me	>355	39	A
2.5u	Me	3-Cl,5-Me	355	34	A
2.5w	Me	4-Cl,3-Me	>360	20	A
2.5x	Me	4-Cl,2-F	274	26	A
2.5y [d]	Me	4-Cl,3-CF_3	>360	44	A
2.5z	Me	3-Br	353 (351-353) [f]	69	B
2.5dd	Me	3,4-F_2	>360	35	A
2.5ee [d]	Me	3-OMe	322-323	48	A
2.5ff [d]	Me	4-OMe	355 (>300) [g]	56	A
2.5gg	Me	3,5-$(OMe)_2$	341	45	A
2.5hh	Me	4-OH	>360 (>300) [g]	24	A
2.5ii	Me	3-NO_2	>360	76	B
2.5jj	Me	4-NMe_2	326	36	B
2.5kk [d]	Me	3-CF_3	346-348	38	A
2.5ll	Me	4-CF_3	>360	15	A
2.5mm [d]	Me	3,5-$(CF_3)_2$	>360	40	A
2.5nn	Me	3-SMe	304-305	35	A
2.5oo	Me	4-SMe	>360	38	A
2.5qq	Me	4-CN	>355 (>360) [b]	21	A
3.4a	Et	H	341	61	A
3.4b	Et	4-Cl	330	15	A
3.4c	Et	4-Br	>360	42	A
3.4d	Et	3-F	>360	33	A
3.4e	Et	4-F	322	25	A
3.4f	Et	3,5-Cl_2	317	44	A
3.4g	Et	3,5-Me_2	276	36	A
3.4h	Et	3-CF_3	297-299	31	A
3.4i	Et	3-CN	349-350	12	A
3.4j	Et	4-CN	>360	23	A
3.4k	Et	3-SMe	341	43	A
3.4l	Et	4-SMe	265	26	A
3.4m	Propyl	4-Cl	>360	34	A
3.4n	Phenyl	4-Cl	>360 (>300)	32	A
3.4o	Benzyl	4-Cl	351	31	A
3.4q	Propyl	H	325	90	B
3.4p	Phenyl	H	dec 280 (>330)	23	A
3.4r	Benzyl	H	327 (325-330) [h]	92	B
3.4s	H	3-CF_3	>360	45	A

