University of Southampton

SYNTHESIS AND ELECTROCHEMICAL PROPERTIES OF BIFUNCTIONALIZED FLAVINS: MODELS OF REDOX COENZYMES

A Thesis Submitted for the
Degree of Doctor of Philosophy

by

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March 1989

Acknowledgements

My thanks go to my supervisor, Dr Dave Gani, for the conception of this project. His enthusiasm and optimism have been of great encouragement throughout the three years. I also thank the SERC and the University of Southampton for financial support.

I should also like to record my appreciation of the help I received from those members of the Wolfson electrochemical unit, particularly Dr. Vinnie Cunnane, who contributed to the setting up and running of the electrochemical experiments.

Finally, many thanks to those who have helped me in the production of this thesis: Neil, Vinnie, Bahar, Nigel and Mahmoud, for proof reading; Martin for macromodel[113] calculations; and Clare for the high quality laser printouts.

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF SCIENCE

CHEMISTRY

Doctor of Philosophy

SYNTHESIS AND ELECTROCHEMICAL PROPERTIES OF BIFUNCTIONALISED FLAVINS: MODELS OF REDOX COENZYMES

by Timothy Richard George Edwards

In biological systems, the flavin nucleus is sandwiched between the oxidised substrate and the reduced cofactor. This led to the development of the unique concept of parallel face bifunctionalised flavin-electrode systems. Biomimetic studies were carried out towards the development of parallel face flavin electrodes.

A number of 10(N) methyl flavins were synthesised, which led to the formation of flavins with terminal amino and thiol functionality. The formation of a series of bifunctionalised flavins based on 7 & N(3) diacid flavins, starting from 4-fluoro-benzoic acid, is presented. Thiourea derivatised flavins led to the formation of covalently modified gold electrodes.

Cyclic voltammetric studies indicated solution state flavin electrode interactions at HMDE, glassy carbon and gold electrodes, for lumiflavin, tetraacetylriboflavin, and non-thiol functionalised flavins. This was in contrast to electrochemical studies with bisthiourea derivatised flavins at gold electrodes. Electrochemical character for an adsorbed species was exhibited and these studies indicated that the flavin was securely attached to the electrode surface. Formal rate constants of 2.92 & 1.98 10^2 s^{-1} for the oxidation and reduction of bisthiourea flavin were determined by cyclic voltammetry. These rates were 100 times faster than reported results, where the flavin lies perpendicular to the electrode surface. This evidence gave support to the concept that rapid electron transfer only occurs at electrodes when the flavin lies parallel to the electrode surface.

CONTENTS

Abbreviations

These symbols agree with the IUPAC Commission on Eiectrochemistry[1].

Symbol	Meaning	Usual dimension
A	area	cm^2
a	anodic	
c	cathodic	
C_j	bulk concentration of species j	M, mol/cm^3, mM
D_j	diffusion coefficient of species j	cm^3/sec
E_{pa}	anodic peak potential	V
E_{pc}	cathodic peak potential	V
ΔE	$\lvert E_{pa} - E_{pc} \rvert$ in CV	V, mV
$E^{o'}$	formal potential of an electrode	V
e	quantity charge on an electron	c
F	faraday	c
i_{pa}	anodic peak current	µA
i_{pc}	cathodic peak current	µA
$k^{o'}$	standard (intrinsic) heterogenous rate constant	cm/s
n	electrons per molecule oxidised or reduced	
R	molar gas constant	$J\ mol^{-1}K^{-1}$
T	absolute temperature	K
v	linear potential scan rate	Vs^{-1}
α_j	transfer coefficient of species j	
Γ_j	surface excess of species j at equilibrium	moi/cm^2
BTBO	1,1-*bis*[6-(trifluoromethyl) benzotriazolyl] oxalate	
DMAP	dimethylaminopyridine	
DMF	N,N-dimethylformamide	
DMSO	dimethylsulphoxide	
FAB	fast atom bombardment	
FAD	flavin adenine dinucleotide	
LDA	lithium diisopropylamide	
$NaBH_4$	sodium borohydride	
NAD	nicotinamide adenine dinucleotide	
TFA	trifluoroacetic acid	
THF	tetrahydrofuran	

CHAPTER 1

Introduction

1.1 Flavin History and Structure

More than a century ago, a yellow, fluorescent pigment was isolated from whey by Blyth[2]. In subsequent years, yellow pigments were extracted from other biological material, such as albumin, a variety of animal organs and vegetable sources.[3-5] These pigments were given names depending on their physical appearance or source of isolation. Later it became evident that all of these compounds were riboflavin (vitamin B_2). It was not until 1934 that the first flavin, lumiflavin, was synthesised[6] and it took another year for the structure to be established by the European chemists R. Kuhn[7] and P. Karrer.[8]

That yellow flavin pigments function as coenzymes had earlier been deduced by H. Theorell[9] of Sweden and O. Warburg[10] of Germany, who found that an enzyme participating in the oxidation of reduced pyridine nucleotides contained a yellow prosthetic group. This was identified by Theorell as riboflavin 5'-phosphate, or flavin mononucleotide (FMN). Later, in 1938, Warburg[11] found a second coenzyme form of riboflavin, flavin adenine dinucleotide (FAD). This was isolated from mammalian liver and kidney and also yeast. Like nicotinamide adenine dinucleotide (NAD^+), figure 1.1, FAD is composed of a nucleoside pyrophosphate linked to a vitamin, which in this case is vitamin B_2 or riboflavin. FMN is not a true nucleotide since it contains no pentose sugar; instead, like FAD, it contains the sugar alcohol ribitol.

Riboflavin, FMN and FAD are the most common flavocoenzymes associated with biological materials. The side chains ribityl, ribityl phosphate and ribityl adenine diphosphate allow the selective binding to a particular flavoprotein. The redox active chemical entity of the flavocoenzymes is the isoalloxazine nucleus. The term isoalloxazine originates from the use of alloxan in the early syntheses of flavins.[12] The IUPAC recommendation for flavin is defined as 7,8-dimethyl-benzo[g]-pteridine-2,4(3H,10H)-dione, where the numbering given in figure 1.2 is internationally accepted. An older numbering system is still sometimes found in the modern literature.

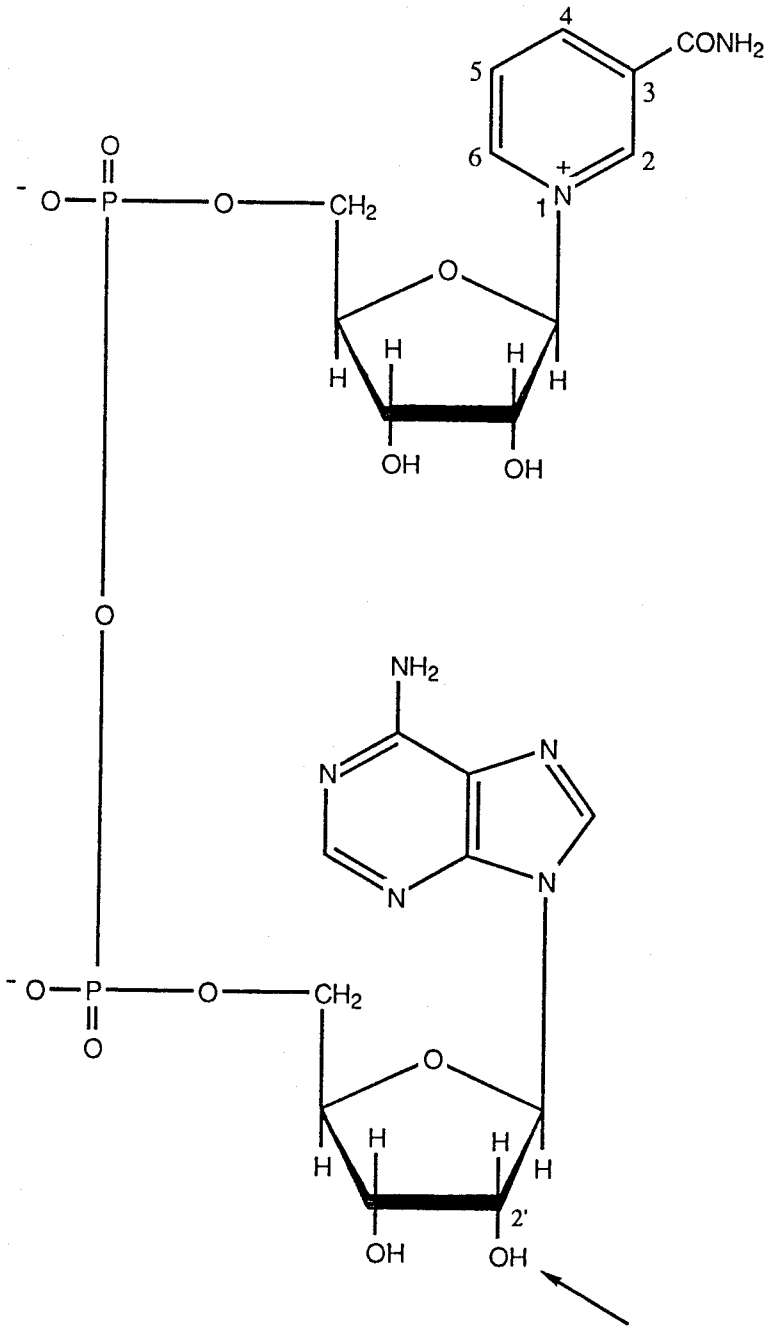


Figure 1.1

Nicotinamide adenine dinucleotide (NAD^+). The nicotinamide moiety, which is the portion undergoing reversible reduction, has been numbered. In nicotinamide adenine dinucleotide phosphate ($NADP^+$) the 2'-hydroxyl indicated by the arrow is esterified with phosphoric acid.