Table 7-4: Analytical data for the 3-substituted 10-(substituted phenyl)flavins

Cmpd no.	Substitutents R	$(X)_n$	Formula	% calculated C	H	N	% found C	H	N
2.5a	Me	H	$C_{17}H_{12}N_4O_2$	67.1	4.0	18.4	66.8	3.8	18.4
2.5b	Me	2-Me	$C_{18}H_{14}N_4O_2$	67.9	4.4	17.6	68.3	4.3	17.8
2.5c	Me	3-Me	"	"	"	"	68.1	4.5	17.8
2.5d	Me	4-Me	"	"	"	"	67.7	4.5	17.5
2.5e	Me	2,4-Me_2	$C_{19}H_{16}N_4O_2$	68.7	4.9	16.9	68.8	4.9	17.0
2.5f	Me	3,4-Me_2	"	"	"	"	68.8	5.1	16.9
2.5g	Me	3,5-Me_2	"	"	"	"	68.3	4.8	16.7
2.5i	Me	2-Et	"	"	"	"	68.7	5.0	16.9
2.5j	Me	3-Et	"	"	"	"	68.4	4.9	17.1
2.5k	Me	4-Et	"	"	"	"	68.4	5.0	17.0
2.5l	Me	4-*n*-butyl	$C_{21}H_{20}N_4O_2$	70.0	5.6	15.6	69.7	5.7	15.3
2.5t	Me	3-Cl,4-Me	$C_{18}H_{13}ClN_4O_2$	61.3	3.7	15.9	61.0	3.6	15.8
2.5u	Me	3-Cl,5-Me	"	"	"	"	61.1	3.9	16.2
2.5w	Me	4-Cl,3-Me	"	"	"	"	61.1	3.6	16.2
2.5x	Me	4-Cl,2-F	$C_{17}H_{10}ClFN_4O_2$	57.2	2.8	15.7	57.5	2.8	15.9
2.5y	Me	4-Cl,3-CF_3	$C_{18}H_{10}ClF_3N_4O_2$	53.2	2.5	13.8	53.2	2.6	13.7
2.5z	Me	3-Br	$C_{17}H_{11}BrN_4O_2$	53.3	2.9	14.6	53.0	2.8	14.7
2.5dd	Me	3,4-F_2	$C_{17}H_{10}F_2N_4O_2$	60.0	3.0	16.5	60.1	2.9	16.5
2.5ee	Me	3-OMe	$C_{18}H_{14}N_4O_3$	64.7	4.2	16.8	64.7	4.3	16.8
2.5ff	Me	4-OMe	"	"	"	"	64.6	4.4	16.8
2.5gg	Me	3,5-$(OMe)_2$	$C_{19}H_{16}N_4O_4$	62.6	4.4	15.4	62.3	4.3	15.4
2.5hh	Me	4-OH	$C_{17}H_{12}N_4O_3$	63.8	3.8	17.5	63.5	3.9	17.5
2.5ii	Me	3-NO_2	$C_{17}H_{11}N_5O_4$	58.5	3.2	20.1	58.3	3.3	19.8
2.5jj	Me	4-NMe_2	$C_{19}H_{17}N_5O_2$	65.7	4.9	20.2	65.7	5.2	20.2
2.5kk	Me	3-CF_3	$C_{18}H_{11}F_3N_4O_2$	58.1	3.0	15.1	57.8	3.1	15.2
2.5ll	Me	4-CF_3	"	"	"	"	58.2	3.1	14.8
2.5mm	Me	3,5-$(CF_3)_2$	$C_{19}H_{10}F_6N_4O_2$	50.5	2.4	13.1	50.4	2.1	12.7
2.5nn	Me	3-SMe	$C_{18}H_{14}N_4O_2S$	61.7	4.0	16.0	61.5	4.1	16.1
2.5oo	Me	4-SMe	"	"	"	"	62.0	3.9	16.1
2.5qq	Me	4-CN	$C_{18}H_{11}N_5O_2$	64.4	3.5	22.1	64.6	3.2	22.1
3.4a	Et	H	$C_{18}H_{14}N_4O_2$	67.9	4.4	17.6	68.0	4.2	17.6
3.4b	Et	4-Cl	$C_{18}H_{13}ClN_4O_2$	61.3	3.7	15.9	61.1	3.6	15.8
3.4c	Et	4-Br	$C_{18}H_{13}BrN_4O_2$	54.4	3.3	14.1	54.0	3.2	13.9
3.4d	Et	3-F	$C_{18}H_{13}FN_4O_2$	64.3	3.9	16.7	64.1	3.9	16.7
3.4e	Et	4-F	"	"	"	"	64.6	3.8	16.7
3.4f	Et	3,5-Cl_2	$C_{18}H_{12}Cl_2N_4O_2$	55.8	3.1	14.5	56.1	3.1	14.6
3.4g	Et	3,5-Me_2	$C_{20}H_{18}N_4O_2$	69.4	5.2	16.2	69.3	5.3	16.4
3.4h	Et	3-CF_3	$C_{19}H_{13}F_3N_4O_2$	59.1	3.4	14.5	59.3	3.4	14.5
3.4i	Et	3-CN	$C_{19}H_{13}N_5O_2$	66.5	3.8	20.4	66.6	3.9	20.6
3.4j	Et	4-CN	"	"	"	"	66.2	3.8	20.3
3.4k	Et	3-SMe	$C_{19}H_{16}N_4O_2S$	62.6	4.4	15.4	62.3	4.6	15.3
3.4l	Et	4-SMe	"	"	"	"	62.8	4.5	15.5
3.4m	Propyl	4-Cl	$C_{19}H_{16}ClN_4O_2$	62.2	4.1	15.3	62.1	4.3	15.1
3.4n	Phenyl	4-Cl	$C_{22}H_{13}ClN_4O_2$	65.9	3.3	14.0	65.6	3.2	13.8
3.4o	Benzyl	4-Cl	$C_{23}H_{15}ClN_4O_2$	66.6	3.7	13.5	66.7	3.6	13.5
3.4q	Propyl	H	$C_{19}H_{16}N_4O_2$	68.7	4.9	16.9	68.6	5.1	17.1
3.4p	Phenyl	H	$C_{22}H_{14}N_4O_2$	72.1	3.9	15.3	71.7	4.2	14.9
3.4r	Benzyl	H	$C_{23}H_{16}N_4O_2$	72.6	4.2	14.7	72.3	4.3	15.0
3.4s	H	3-CF_3	$C_{17}H_9F_3N_4O_2$	57.0	2.5	15.6	56.6	2.5	15.6

Substituted nitrosobenzenes (4.1a-c)

Caro's acid - A paste of ground potassium persulfate (60 g, 0.22 mol) and concentrated sulfuric acid (60 ml) was well stirred for 1 hr at room temperature before being mixed with ice (1200 g). The solution was neutralized to a pH of 7 with solid potassium carbonate and acetic acid.

The Caro's acid solution from above was cooled to 0° before adding it to a cooled (0°) mixture of the appropriate aniline (60 mmol) in water (600 ml). The reaction mixture was allowed to stir for 5 hrs at 0°. The solution was filtered and the residue was then washed with water (2 litres), 1 M hydrochloric acid (800 ml) and water (500 ml). Steam distillation of the residue yielded the desired nitrosobenzene. Melting points of samples were determined on compounds recrystallization from ethanol: 4-chloronitrosobenzene (**4.1c**) mp 87° (lit mp 90°, prepared by the same method as above [137]); 3-chloro-nitrosobenzene (**4.1b**) mp 70° (lit mp 72° [164]); and 3,4-dimethylnitrosobenzene (**4.1a**) mp 44° (lit mp 44-45° [155]).

8-Chloro-10-(4'-chlorophenyl)-3-methylflavin (4.2c)

The title compound was prepared by a method similar to that of Yoneda *et al.* [61]. The crude material was recrystallized from acetic acid to give the title compound (**4.2c**) (0.8 g, 37%) dec 346° (lit mp >360° [61]). (Found: C, 54.4; H, 2.6; N, 15.0. $C_{17}H_{10}Cl_2N_4O_2$ requires C, 54.7; H, 2.7; N, 15.0%). m/z (rel. int.) 374 (18), 373 (56), 372 M (27), 371 (83), 315 (18), 287 (25). ^{1}H n.m.r. spectral data are presented in Table 4-1 (pg 83).

***9-Chloro-10-(4'-chlorophenyl)-3-methylflavin* (4.3b)**

A solution of 3-chloronitrosobenzene (**4.1b**) (2.6 g, 18 mmol) and 6-(4'-chloroanilino)-3-methyluracil (**2.4o**) (1.5 g, 6 mmol) in acetic anhydride (12 ml) and acetic acid (4 ml) was refluxed for 35 min. After crystallization was complete the solid was filtered off, washed with ether and recrystallization from acetic acid. The ^{1}H n.m.r. spectrum of the recrystallized material indicated it to be a mixture of the 7- and 9- chloro isomers. The mixture was twice recrystallized from dimethylformamide to give the *title*

compound (**4.3b**) (0.4 g, 18%) mp >360°. (Found: C, 54.7; H, 2.8; N, 15.1. $C_{17}H_{10}Cl_2N_4O_2$ requires C, 54.7; H, 2.7; N, 15.0%). The above recrystallization filtrates were combined and retained for use below. m/z (rel. int.) 374 (18), 373 (29), 372 M (20), 371 (41), 315 (9), 287 (33). ^{1}H n.m.r. data are presented in Table 4.1 (pg 83).

7-Chloro-10-(4'-chlorophenyl)-3-methylflavin **(4.2b)**

The filtrate from above was diluted with ether and the resulting solid was filtered off and recrystallized from acetic acid to give the *title compound* (**4.2b**) (0.25 g, 11%) dec 335°. (Found: C, 54.7; H, 2.8; N, 15.1. $C_{17}H_{10}Cl_2N_4O_2$ requires C, 54.7; H, 2.7; N, 15.0%). m/z (rel. int.) 374 (15), 373 (41), 372 M (20), 371 (64), 315 (14), 287 (25). ^{1}H n.m.r. data are presented in Table 4.1 (pg 83).

10-(4'-Chlorophenyl)-3,8,9-trimethylflavin **(4.3a)**

3,4-Dimethylnitrosobenzene (**4.1a**) (2.4 g, 18 mmol) was treated as for **4.3b** above. The ^{1}H n.m.r. spectrum of the recrystallized material indicated it to be a mixture of the 7,8- and 8,9- dimethyl isomers. The mixture was recrystallized twice from acetic acid and then dimethylformamide to give the *title compound* (**4.3a**) (0.5 g, 23%) dec 304°. (Found: C, 62.0; H, 4.0; N, 15.1. $C_{19}H_{15}ClN_4O_2$ requires C, 62.2; H, 4.1; N 15.3%). ^{1}H n.m.r. data are presented in Table 4.1 (pg 83).

5-Bromo-6-(4'-chloroanilino)-3-methyluracil **(4.4b)**

To a suspension of 6-(4'-chloroanilino)-3-methyluracil (**2.4o**) (3.8 g, 15 mmol) in methanol (50 ml), bromine (0.9 ml, 2.4 g, 18 mmol) was added and the mixture stirred for 1.5 hrs at room temperature. The resulting crystals were filtered off and recrystallized from acetic acid to give the *title compound* (**4.4b**) (3.2 g, 64%) mp 236°. (Found: C, 39.9; H, 2.7; N, 12.7. $C_{11}H_9BrClN_3O_2$ requires C, 40.0; H, 2.7; N, 12.7%.) ^{1}H n.m.r. (CD_3SOCD_3) δ 3.14, s, CH_3N; 7.16, d, 7.38, d, $J_{2',3'}$ 8.8 Hz, H 2', 3', 5', 6'; 8.72, s, NH.

6-(4'-Chloroanilino)-5-(3",4"-dimethylanilino)-3-methyluracil **(4.5b)**

A mixture of 5-bromo-6-(4'-chloroanilino)-3-methyluracil (**4.4b**) (1.7 g, 5 mmol) and 3,4-dimethylaniline (6.1 g, 50 mmol) in dimethyl sulfoxide (20 ml) was stirred at room temperature for 17 hrs. The resulting crystals were filtered off and recrystallized from acetic acid to give the *title compound* (**4.5b**) (1.2 g, 65%) dec 250°. (Found: C, 62.0; H, 4.1; N, 15.4. $C_{19}H_{19}ClN_4O_2$ requires C, 62.2; H, 4.1; N, 15.3%). ^{1}H n.m.r. (CD_3SOCD_3) δ 2.06, s $(CH_3)_2C_6H_3$; 3.10, s, CH_3N; 6.12, s, NH; 6.18-6.82, complex, $(CH_3)_2C_6H_3$; 7.11, d, 7.31, d, $J_{2',3'}$ 8.5 Hz, C_6H_4Cl; 8.47, s, NH.