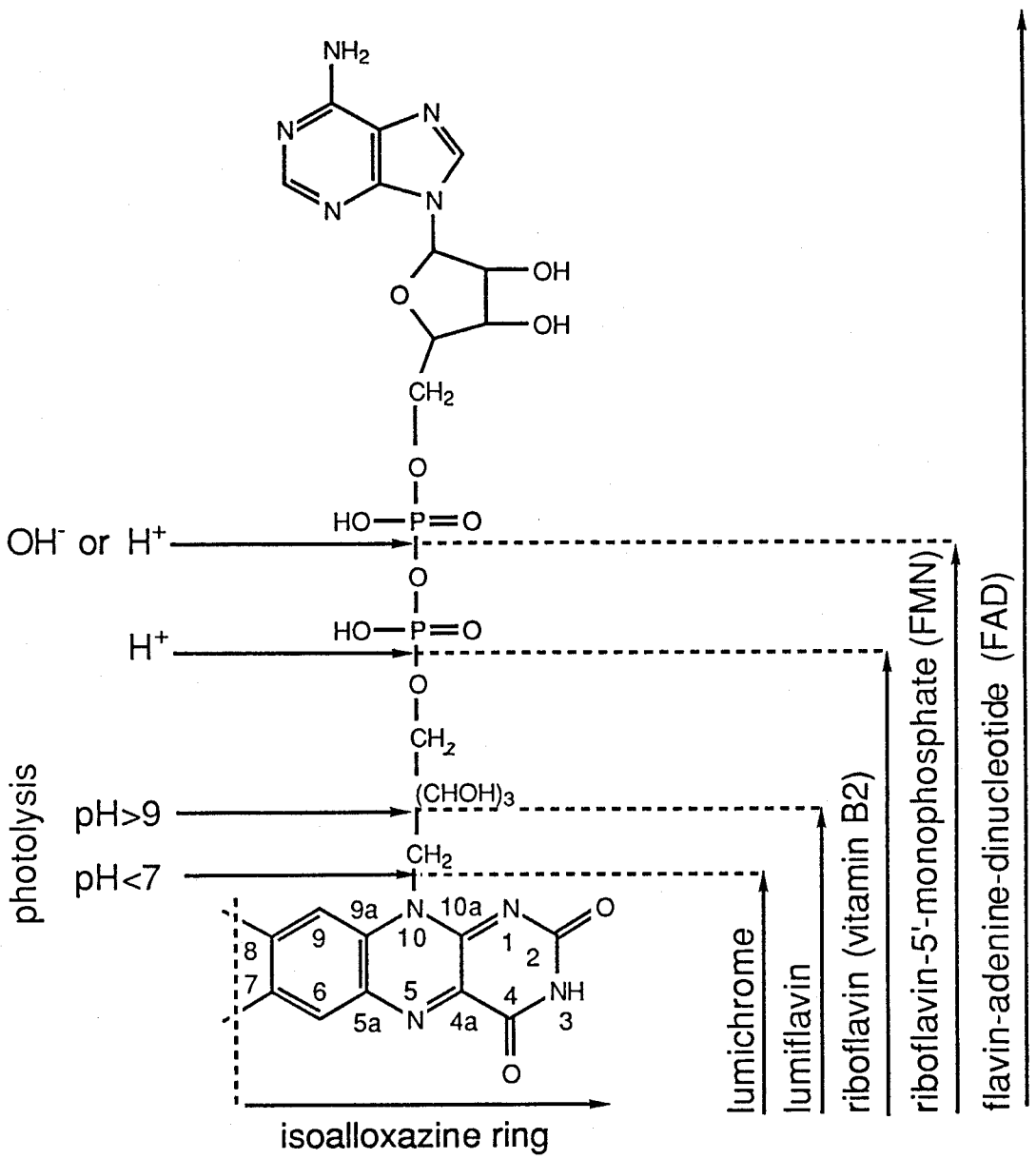


Figure 1.2

Flavin adenine dinucleotide (FAD). The redox active centre is numbered.

1.2 Flavin Dehydrogenases and Oxidases

The flavin nucleotides function as the prosthetic groups for the oxidation-reduction enzymes known as flavoenzymes or flavoproteins. These enzymes function in the oxidative degradation of pyruvate, fatty acids and amino acids, and also in the process of electron transport. In most flavoenzymes the flavin nucleotide is tightly but not covalently bound to the protein; an exception is succinate dehydrogenase, in which the flavin nucleotide FAD is covalently bound to a histidine residue of the polypeptide chain. Flavin nucleotides undergo reversible reduction of the isoalloxazine ring in the catalytic cycle of flavoproteins, to yield the reduced nucleotides, symbolised $FMNH_2$ and $FADH_2$.

The most important flavin-linked dehydrogenases in the main stream of respiration and electron transport, all localised in the mitochondria, are: (1), NADH dehydrogenase which contains FMN and catalyses transfer of electrons from NADH to the next member of the electron-transport chain; (2), succinate dehydrogenase, active in the tricarboxylic acid cycle; (3), dihydrolipoyl dehydrogenase, a component of the pyruvate and α-ketoglutarate dehydrogenase systems; and (4), acyl-Co A dehydrogenase, which catalyses the first dehydrogenation step of fatty acid oxidation.

Flavin-linked oxidation-reduction enzymes may be placed into two classes, dehydrogenases and oxidases, according to their ability to react with electron acceptors. In the flavin dehydrogenases, such as NADH dehydrogenase and succinate dehydrogenase, there is little or no tendency of the reduced form of the flavin nucleotide to be reoxidised by molecular oxygen. Reduced flavin oxidases, in contrast are reoxidised by oxygen to yield hydrogen peroxide. In members of this group, which includes D-amino acid oxidase and xanthine oxidase, the flavin nucleotide is bound to the protein in such a way that the reduced form of the nucleotide is available to react with oxygen.

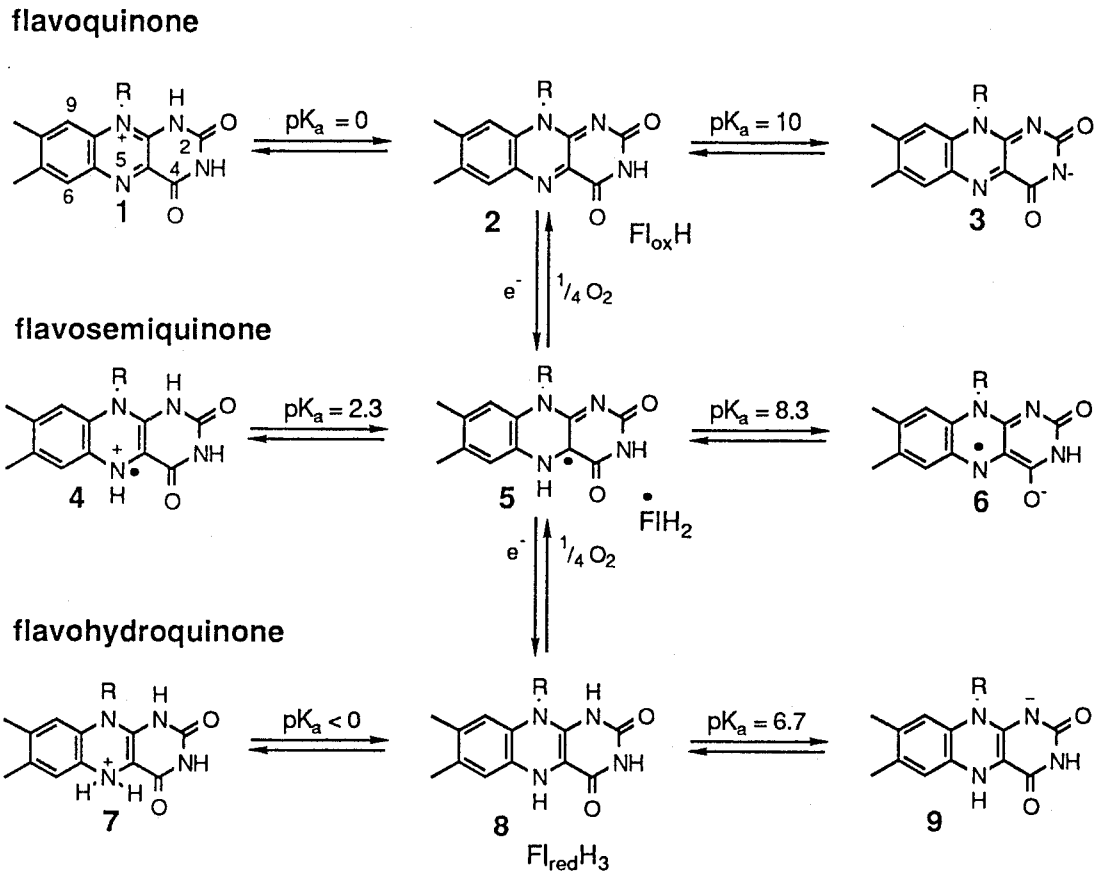
1.3 Flavocoenzymes

It has been shown that the prosthetic groups of flavoenzymes can be released reversibiy without proteolytic digestion of the protein.[11,13,14] Most flavoproteins known today show a similar behaviour. Although very large coenzyme association constants are observed for many flavoproteins, the prosthetic group is usually released from the flavoprotein under relatively mild denaturing conditions. It is known that the tight binding is a result of electrostatic and hydrophobic interactions between the phosphate moiety and the dimethylbenzo moiety of the prosthetic group and the apoflavoprotein. In 1955 Green *et al.*[15] discovered that only part of the total tissue flavin was released upon treatment with the usual techniques and concluded from this observation that part of the flavin was covalently bound. This proposal was supported by the work of Kearney[16] who succeeded in isolating a peptide on the level of lumiflavin after proteolytic digestion of succinate dehydrogenase and alkaline photolysis of the isolated flavin peptide. Later Wang *et al.*[17] eliminated the 1-, 2- and 3-positions as the sites of attachment, since hydrolysis in mild alkali yields urea, not a ureido peptide.