6-Anilino-5-bromo-3-methyluracil **(4.4a)**

A solution of 6-anilino-3-methyluracil (**2.4a**) (2.0 g, 9.2 mmol) in methanol and acetic acid (1:4, 1500 ml) was cooled to -7° in an ice salt/ethanol bath. A solution of bromine (1.8 g, 11 mmol) in acetic acid (30 ml) was added at once to the cold solution and stirred for 20 min. The reaction mixture was allowed to come to room temperature over 2 hrs and the solution was evaporated to dryness under vacuum. The residue was recrystallized from ethanol to give the *title compound* (**4.4a**) (1.9 g, 69%) mp 243°. (Found: C, 44.9; H, 3.5; N, 14.1. $C_{11}H_{10}BrN_3O_2$ requires C, 44.6; H, 3.4; N, 14.2%). ^{1}H n.m.r. (CD_3SOCD_3) δ 3.13, s, CH_3N; 7.20-7.34, m, C_6H_5; 8.63, s, NH.

5-Bromo-6-(4'-bromoanilino)-3-methyluracil **(4.4c)**

When 6-anilino-3-methyluracil (**2.4a**) (1.8 g, 8.3 mmol) was treated as for **4.4a** above, except that the reaction was kept at 0°, the crude product was seen (^{1}H n.m.r.) to contain a mixture of starting material (**2.4a**), 6-anilino-5-bromo-3-methyluracil (**4.4a**) and the *title compound* (**4.4c**). Recrystallization of this crude material from ethanol gave the *title compound* (**4.4c**) (0.5 g, 16%) mp 222°. (Found: C, 35.1; H, 2.6; N, 10.9. $C_{11}H_9Br_2N_3O_2$ requires C, 35.2; H, 2.4; N, 11.2%). ^{1}H n.m.r. (CD_3SOCD_3) δ 3.13, s, CH_3N; 7.09, d, 7.50, d, $J_{2',3'}$ 9 Hz, H 2', 3', 5', 6'; 8.71, s, NH.

6-Anilino-5-(3',4'-dimethylanilino)-3-methyluracil (**4.5a**)

A mixture of 6-anilino-5-bromo-3-methyluracil (**4.4a**) (1.0 g, 3.4 mmol) and 3,4-dimethylaniline (4.1 g, 34 mmol) in dimethyl sulfoxide (20 ml) was stirred at room temperature for 17 hrs. The solution was poured into cold 0.5 M hydrochloric acid (100 ml) and stirred for 20 min. The crystalline mass was filtered off, washed with ether (200 ml) and recrystallized from ethanol to give the *title compound* (**4.5a**) (1.0 g, 84%) mp 228°. (Found: C, 67.6; H, 6.0; N, 16.5. $C_{19}H_{20}N_4O_2$ requires C, 67.8; H, 6.0; N, 16.7%). ^{1}H n.m.r. (CD_3SOCD_3) δ 2.06, s, $(CH_3)_2C_6H_3$; 3.10, s, CH_3N; 6.12, s, NH; 6.28-6.83, complex, $(CH_3)_2C_6\mathbf{H}_3$; 7.14-7.40, m, C_6H_5; 8.35, s, NH.

3,7,8-Trimethyl-10-phenylflavin (4.2d)

The title compound was prepared by a similar method to that of Sako *et al.* [62]. The ^{1}H n.m.r. spectrum of the crude material indicated the presence of only the 7,8-dimethyl isomer. The crude material (0.79 g) was recrystallized from acetic acid to give the title compound (**4.2d**) (0.52 g, 37%) dec 330° (lit mp >300° [62]). (Found: C, 69.0; H, 4.6; N, 16.7. $C_{19}H_{16}N_4O_2$ requires C, 68.7; H, 4.9; N 16.9%). ^{1}H n.m.r. data are presented in Table 4-1 (pg 83).

10-(4'-Chlorophenyl)-3,7,8-trimethylflavin (**4.2a**)

A solution of 6-(4'-chloroanilino)-5-(3",4"-dimethylanilino)-3-methyluracil (**4.5b**) (1.1 g, 3 mmol) in dimethylformamide (30 ml) was heated at 120° for 2 hrs under oxygen. The solution was cooled and diluted with ether (100 ml) and the resulting yellow crystals were filtered off. The ^{1}H n.m.r. spectrum of the crude material indicated the presence of both the 7,8-dimethyl and 8,9-dimethyl isomers. The crude material (0.3 g) was recrystallized from acetic acid and then dimethylformamide to give the *title compound* (**4.2a**) (0.1 g, 9%) dec 330°. (Found: C 62.0; H, 4.1; N, 15.4. $C_{19}H_{15}ClN_4O_2$ requires C, 62.2; H, 4.1; N, 15.3%). ^{1}H n.m.r. data are presented in Table 4-1 (pg 83).

7-(4'-Chlorophenyl)purine (4.7)

A solution of 4'-chloroanilinoacetonitrile [142,143] (3.3 g, 20 mmol), formamidine acetate [165] (15.6 g, 150 mmol) and 1-butanol (20 ml) was refluxed for 5 hrs. The butanol was distilled off under reduced pressure and the residue taken up in a mixture of chloroform (50 ml) and water (50 ml). The aqueous fraction was separated and extracted with chloroform (2 x 20 ml). The chloroform extracts were combined, dried (Na_2SO_4), filtered and evaporated to dryness. The residue was washed with ether (100 ml) and allowed to air dry. The dry residue was sublimed at 220-240° (1 mm Hg) to give white crystals of the *title compound* (**4.7**) (2.4 g, 52%) mp 223°. (Found: C, 56.9; H, 3.0; N, 24.1. $C_{11}H_7ClN_4$ requires C, 57.3; H, 3.1; N, 24.3%). ^1H n.m.r. ($CDCl_3$) δ 7.49, d, 7.65, d, $J_{2',3'}$ 8.8 Hz, H 2', 3', 5', 6'; 8.44, s, 9.04, s, 9.22, s, H 2, 6, 8.