A collaboration, initiated in 1965 between the laboratories in Konstanz, San Francisco and Stockholm resulted in the unambiguous demonstration that, in contrast to alternative speculations,[18] the peptide chain is linked to the 8α-methylene on the benzene ring of riboflavin.[19,20] It was demonstrated[21] that histidine is the adjacent amino acid, linked through an imidazole ring nitrogen. The peptide sequence at the active centre was finally elucidated in 1970.[22, 23]

1.4 The Flavin Redox System

The most prominent features of the chemistry of flavin are its redox properties. These properties make flavin especially suitable for its broad involvement in biological reactions. The structures of the various ionic forms of the isoalloxazine nucleus that could exist in the oxidised, semi-quinone and hydroquinone forms are shown in scheme 1.1.



Scheme 1.1

In all three redox states, flavin is an amphoteric molecule. Flavoquinone, i.e. oxidised flavin, $Fl_{ox}H$, possesses pKa's of ≃0 and ≃10. It should be noted that the first protonation occurs at N(1), which is the most basic nitrogen in $Fl_{ox}H$ and not N(5). In very strong acidic solutions, however, N(5) can also be protonated reversibly. The addition of one half equivalent of for example dithionite to a solution of (**2**) yields a so called 'half-reduced' system. 'Half-reduced' is not a very meaningful term, it only implies that one electron is added to flavin without any indication of the spin distribution.

$$Fl_{ox}H + Fl_{red}H_3 \rightleftharpoons 2\,\dot{F}lH_2 \qquad 1.1$$

This equation (1.1) shows that under unfavourable conditions only a very small concentration of flavosemiquinone may be formed. In neutral aqueous solution the system is rapidly disproportionated to yield only a few percent of flavosemiquinone. Reduction of flavin by two electrons yields the 1,5-dihydroflavin, often called 'reduced flavin'. The majority of flavin redox species are highly coloured, this is in contrast to a solution of 1,5-dihydroflavin (**8**) which is devoid of a strong colour, but is not colourless. For all one- or two-electron reduced species various tautomeric forms can exist, e.g. iminol tautomers. Such tautomers do not exist in flavoquinone. In addition in (**6**) and (**9**) the negative charge could also be placed on the other hetero atoms in the pyrimidine subnucleus of the flavin system, except on N(3). Finally it is important to note that the formation of all one- or two-electron reduced species is thermodynamically fully reversible.

1.5 Flavoproteins-Redox Shuttles

Flavoproteins occupy a key position among the oxidising enzymes. No significant extent of biological oxidation occurs except through flavoproteins as part of the electron transfer mechanism. Among the great number of flavoproteins, there are three which are as yet not believed to be involved in biological oxidation: oxynitrilase which catalyses the formation of dextrorotatory oxynitriles;[24] glyoxylate carboligase which catalyses the formation of hydroxymalonic semialdehyde[25] and CO_2 from glyoxylate and finally, riboflavin-binding protein from eggs which is believed to function as a storage for vitamin B_2 and as a riboflavin carrier.[26]

Flavoproteins could make use of all three redox states during catalysis but it turns out that flavoproteins use selectively only certain redox states, depending on their biological function. Flavoproteins not containing a metal prosthetic group, shuttle between the flavoquinone state forming hydrogen peroxide (1.2).

$$Fl_{red}H_3 + O_2 \rightarrow Fl_{ox}H + H_2O_2 \qquad 1.2$$

Examples of this class of enzymes are glucose oxidase and D-amino acid oxidase.[27] In flavoprotein oxidases, the 1,5-dihydroflavin is very reactive towards O_2, whilst the two-electron reduced form of flavoprotein oxidases react slowly with pure one-electron acceptors, e.g. ferricyanide. That the two-electron transition is biologically favoured in these enzymes explains why they can react easily with sulphite.[28]

The majority of flavoprotein dehydrogenases contain one or more types of metal prosthetic groups, e.g. xanthine oxidase contains Fe and Mo. Since these metal ions are involved in electron flux, their possible participation in the reaction with O_2 cannot be excluded. Evidence indicates that the flavin is involved in the one-electron reduction of O_2 (1.3).

$$Fl_{red}H_3 + \tfrac{1}{4}O_2 \rightarrow \dot{F}lH_2 + \tfrac{1}{2}H_2O \qquad 1.3$$

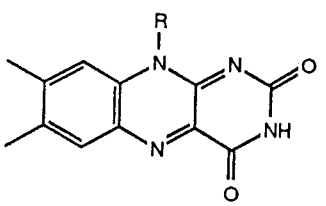
Besides flavocoenzymes, other prosthetic groups or coenzymes can be involved in the biological oxidation reactions, i.e. metal ions. Whenever electrons must be transported from reduced pyridine nucleotides to metal containing enzymes, which are one-electron acceptors or donators, then a flavoprotein is required as a mediator. In such cases the flavoprotein receives two electrons and one proton from NAD(P)H yielding the flavohydroquinone state of the protein which in turn splits the electron pair into two electron equivalents which can be accommodated by the metal-containing enzyme. The entry point of electrons is FAD shuttling between the flavoquinone and flavohydro-quinone state during catalysis, but making use of the intermediate flavosemiquinone level. FMN, on the other hand, forms a rather stable semiquinone and shuttles between the semiquinone and hydroquinone state.

$$NADPH + FAD \rightarrow NADP^+ + FADH_2 \qquad 1.4$$

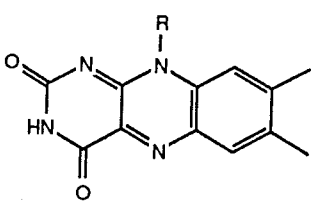
$$FADH_2 + 2FMNH \rightarrow FAD + 2FMNH_2 \qquad 1.5$$

$$2FMNH_2 + 2A_{(acceptor)} \rightarrow 2FMNH + 2A^- \qquad 1.6$$

Enzymatic[29-32] as well as bio-organic model studies[33,34] have made it clear that positions C(4a) and N(5) are the key loci of interaction between flavins and substrates. Crystallographic studies show that the C(4) and N(5) positions of the respective co-enzymes are optimally aligned for hydride transfer to occur.[35] As the two faces of the flavin are stereoheterot opic (figure 1.3), the question arises whether only one of them is used by any particular enzyme and if so which one.



N(5) *si* - face



N(5) *re* - face

Figure 1.3

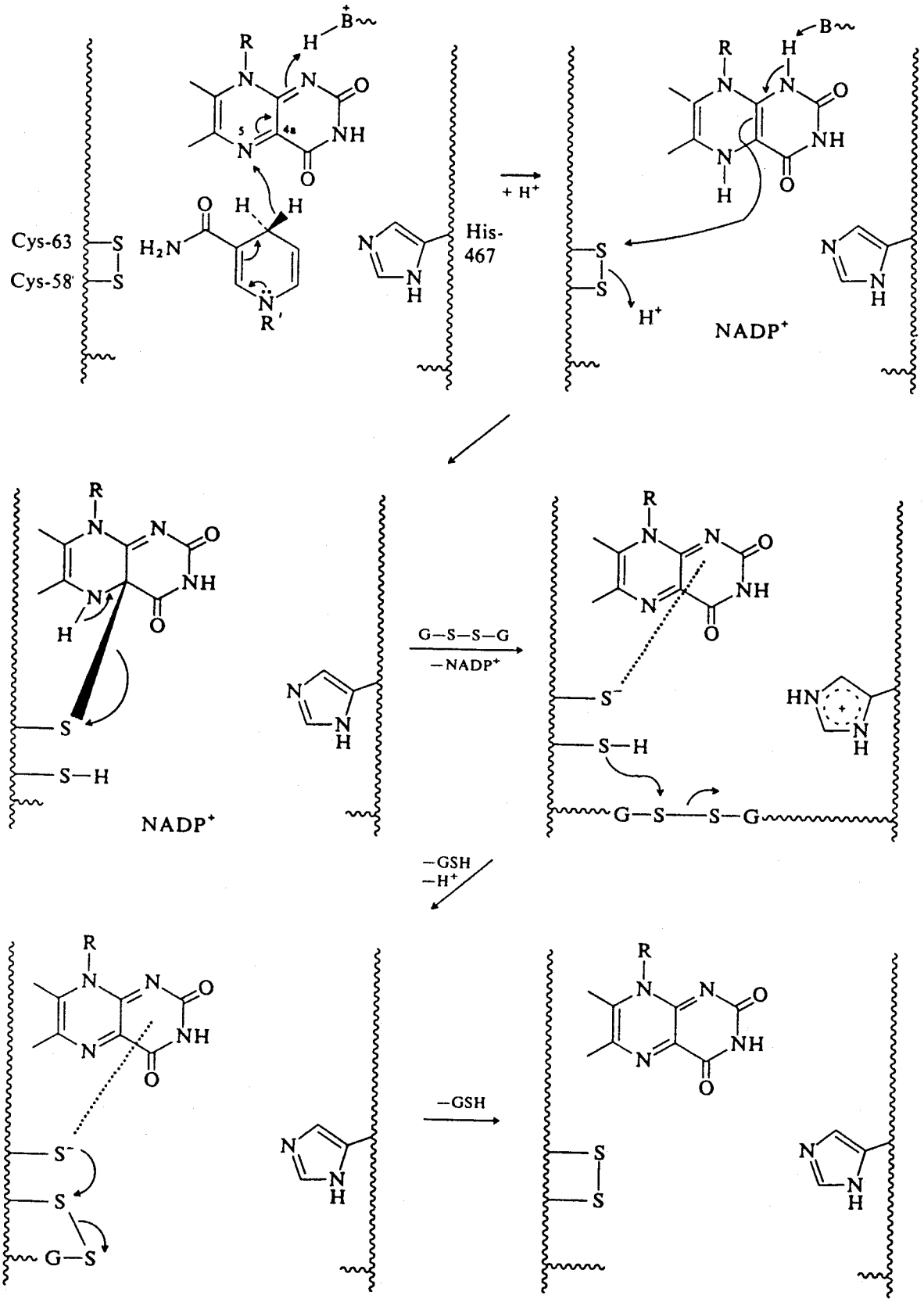
Determination of the stereochemistry of the substrate- flavin interaction in flavoproteins relies on comparison of results obtained with different flavoenzymes to the results obtained with human erythrocyte glutathione reductase. Glutathione reductase from human erythrocytes was the first flavoenzyme for which the absolute stereochemistry of its prosthetic group became known. X-ray crystallography established that the nicotinamide ring of its substrate NADPH interacts with the N(5) *re*-face of the isoalloxazine ring of its prosthetic group FAD.[36] On reducing 5-deaza-FAD-reconstituted acyl-CoA dehydrogenase with NaB^3H_4, Ghisla *et al.* [37] found that about 90% of the tritium label had been incorporated into the N(5) *re*-face of the flavin.

The stereochemistry of flavin-pyridine nucleotide interaction in the latter enzyme is known from the X-ray crystal structures of several reaction intermediates.[36] By use of this approach the stereochemistry of eight flavoenzymes has been reported: glutathione reductase, mercuric

reductase, thioredoxin reductase, p-hydroxybenzoate hydroxylase, melilotate hydroxylase, anthranilate hydroxylase, glucose oxidase and general acyl-CoA dehydrogenase. All flavoenzymes so far tested which employ the traditional flavin coenzymes showed N(5) *re*-face stereospecificity towards transfer of hydrogen from NAD(P)H.[38,39]

Glutathione reductase converts glutathione (γ-Glu-Cys-Gly), an intracellular tripeptide coenzyme, through reduction from its inactive disulphide form into its active free thiol form. The first reaction involves the reduction of the flavin by NADPH (eq. 1.4). Glutathione reductase catalyses transfer of the 4-pro-*s*-hydrogen of NADPH to N(5) of the flavin.[40] In the absence of NADPH, the 4a-*re* face of the flavin is covered by Tyr-197. It is thought that this prevents the reduced flavin from undergoing oxidation. The N(5) atom is close to the C(6) of Lys-66, this protects the flavin from transferring single electrons so that the two-electron transfer process observed with glutathione reductase is favoured. On the opposite face of the flavin, the 4a-*si* face, there is a redox active Cys-63 Cys-58 disulphide bridge. The sulphur atom of Cys-63 is positioned so that nucleophilic attack by the reduced flavin at C(4a) allows formation of a C(4a)-flavin-Cys-63 bond and a free thiolate at Cys-58, (Scheme 1.2).[41] The flavin is reoxidised to form a charge-transfer anion while the N(5) proton is transferred, probably *via* Cys-63, to the imadazole moiety of His-467. The Cys-58 thiol can then transfer electrons to glutathione disulphide to form a new disulphide bridge and one molecule of glutathione. Release of the product from the active-site followed by reformation of the original disulphide bridge and collapse of the charge transfer complex results in reduction of the remaining glutathione molecule and reoxidation of the flavoprotein.

Recent stereochemical studies of flavoenzyme reactions for D-lactate dehydrogenase and L-lactate oxidase indicate that the substrate reacted with the N(5) *si*-face of the flavin.[39] Support for this unusual mode of reaction comes from X-ray crystallographic studies on glycolate oxidase from spinach[42] and on flavocytochrome B_2 from bakers' yeast,[43] both of which utilize the N(5) *si*-face for reaction with their α-hydroxy acid substrate. All four enzymes are irreversibly inhibited by α-hydroxy-butynoate.[44-46] The three L-specific enzymes (L-lactate oxidase, glycolate oxidase and flavocytochrome b_2) form a flavin C(4a), N(5) covalent adduct with this inhibitor,[45] while the D-specific enzyme D-lactate dehydrogenase reacts similarly but at positions N(5), C(6).[45]



Scheme 1.2

On the basis of this information and general mechanistic similarities, a detailed geometry was proposed for the interaction of α-hydroxy acid, flavin, and protein functionalised groups. The hypothesis was put forward that during evolution the relative orientation of substrate and flavin face was retained, but that changes in the amino acid residues governing substrate specificity occurred.[45,48] This prediction is substantiated by the fact that the D- and L- specific α-hydroxy acid oxidising enzymes all use the *si*-face of the flavin ring for catalysis.

The substrate α-position must be located close to N(5) of the flavin since a covalent bond is formed between the two positions in the case of L-lactate oxidase.[48]

The reactivity of α-hydroxybutynoate with the D- and L- specific enzymes, respectively, fixes the orientation of the substrate α-carbon substituent above the plane of the flavin. The carboxylate residue has been proposed to point away from N(5) parallel to the N(1)-N(5) axis,[49] figure 1.4. However, if the substrate α-hydrogen is placed as shown in figure 1.4A, i.e. on the substrate face distal to the flavin, then it is difficult to envisage the transfer of the abstracted proton from the base to the flavin N(5) position. A solution to this dilemma exists if it is assumed two distinctly different mechanisms occur. A carbanion mechanism for the normal flavin, figure 1.4A, and a hydride-transfer mechanism for deazaflavin, figure 1.4B. For the hydride mechanism, the substrate would need to be inserted with the α-hydrogen sandwiched between the central carbon of the lactate and the N(5) position of the flavin.