4-Amino-5-(4'-chloroanilino)pyrimidine (4.8)

A suspension of 7-(4'-chlorophenyl)purine (**4.7**) (2 g, 8.7 mmol) in 0.5 M sodium hydroxide (80 ml) was refluxed for 10 min. The precipitate was filtered off and recrystallized from ether to give the *title compound* (**4.8**) (1.7 g, 89%) mp 204°. (Found: C, 54.7; H, 4.1; N, 25.4. $C_{10}H_9ClN_4$ requires C, 54.4; H, 4.1; N, 25.4%). ^1H n.m.r. (CD_3SOCD_3) δ 6.70, d, 7.18, d, $J_{2',3'}$ 8.8 Hz, H 2', 3', 5', 6'; 7.40, s, NH; 8.00, s, 8.18, s, H 2, 6.

10-(4'-Chlorophenyl)-3-methyl-6,8-diazaflavin (4.9)

A mixture of 4-amino-5-(4'-chloroanilino)pyrimidine (**4.8**) (1.7 g, 7.7 mmol), N-methylalloxan [144] (1.5 g, 7.8 mmol), boric acid (0.6 g, 9.7 mmol) and acetic acid (130 ml) was heated and stirred at 60° for 30 min. After cooling, the solvent was removed under vacuum to give a dark syrup. The syrup was redissolved in acetic acid (20 ml) and diluted with ether (50 ml), the resulting precipitate was filtered off and recrystallized from isopropanol to give the *title compound* (**4.9**) (0.4 g, 15%) dec 298°. (Found: C, 53.3; H, 3.1; N, 24.3. $C_{15}H_9ClN_6O_2$ requires C, 52.9; H, 2.7; N, 24.7%). m/z (rel. int.) 342 (15), 341 (26), 340 M (27), 339 (67), 283 (18), 255 (56). MSCl m/z

(rel. int.) 345 (36), 344 (25), 343 (100), 342 (26), 231 (24). ^{1}H n.m.r. ($CDCl_3$) δ 3.47, s, CH_3N; 7.34, d, 7.70, d, $J_{2',3'}$ 8.6 Hz, H 2', 3', 5', 6'; 8.64, s, 9.35, s, H 7, 9.

5-Amino-6-(4'-chloroanilino)-3-methyluracil **(5.2a)**

To a stirred cooled solution of 6-(4'-chloroanilino)-3-methyluracil (**2.4o**) (7.7 g, 30 mmol) in trifluoroacetic acid (50 ml) was added a sodium nitrite solution (3.2 g, 46 mmol) in water (20 ml) dropwise over 15 min. After stirring for an additional 10 min the resulting 6-(4'-chloroanilino)-3-methyl-5-nitrosouracil trifluoroacetic acid salt was filtered off, washed with ether, dried, pulverized and suspended in a solution of 1 M sodium hydroxide and methanol (4:1, 250 ml). Sodium dithionite was added with stirring until the suspension's red colour disappeared. The product was filtered off, washed with water and ether, and recrystallized from methanol to give the *title compound* (**5.2a**) as a white powder (4.6 g, 56%) mp 230-232°. (Found: C, 49.6; H, 4.2; N, 20.9. $C_{11}H_{11}ClN_4O_2$ requires C, 49.5; H, 4.2; N, 21.0%). m/z (rel. int.) 268 (32), 267 (15), 266 M (100), 154 (16), 138 (26). ^{1}H n.m.r. (CD_3SOCD_3) δ 3.15, s, CH_3N; 6.80, d, 7.25, d, $J_{2',3'}$ 8.9 Hz, H 2', 3', 5', 6'.

5-Amino-6-(3',5'-dimethylanilino)-3-methyluracil **(5.2b)**

6-(3',5'-Dimethylanilino)-3-methyluracil (**2.4g**) (7.35 g, 30 mmol) was treated as for **5.2a** above to give the *title compound* (**5.2b**) (5.2 g, crude yield 67%).

3,6,8-Trimethylbenzo[*g*]pteridine-2,4(1*H*,3*H*)-dione (5.4)

(A) Recrystallization of crude 5-amino-6-(3',5'-dimethylanilino)-3-methyluracil (**5.2b**) (0.5 g, 2 mmol) from methanol gave 3,6,8-trimethylbenzo[*g*]pteridine-2,4(1*H*,3*H*)-dione (**5.4**) (0.3 g, 60%) mp 310-312° (lit mp 295-298° [160]). (Found: C, 61.2; H, 5.0; N, 21.9. $C_{13}H_{12}N_4O_2$ requires C, 60.9; H, 4.7; N, 21.9%). m/z (rel. int.) 257 (15), 256 M (100), 199 (20), 171 (40), 156 (19). ^{1}H n.m.r. (CD_3SOCD_3) δ 2.51, s, 2.70, s, 6-, 8-CH_3; 3.30, s, CH_3N; 7.47, s, 7.51, s, H 7, 9.