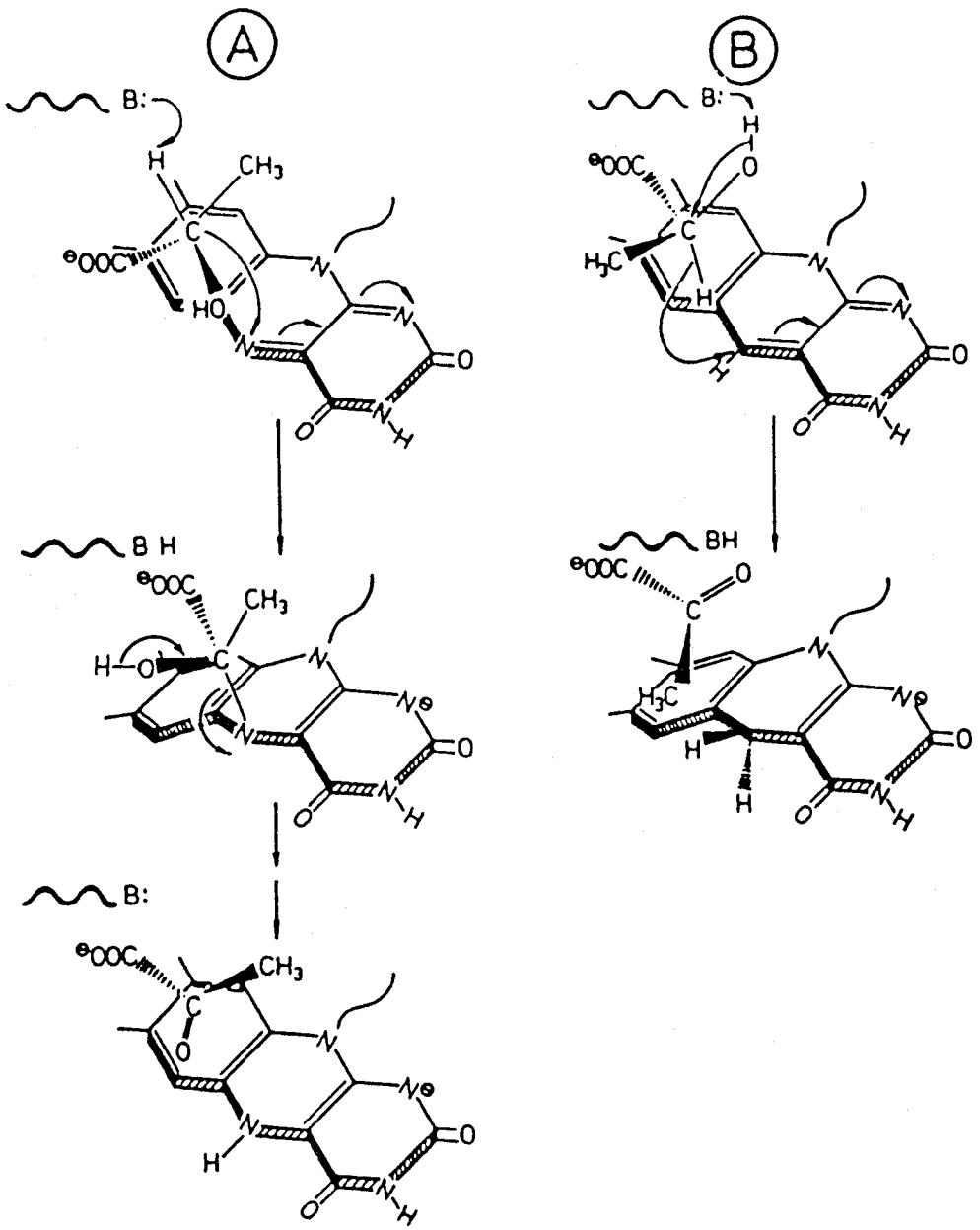


Figure 1.4

Relative orientation of substrate, flavin and proton-abstracting base in α-hydroxy carboxylic acid oxidising enzymes: (A) for carbanion mechanism; (B) for hydride-transfer mechanism.

An alternative explanation would be to have the substrate α-hydrogen orientated toward the face of the flavin. This would require that the base abstracting the α-hydrogen is close to N(5), roughly in plane with the flavin, figure 1.5.

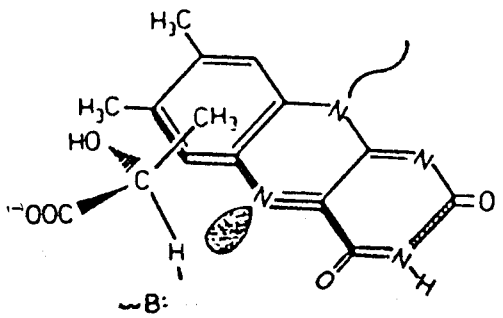


Figure 1.5

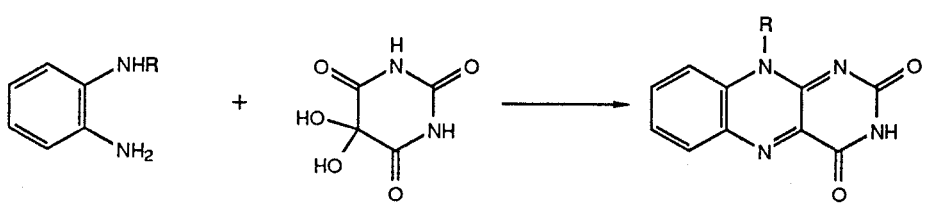
Alternative orientation of substrate, flavin and protein base. This alignment would make it difficult to differentiate between a hydride-transfer mechanism and carbanion mechanism.

Space-filling models suggest that accommodation of both a monofunctional base and the substrate around the flavin N(5) is possible. Such a juxtaposition of reactants would make it difficult to differentiate between a hydride transfer and a carbanion mechanism.

1.5 Flavin Synthesis

In view of the presence of the isoalloxazine nucleus in riboflavin and its coenzyme derivatives FMN and FAD and the importance of flavin-dependent enzymes in biology,[50-56] much effort has been devoted to chemical synthesis of riboflavin analogues as model systems for mechanistic studies of the natural co-enzyme.[8,59-60] Since the first synthesis of riboflavin in 1935,[9] the routes to flavin synthesis have increased enormously. The synthetic routes to isoalloxazine formation have been

extensively reviewed by J.P. Lambooy.[57] Efficient synthesis of isoalioxazines has been found by the condensation of 2-amino N-alkylanilines with alloxan, alloxantin, isodialuric acid, or 5-halobarbituric acid.[57] More recent syntheses include: the nitrosative and nitrative cyclisation, followed by reduction of 6-(N-alkylanilino) uracils[61-63] with concentrated sulphuric acid[64,65] and finally from a number of other 5- and 6- anilino uracils by oxidative cyclisation.[66-69] Of these methods, the treatment of 2-amino-N-alkylanilines with alloxan has been by far the most widely employed, scheme 1.3, and has been used to prepare a variety of riboflavin analogues[58,59] However, the yields obtained were highly variable, depending strongly and unpredictably on the number, nature and placement of substitutes on the 2-phenylene diamine nucleus.



Alloxan monohydrate

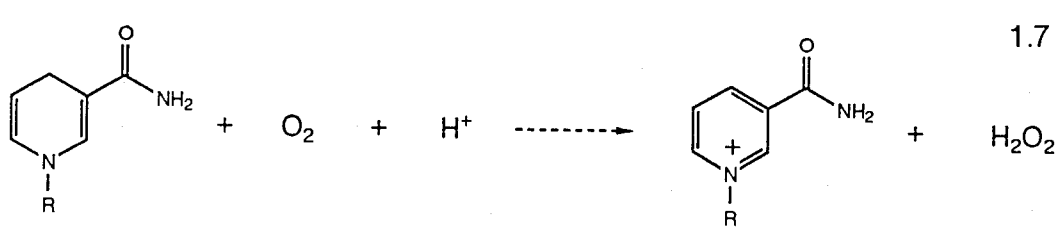
Scheme 1.3

1.5.1 Semisynthetic flavoenzymes

Microbiological processes frequently used in the pharmaceutical industry involve enzymatic reactions. However, the applications of purified enzymes have been limited in general because of the enzymes' cost, lack of stability, or insufficient catalytic efficiency and versatility.

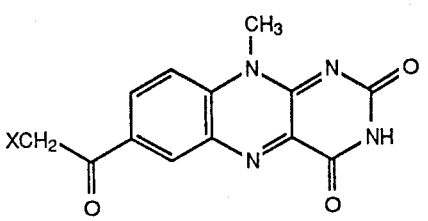
Very recently, two approaches have been pursued actively in an attempt to improve the stability, catalytic efficiency, and even catalytic versatility of naturally occurring enzymes. On the one hand, there is the approach involving genetic engineering techniques;[70] more specifically, the method of site-directed mutagenesis; and on the other, the technique of 'chemical mutation' of enzyme active sites.[71] In the case of site-directed mutagenesis, individual amino acids of an enzyme molecule can be replaced by essentially any other naturally occurring amino acid in locations chosen by the experimentor.[72] in the 'chemical mutation' approach new residues or new catalytic groupings are introduced by chemical modification of the enzyme.[73,74]

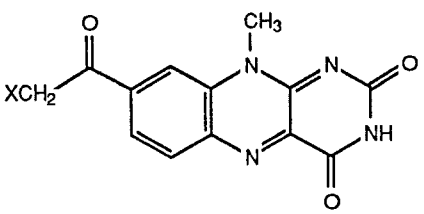
Slama *et al.*[75] have attempted to design catalysts upon the premise that a substrate can be transformed into a specific biomimetic catalyst. They have used the chemical alteration approach of modifying an existing enzyme at or near its binding site with a reactive coenzyme analogue. The work has centred on a series of flavopapains,[76-81] a family of semisynthetic enzymes in which reactive flavin analogues are attached to the active site cysteine-25 of the simple hydrolytic enzyme papain [E.C.3.4.4.10]. These semisynthetic enzymes have been found to be effective redox catalysts for the oxidation of 1-alkyl-1,4-dihydronicotinamides, according to equation (1.7).



1.7

Alkylation of the active site cysteine-25 of papain with the flavin analogue 7α-(bromoacetyl)-10-methylisoalloxazine (**10**) has been demonstrated to yield a flavopapain (**11**) which is an effective oxidoreductase, which catalyses the oxidation of 1-alkyl-dihydronicotinamides. The reaction rates are up to 20 times greater than the rates of oxidation of the same substrates using the unsubstituted flavin (**12**), Figure 1.6. The second order rate constant for the dehydronicotinamide, NADH, with the unsubstituted flavin (**12**) was 13 $M^{-1}s^{-1}$.



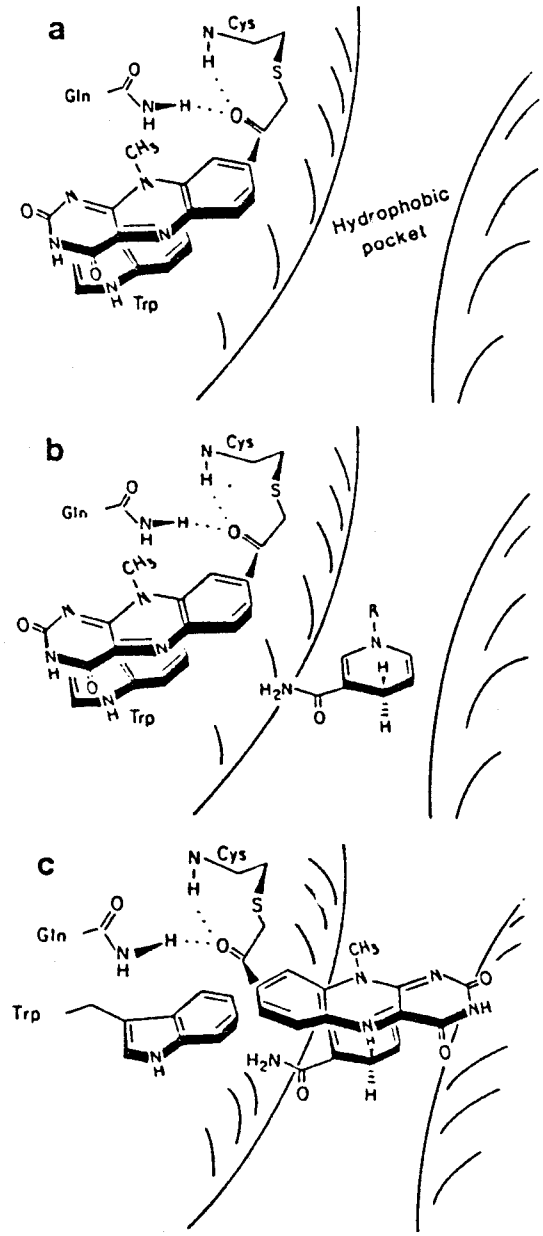


10 *X = Br*
11 *X = papain Cys-25-S*
12 *X = H*

13 *X = Br*
14 *X = papain Cys-25-S*
15 *X = H*

Figure 1.6

Papain has been successfully modified with a series of isoalloxazines which are related as geometric isomers. The resultant flavopapains show widely different rate enhancements for catalysis of the oxidation of 1-benzyl-1,4- dihydronicotinamide by oxygen.[75] The differences in the efficacy of the flavopapains is best explained in terms of the differing geometries in the active site of the semisynthetic enzymes. The activity of the semisynthetic enzymes within this series lends support to the hypothesis that proper positioning of the flavin in the active site is crucial for the production of an effective semi-synthetic active site, scheme 1.4. It could be that hydrogen bonding of the flavin acetyl group to residues in the protein backbone is an important contribution to the correct orientation of the flavin.



(a) Active site of the semisynthetic enzyme (**11**). *The acetyl side chain of the flavin moiety is hydrogen bonded to the Gln-19 and Cys-25 backbone of the polypeptide. The flavin is participating in a charge-transfer complex with Trp-26. (b) Michaelis complex. The dihydronicotinamide is embedded within the hydrophobic groove of the flavoenzyme. (c) ES ' intermediate. The flavin-Trp-26 charge-transfer complex has been disrupted and the flavin now lies directly over the nicotinamide substrate. The pro-R hydrogen is shown as the species being transferred to the N(5) position of the flavin. This corresponds to the same transfer preference found for the oxidation of labelled NADH by flavopapain* (**14**).

Scheme 1.4

1.6 Electrochemical Sensors

Biosensor design and technology is a developing area in the quest for innovative approaches to molecular detection and analysis. Sensors offer the possibility of real-time measurements during analysis, which is particularly important for the rapid measurement of body analytes. The largest potential markets for biosensors currently are the clinical and health care fields.

A biosensor is a device which converts a biological recognition process into an electrical signal, the amplitude of which is related to the concentration of analyte in the solution. A biosensor may be considered as a multi-component system, where the complete sensor requires the solution and the union of the individual subunits. Biosensors have two principal components: a molecular recognition element (the biological component) and a transducing or signal-generating element (the instrumental component). Because the biologically sensitive material is responsible for recognising the analyte, it also regulates, to a large extent, the specificity and sensitivity of the device. The purpose of the transducing element is to convert the biochemical signal into an electronic signal that can be suitably processed and outputted. The transducer can take many forms, but the emphasis to date has been on the following electronic configurations: optoelectronic detectors, field-effect transistors, potentiometric or amperometric electrodes and thermistors. Many aspects of biosensors based on these different transducing systems have been reviewed in the literature.[82-89] Much of the art of biosensor research involves optimising the union of these components and creating new designs for use as biosensors.

The intimate contact between the biological component and the transducer facilitates both a rapid response and high selectivity. The immobilisation of the biocatalyst to the transducer can be achieved in a number of ways:

(1) By covalently linking the biocatalyst to an insoluble matrix *via* a bidentate ligand.

(2) By occluding the biocatalyst within a polymer matrix such as polyacrylamide or agarose.

(3) By trapping the biocatalyst behind a semipermeable membrane such as nylon, cellophane or cellulose acetate.

(4) By covalently attaching the biocatalyst directly to the transducer. In this configuration not only is an intimate contact established but also diffusional constraints observed with polymer and membrane systems can sometimes be eliminated.

1.6.1 Amperometric Sensors

Amperometry involves maintaining the potential of the indicator electrode at a constant value that will reduce or oxidise a species of interest at the electrode surface, and measure the resulting current.[90] The magnitude of the current is proportional to the concentration of electroactive material present in the solution.

The Clark pO_2 electrode is perhaps the best known example of a conventional amperometric sensor. Oxygen diffuses through a gas permeable teflon membrane and is reduced to hydrogen peroxide at a platinum electrode kept at a fixed potential. Since many enzymes (oxidases and oxygenases) carry out their function with the net consumption of oxygen, this system has proved useful for the development of amperometric biosensors. Oxalate oxidase, for example, immobilised on a membrane proximate to an oxygen electrode, by glutaraldehyde cross linking, was developed to monitor oxalate concentrations in urine.[91]

The problem of modification of an electrode surface in order to attach the enzyme and make a direct measurement of enzyme activity, and ultimately achieve the reagentless biosensor, is now being approached from many directions. Direct electron transfer between the electrode and enzyme has yet to be reported with the desirable efficiency. In general, modification of graphite and gold electrodes have been reported most often, and a dual function requirement has been established for the

modifier. Interaction with the electrode surface must be through a group on the modifier which allows electron transfer, while the modifier must contain a group which will interact with the redox centre of the protein.[92,93] Quasi-reversible electron transfer for example has been reported for electrodes modified with (pyridinylmethylene) hydrazine carbothioamides,[94] while well behaved electrochemistry was observed for cytochrome *c* with pyrolytic graphite electrodes where the edge plane was exposed.[95]

1.6.2 Potentiometric Sensors

A potentiometric sensor utilises an ion specific membrane or sensing surface to generate a potential difference which is proportional to the logarithm of analyte concentration in the solution. Potentiometric sensors are based upon the measurement of a zero-current potential developed across a permiselective membrane, these conditions are used because of the poor conductivity of the membrane. The best known examples of these sensors are the ion selective electrodes (ISEs). Examples of these include the pH probe, pCO_2 and O_2 monitors and the K^+ electrode. A multi-composite membrane containing one or more immobilised enzymes is used in a potentiometric enzyme electrode. When placed in contact with a suitable substrate, the analyte, the membrane catalyses the conversion of the substrate into an electroactive species. In 1973, Nagy *et al.*[96] described an enzyme electrode for glucose based on the following sequence of reactions.

$$\text{glucose} + O_2 \rightleftharpoons \text{gluconic acid} + H_2O_2 \qquad 1.8$$

$$H_2O_2 + 2I^- + 2H^+ \rightleftharpoons I_2 + 2H_2O \qquad 1.9$$

Horse radish peroxidase was used in stage 1.9 to give high efficiency at low concentrations (10^{-4} M) of iodide. The enzyme catalyses the oxidation of I^- by the peroxide produced from the oxidation of glucose. This results in a depletion of I^- which can be monitored with an I^- ISE.

1.6.2.1 Pyrrole Electrodes

The development of a technique for the efficient capture of the biomolecule at the electrode surface has attracted the attention of a number of groups. The electrochemical polymerisation of pyrrole has appeared particularly suitable for this application.[97-99] The polymerisation occurs during the electrochemical oxidation of a solution containing pyrrole monomers. The generation of an extremely reactive radical cation that reacts with neighbouring pyrrole species produces a polymer that is predominantly α,α'-coupled, figure 1.7.

A linear relationship was established between the enzyme activity of the polymer film and the concentration of enzyme in the polymerisation solution. Glucose oxidase (GOD) is an example of an enzyme that has been employed in this immobilisation procedure. Like many other reductases, the overall reaction of GOD with its substrate, utilises the natural mediator, oxygen (1.10).

$$\beta\text{-glucose} + O_2 \xrightarrow[\text{oxidase}]{\text{glucose}} \beta\text{-gluconic acid} + H_2O_2 \qquad 1.10$$

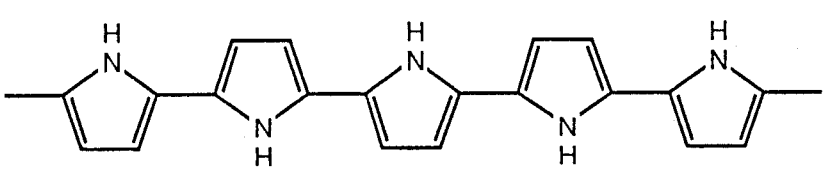


Figure 1.7

The direct electron transfer between electrode and enzyme is monitored by the secondary production of hydrogen peroxide. This is, however, far from ideal since it relies on a constant oxygen supply, and produces H_2O_2 which has been reported to lead to various undesirable side reactions.