(B) Sodium nitrite solution (1.7 g, 25 mmol) in water (10 ml) was added to a hot (100°) stirred solution of 6-(3',5'-dimethylanilino)-3-methyluracil (**2.4g**) (1.2 g, 5 mmol) in acetic acid (50 ml); the mixture went fleetingly red before becoming yellow. On cooling crystals formed, which were filtered off, and washed with water and methanol. Recrystallization from acetic acid gave light yellow crystals of the title compound (**5.4**) (0.9 g, 70%) identical with the compound from (A) above by mp, mass spectrum and ^{1}H n.m.r. comparison (Found: C, 60.8; H, 5.0; N, 21.8).

8-(4'-Chlorophenyl)-3-methylpteridine-2,4(3H,8H)-dione **(5.3a)**

A glyoxal solution (1 g of 30% glyoxal solution, 5.6 mmol) in methanol (10 ml) was added to a suspension of 5-amino-6-(4'-chloroanilino)-3-methyluracil (**5.2a**) (1.5 g, 5.6 mmol) in water (50 ml); after briefly stirring at room temperature the mixture was refluxed for 30 min. After crystallization was complete the solid was filtered off, washed with ether, and recrystallized from acetic acid to give yellow crystals of the *title compound* (**5.3a**) (0.75 g, 46%) dec 333°. (Found: C, 54.1; H, 3.2; N, 19.3. $C_{13}H_9ClN_4O_2$ requires C, 54.1; H, 3.1; N, 19.4%). m/z (rel. int.) 289 (9), 288 M (35), 287 (28), 286 (100), 230 (34), 202 (27). ^{1}H n.m.r. data are presented in Table 5-1 (pg 92o).

8-(3',5'-Dimethylphenyl)-3-methylpteridine-2,4(3H,8H)-dione **(5.3b)**

5-Amino-6-(3',5'-dimethylanilino)-3-methyluracil (**5.2b**) (1.5 g, 5.6 mmol) was treated as for **5.3a** above. The product was recrystallized from 70% ethanol to give brown orange crystals of the *title compound* (**5.3b**) (0.25 g, 16%) dec 280°. (Found: C, 64.1; H, 5.0; N, 20.1. $C_{15}H_{14}N_4O_2$ requires C, 63.8; H, 5.0; N, 19.9%). m/z (rel. int.) 282 M (53), 281 (100), 267 (73), 225 (46), 224 (54), 210 (50), 197 (43), 196 (55). ^{1}H n.m.r. data are presented in Table 5-1 (pg 92o).

***8-(4'-Chlorophenyl)-3,6,7-trimethylpteridine-2,4(3H,8H)-dione* (5.3c)**

Biacetyl (0.5 g, 5.6 mmol) in methanol (10 ml) was added to a suspension of 5-amino-6-(4'-chloroanilino)-3-methyluracil (**5.2a**) (1.5 g, 5.6 mmol) in water (50 ml) and was refluxed for 1 hr. After crystallization was complete the solid was filtered off, washed with water and then ether, and recrystallized from methanol to give yellow crystals of the *title compound* (**5.3c**) (0.5 g, 28%) dec 261-263°. (Found: C, 56.6; H, 4.1; N, 17.6. $C_{15}H_{13}ClN_4O_2$ requires C, 56.9; H, 4.1; N, 17.7%). m/z (rel. int.) 318 (18), 317 (39), 316 M (59), 315 (100), 258 (18), 231 (23). ^{1}H n.m.r. data are presented in Table 5-1 (pg 92o).

***8-(3',5'-Dimethylphenyl)-3,6,7-trimethylpteridine-2,4(3H,8H)-dione* (5.3d)**

5-Amino-6-(3',5'-dimethylanilino)-3-methyluracil (**5.2b**) (1.5 g, 5.6 mmol) was treated as for **5.3c** above. The product was recrystallized from 70% ethanol to give brown orange crystals of the *title compound* (**5.3d**) (0.3 g, 17%) dec 280°. (Found: C, 65.8; H, 6.0; N, 18.3. $C_{17}H_{18}N_4O_2$ requires C, 65.8; H, 5.9; N, 18.1%). m/z (rel. int.) 310 M (88), 309 (100), 295 (36), 253 (15), 252 (16), 238 (10), 225 (17), 224 (21). ^{1}H n.m.r. data are presented in Table 5-1 (pg 92o).

***8-(4'-Chlorophenyl)-3-methyl-6,7-diphenylpteridine-2,4(3H,8H)-dione* (5.3e)**

A solution of benzil (1.3 g, 6 mmol) in methanol (30 ml) was added to 5-amino-6-(4'-chloroanilino)-3-methyluracil (**5.2a**) (1.5 g, 5.6 mmol) in 50% acetic acid (50 ml), and refluxed for 2 hrs. After crystallization was complete the solid was filtered off, washed with water and then ether, and recrystallized from 70% ethanol to give bright yellow crystals of the *title compound* (**5.3e**) (1.3 g, 49%) mp 313-314°. (Found: C, 67.9; H, 3.9; N, 12.8. $C_{25}H_{17}ClN_4O_2$ requires C, 68.1; H, 3.9; N, 12.7%). m/z (rel. int.) 441 (16), 440 M (43), 439 (51), 438 (100), 353 (24). ^{1}H n.m.r. data are presented in Table 5-1 (pg 92o).

***8-(3',5'-Dimethylphenyl)-3-methyl-6,7-diphenylpteridine-2,4(3H,8H)-dione* (5.3f)**

5-Amino-6-(3',5'-dimethylanilino)-3-methyluracil (**5.2b**) (1.6 g, 6 mmol) was treated as for **5.3e** above. The product was recrystallized from 70% ethanol to give bright yellow crystals of the *title compound* (**5.3f**) (0.6 g, 24%) mp 331-333°. (Found: C, 75.0; H, 5.1; N, 12.8. $C_{27}H_{22}N_4O_2$ requires C, 74.6; H, 5.1; N, 12.9%). m/z (rel. int.) 434 M (65), 433 (100), 419 (21), 349 (11), 348 (33). ^{1}H n.m.r. data are presented in Table 5-1 (pg 92o).

7-3 Biological activity experimental

7-3.1 Biological evaluation of antimalarial activity

(i) Antimalarial activity *in vivo* testing

Experimental animals

Female CBA/CaH mice (6-8 weeks old) were used in all experiments. These were bred and maintained in the John Curtin School of Medical Research Animal Breeding Establishment (under specific pathogen free conditions) and fed normal laboratory diet pellets and tap water *ad libitum*. Mice were usually put into groups of 4 to 6 animals in which the weight range was no greater than 1 g.

Parasite

Plasmodium vinckei subsp. *vinckei* (originally from Dr D. Walliker, Institute of Animal Genetics, Edinburgh) was stored frozen in liquid nitrogen and had been passaged several times before experimental use in CBA/CaH mice. All infections were initiated by intraperitoneal injection of 1×10^6 or 1×10^5 parasitized erythrocytes in sterile saline to provide infected mice (15-35% parasitemia) in 5 or 6 days time, respectively. The percentage parasitemia was monitored by thin blood smears taken from the tail vein and stained with Diff-Quik stain (Australian Hospital Supply, Sydney) and examined under oil immersion microscopy. Counts of parasitized red blood cells were determined in microscopic fields with approximately 200 cells per field in which there were few overlapping erythrocytes.

Drug treatment

Purified drugs were ground to a fine powder using an agate mortar and pestle. If the compound failed to give a well separated fine powder at this stage it was put through a sieve (0.15 mm screen).

The doses given were calculated on the average weight of the group (when the weight range of the group was less than 1 g, otherwise the dose was based on the weight of individual mice). Test compounds were then evenly suspended in olive oil using a

vortex mixer immediately prior to injection. Compounds were administered as a single 100 µl (or 200 µl in cases where the dose was greater than 70 mg/kg) intraperitoneal injection when parasitemias were between 15 and 35%. Glass syringes (1 ml) and 23 gauge needles were used to prevent clogging of the suspension during injection.

In control mice treated with olive oil alone, parasitemias rose to 40-60% one day post treatment and to 70-90% after 2 days. Death inevitably occurred on day 3.

The efficacy of the compounds tested was determined by the percentage of the animals in the group cured, the mean extension in lifespan and the percentage parasitemia two days after treatment (or 1 day after treatment if death occurred before day 2).

(ii) *In vitro* inhibition of *P. falciparum* growth

(a) Method 1

This method was used in collaboration with Dr G. A. Butcher. It was similar to that quoted in reference [41]. A brief description of the method including differences from the referenced method are outlined below.

Parasites

A Papua New Guinea strain of *P. falciparum* (FC-27) isolated at the Walter and Eliza Hall Institute, Melbourne, and maintained in continuous culture by Dr G. A. Butcher [41,166] (Department of Pure and Applied Biology, Imperial College, Prince Consort Rd, London, SW7 2BB) was used. Parasites were maintained using group O erythrocytes at a hematocrit of 5%.

Media

Complete medium: The complete medium was RPMI 1640 supplemented with 25 mM HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]), 26 mM sodium bicarbonate and 100 ng/ml gentamicin.

Culturing medium: Complete medium was supplemented with 10% (v/v) heat-inactivated human group O blood serum to produce normal culturing medium.

Drug preparation

The insolubility of the flavins required that they were first dissolved in dimethyl sulfoxide to obtain a stock solution which was diluted with complete medium supplemented with 40% (v/v) group O blood serum (the high serum concentration facilitated the solubilization of the drugs). A four fold dilution with complete medium gave culturing media with the highest drug concentration. Dilution of this with normal culturing medium gave the range of drug concentrations required for the assay.

Drug preparation was always carried out on the same day as the assay was begun. All drug treated culturing media were passed through millipore filters (0.22 μm) for sterilization and to ensure that all the test compounds were in solution.

Preparation and processing of microtiter plates

The drug treated culturing medium (100 μl) was added in triplicate to the flat bottom wells of 96-well microtiter plates (Nunc). Control wells consisted of 100 μl of culturing medium containing equivalent volumes of dimethyl sulfoxide (max 0.25% v/v). This was followed by the addition of 1 μl of parasitized erythrocytes (0.5% - 5% parasitemia, asynchronous parasites) and 20 μl of ^{3}H-hydroxanthine (1:100 dilution of 1 mCi/ml, 2.8 Ci/mmol, Amersham, Australia) in RPMI as a combined mixture (21μl) to all wells. The final drug concentration range in the wells was 1-20 μM.

The microtiter plates were incubated for 48 hours in the gas mixture 5% CO_2, 5% O_2, 90% N_2 at 37°.