1.6.2.2 Ferrocene Sensors

More promising as substitute mediators are those based on ferrocene, (bis(η5-cyclopentadienyl)iron, $Fecp_2$). Ferrocene is a transition metal π-arene complex which consists of an iron atom sandwiched between two cyclopentadienyl rings. Many substituted ferrocenes are available with different overall charges and a wide range of solubilities in various solvents. The formal potential of the ferrocene is responsive to the substituents on either or both of the cyclopentadienyl rings, but the electron transfer reactions retain their desirable characteristics of rapidity and reversibility. However, most significant in the context of biosensor design is the fact that they can be used to modify other molecules, including proteins, and retain the properties of a simple, one-electron redox couple. A glucose sensor has been successfully developed utilising the ferrocene mediator.[100]

A substituted ferrocene ($Fecp_2R$) mediates electron transfer between immobilised glucose oxidase and a graphite electrode. The system is not dependent on oxygen; the ferricinium ion replaces oxygen as the cofactor for glucose oxidase. Once reduced, the ferricinium ion can be regenerated at the electrode.

$$\text{Glucose} + GOD_{ox} \rightarrow \text{Gluconolactone} + GOD_{red} \qquad 1.11$$

$$GOD_{red} + 2Fecp_2R^+ \rightarrow GOD_{ox} + 2Fecp_2R + 2H^+ \qquad 1.12$$

$$2Fecp_2R \rightarrow 2Fecp_2R^+ + 2e^- \qquad 1.13$$

A linear response, proportional to glucose concentration, is observed over the range commonly found in diabetic blood samples (1 - 30 mM), and measurements can be made in plasma and whole blood.

A combined ferrocene/pyrrole electrode utilising N-substituted pyrrole monomers of the form shown in figure 1.8 have been prepared. Co-polymerised with unsubstituted pyrrole, in the presence of oxidoreductase, these amperometric devices show all the signs of realising the amperometric biosensor.[101] The ferrocene functionality acts here in its oxidised form, the ferrocinium ion, as an electron acceptor for the enzyme, transferring electrons to the electrode as shown, scheme 1.5 (a).

The mediator properties of ferrocene have been demonstrated where electron transfer in the opposite direction is required (b); for example in the enzymatic reduction of peroxide by peroxidase,[102,103] hydrogen peroxide can be detected at 10^{-8} M.

1.6.2.3 Flavin electrodes

There is considerable research interest in the development of electrodes based on oxidation-reduction enzymes.[104,105] Such enzymes consist of a cofactor plus an apoenzyme (protein component). The chemical modification of electrode surfaces with biological redox components provides a novel environment for fundamental[51,106] and applied[103,107,108] studies of biological processes. In particular, attachment of an oxidase enzyme or its cofactor FAD to an electrode surface is an attractive field of investigation.

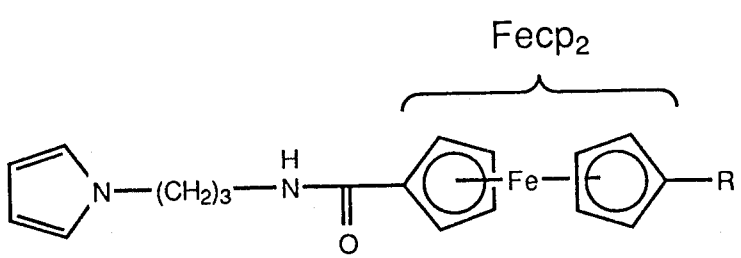
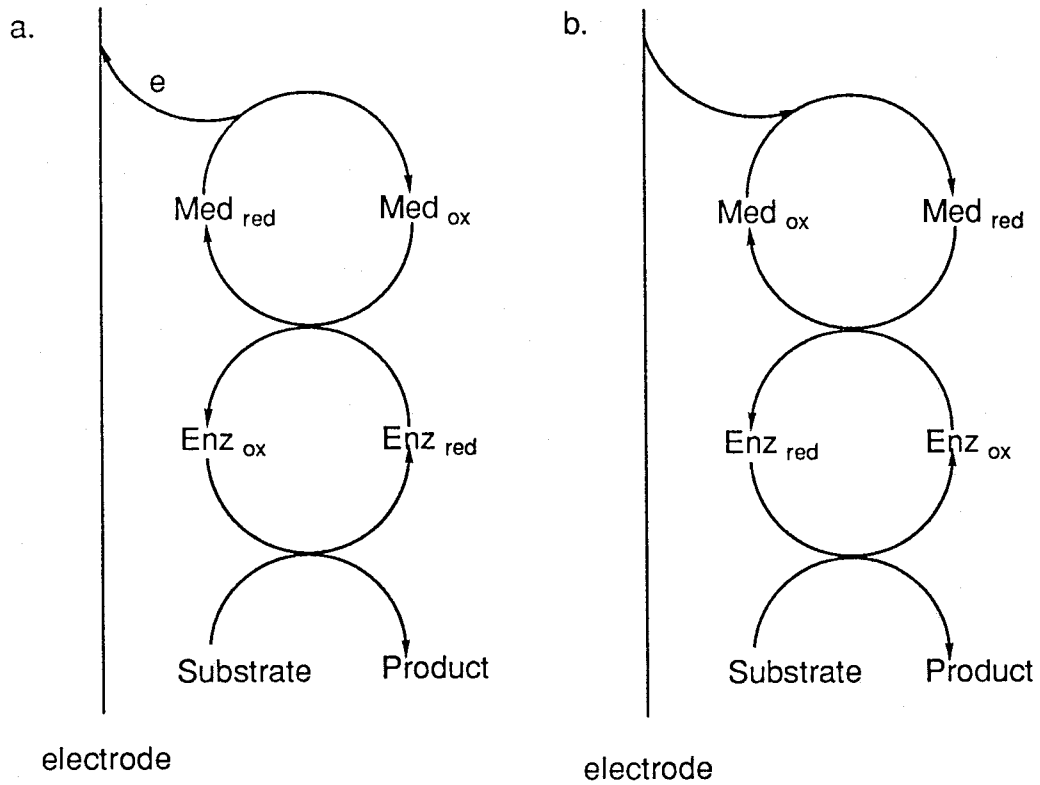


Figure 1.8



Scheme 1.5

Schemes for mediation of a redox enzyme by a low molecular redox mediator in an ampherometric sensor.

L.B Wingard Jr. has addressed the fundamental spatial aspects of FAD-apoenzyme association and of flavin electron transfer kinetics.[107,108,109] Of all the natural redox cofactors, only the flavin nucleotides have the unique ability to transfer one or two electrons and to promote the facile reduction of molecular oxygen. Wingard has developed structured enzyme-cofactor electrodes, where the relative spatial orientations of the cofactor, the electrode surface and the apoenzyme are clearly defined.[110] Covalent attachment of FAD to the electrode surface through the 8-methyl position of the isoalloxazine ring was attempted, since several natural flavoenzymes have been found in which FAD is covalently attached to the apoenzyme *via* histidyl or imidazoyl functions through the 8-methyl group.[111]

It was believed that position 8 must be close to the protein surface at least in these natural cofactor-attached enzymes. The attachment through position 8 would allow π-orbital delocalisation with the N(1)/N(5) redox centre, so that attachment of FAD to an electrode at position 8 through electron delocalising functions, such as a double bond, could result in convenient electron transfer between the FAD isoalloxazine redox centre and the electrode surface (Figure 1.9).

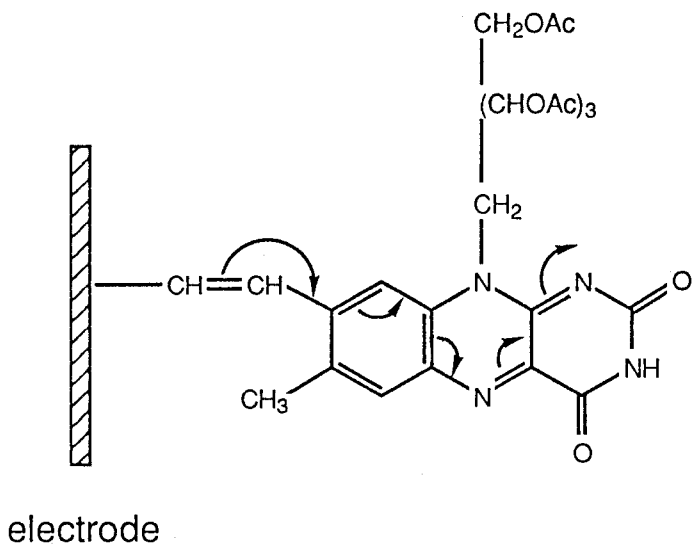


Figure 1.9

Proposed structure of immobilised tetraacetylriboflavin.