At the end of incubation the cells were harvested onto filters with a semi-automated cell harvester using distilled water as the washing fluid. The filters were then

counted by liquid scintillation to determine the incorporation of ^{3}H-hypoxanthine into the nucleic acid of the parasites.

(b) Method 2

This method, used by Dr K. A. Rockett, is similar to that quoted in reference [167]. A brief description of the method and the differences from the referenced method are outlined below.

Parasites

The strains of *P. falciparum* used were FC-27 as in method 1 and the multi-drug resistant K-1 strain (a generous gift from Dr K. H. Rieckmann, Army Malaria Research Unit, Milpo, Ingleburn, NSW, 2174, Australia) isolated in Bangkok, Thailand, and originating from Kanchanaburi, Thailand [168]. Parasites were maintained using group A^{+} erythrocytes at a hematocrit of 5%.

Media

As in method 1.

Drug preparation

Flavins were made up as stock solutions in dimethyl sulfoxide. Dilution of the stock solution followed by serial dilution with culturing media gave the desired concentrations of drug treated culturing media.

The antimalarials, chloroquine, pyrimethamine and quinine were dissolved in culturing media to the appropriate dilution and likewise serially diluted.

Preparation and processing of microtiter plates

To triplicate wells in 96-well microtiter plates were added 50 µl of each dilution of the drug treated culturing medium followed by the addition of another 50 µl culturing medium containing synchronized parasitized cells (at ring stage) adjusted to a parasitemia

of between 0.5 and 1.0% at a hematocrit of 5%. The concentration range tested for flavins against the FC-27 strain was 0.756-48.4 μM (in 4-fold dilution intervals) and against the K-1 strain was 0.756-96.8 μM (in 2-fold dilution intervals). The other antimalarials were tested in the concentration range 0.001 to 100 μM. Controls consisted of wells treated with normal culturing medium and infected erythrocytes (100% control) or with normal culturing medium and non-infected erythrocytes (0% control). It had been previously shown that dimethyl sulfoxide at the concentration (max 0.2% v/v) used had no effect on parasite growth.

The plates were incubated under the same conditions as in method 1 for 24 hrs when ^{3}H-hypoxanthine (0.4 μCi per well) was added to each well. After a further 24 hrs of incubation, the plates were treated as in method 1.

7-3.2 Antibabesial screen in mice

Experimental animals

The same as used in section 7-3.1(i).

Parasite

Babesia microti (King strain originally from Dr F. E. G. Cox, King's College, London) was stored frozen in liquid nitrogen and had been passaged several times in CBA/CaH mice before experimental use. Infections were initiated by intraperitoneal injection of 1 x 10^{7} parasitized erythrocytes in sterile saline and became patent 3 days later. The percentage parasitemia was monitored by tail vein thin blood smears stained with Giemsa's stain and examined under oil immersion microscopy. Parasitemias were expressed as the percentage of erythrocytes infected.

Drug treatment

The test compounds were prepared and injected as described in section 7-3.1(i). Four groups of mice were treated as follows:

Control group - Olive oil (100 µl) was injected daily for 5 days starting on day 3 after inoculation;

Group 1 - 15 Mg/kg of 10-(3',5'-dichlorophenyl)-3-methylflavin (**2.5s**) in olive oil (100 µl) was injected daily for 5 days starting on day 3 after inoculation;

Group 2 - 5 Mg/kg of 10-(3',5'-dichlorophenyl)-3-methylflavin (**2.5s**) in olive oil (100 µl) was injected daily for 5 days starting on day 3 after inoculation; and

Group 3 - 10 Mg/kg of 10-(4'-chlorophenyl)-3-methylflavin (**2.5o**) in olive oil (100 µl) was injected daily for 4 days starting on day 3 after inoculation. Only four injections were given to this group as signs of toxicity were noted after the fourth injection.

The course of the disease was monitored by daily blood smears for 10 days after the first injections.

7-3.3 Anticoccidial screen in mice

The testing of 3-ethyl-10-(3'-trifluoromethylphenyl)flavin (**3.4h**) against the *Coccidia* species, *Eimeria vermiformis*, a species of rodent *Eimeria*, was kindly carried out by Mr C. Reiger and Dr K. Ovington (Department of Zoology, The Australian National University, Canberra, ACT, 2601, Australia). Female C57/BL6 mice weighting approximately 18 g were infected orally with 1 x 10^3 oocysts. The infection was monitored by daily determinations of the numbers of oocysts present in the total faecal output of individual mice. Methods used for routine maintenance of the parasite, infection with oocysts and counting of oocysts were the same as those described by Rose, Owen and Hesketh [169]. Infections became patent on the seventh day after infection and continued for a further seven days.

To test for anticoccidial activity eight mice were fed a daily ration of 3 g of laboratory diet pellets into which the test compound was incorporated at a level of

150 ppm. Treatment began on the day of infection and continued until day 14, excepting days 9 and 13 on which the mice were fed untreated food because of apparent signs of toxicity. The control group of 6 mice were also fed a daily ration of 3 g laboratory diet pellets, identical to the treated pellets except for the incorporated test compound. Both groups were given tap water *ad libitum*.

At the end of the experiment animals were kept for 7 days during which time no adverse effects were noted.

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