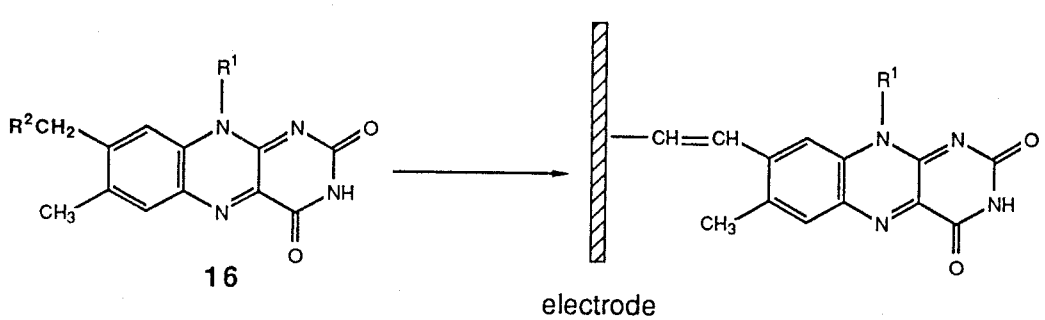
CHAPTER 2

Approaches Towards Synthetic Bifunctional Flavins

Approaches towards synthetic bifunctionalised flavins

The most recent and most encouraging results in flavin electrode development have come from Wingard and his group.[109,110] Their approach has been to covalently attach a flavin nucleus to an activated electrode *via* a single point of attachment, such that the isoalloxazine nucleus lies perpendicular to the electrode surface. The points of attachment have varied from the 8-methyl group of tetraacetylriboflavin, through to the adenine moiety of FAD,[109,110] though the attention has been directed towards the 8 methyl group attachment.

Condensation of the tetraacetylriboflavin organo lithium intermediate (**16**) with aldehyde derivatised glassy carbon (GC) electrodes led to the coupling of the isoalloxazine ring system to the GC electrode, scheme 2.1



$R^1 = CH_2(CHOAc)_3CH_2OAc$
$R^2 = BrLi$

Scheme 2.1

Voltammetric measurements were carried out to determine whether the isoalloxazine was indeed bound to the GC electrode as predicted[109,110]. The detailed results will be discussed fully in a later chapter, however, the voltammograms recorded for these systems were not encouraging, see Figure 2.1.

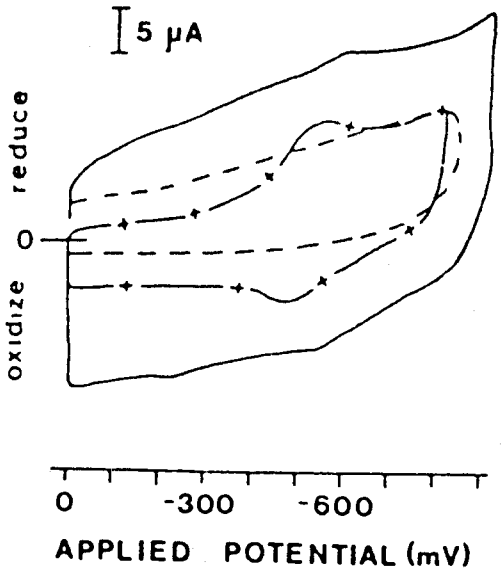


Figure 2.1

Cyclic voltammograms of GC electrodes. Data obtained under anaerobic conditions. Potentials vs Ag/AgCl. Untreated control GC (— — —), GC with attached riboflavin tetraacetate (———), and in solution (—— + ——).

Any conclusions drawn from these results must be treated with scepticism, and the results may indicate that the proposed method of electron transfer may be inaccurate.

2.1 Design strategy

Glutathione reductase catalyses the transfer of electrons from NADPH to the peptide disulphide, oxidised glutathione (GS-GS). The X-ray crystal structure has been determined at ~2 Å by Schulz and co-workers[35,36,112] and the mechanism, derived from chemical and X-ray studies from this and related flavoproteins have been summarised by Gani in a recent review.[41] The single striking feature of the tertiary protein structure is that the reductant (NADPH) and the electron acceptor (GS-GS), sandwich the flavin and that the spent species are replaced by NADPH and GS-GS on both sides of the prosthetic group. We have now analysed the Brookhaven structure in detail using Macromodel[113] on the Department's Evans and Sutherland PS390, see figure 2.2. It occurred to us that the geometry of this arrangement should provide an efficient pathway for electron transfer and

that one of the species could be replaced by an electrode to test this idea. In view of the poor electron efficiencies reported by Wingard[114,115] for glassy carbon-flavin electrodes in which the flavin is attached at only one position and thus could be in a sterically unfavourably position with respect to the electrode surface, we set out to develop and study parallel-face flavin electrodes in which the interfacial distance could be fine tuned.

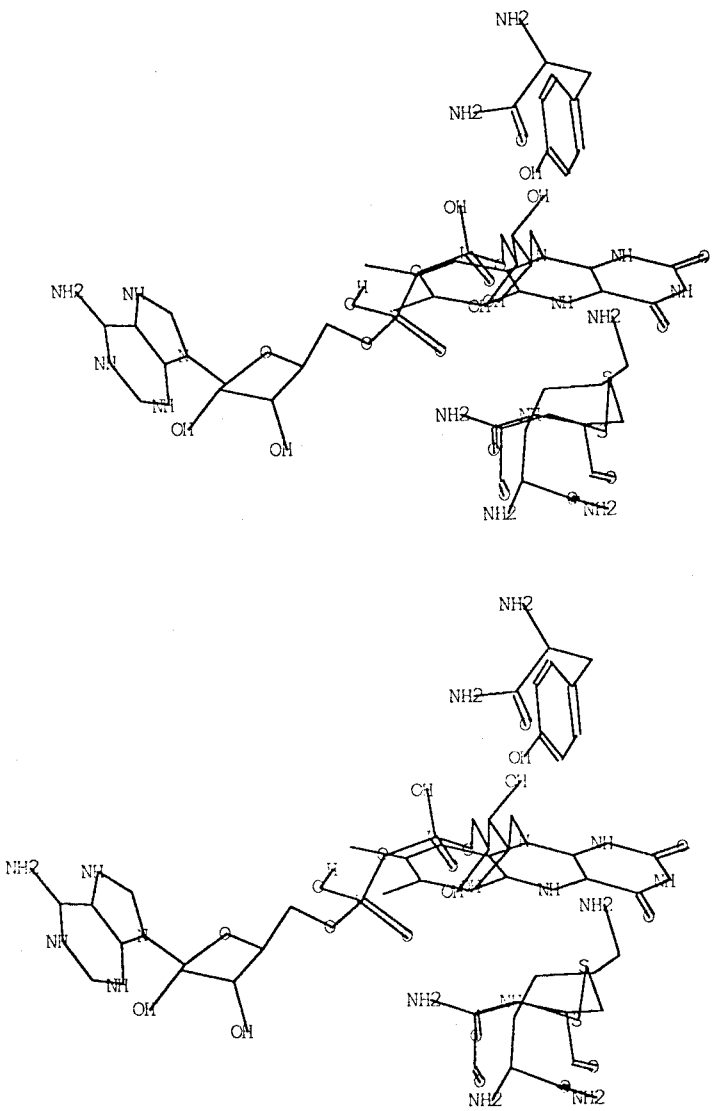


Figure 2.2

Macromodel[113] *representation of the active site structure of glutathione reductase, showing the flavin redox centre.*

This le d us to design our flavin electrodes in such a way that the flavin geometry would be similar to that within glutathione reductase and other flavin-based enzyme systems: the electrode surface taking the place of the substrate (Figure 2.3).

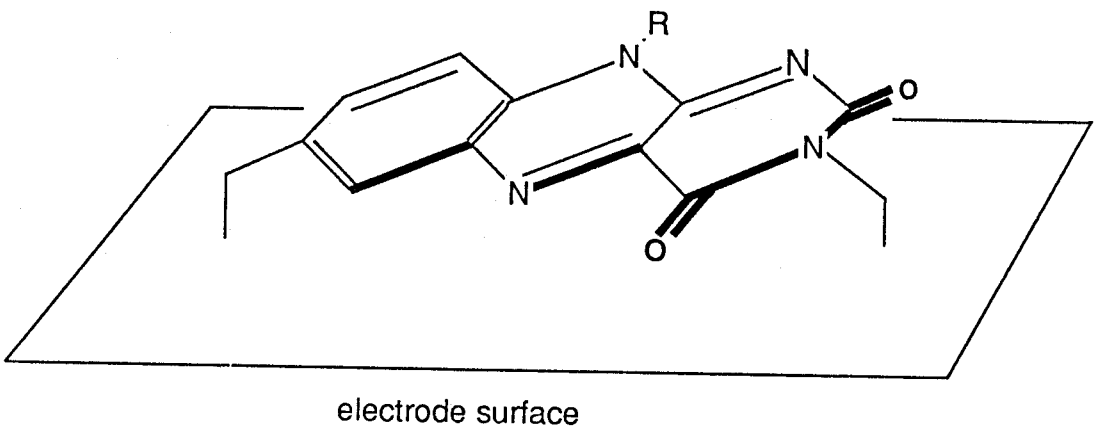


Figure 2.3

Although dioxygen is the physiological electron acceptor for oxidases such as glucose oxidase, in the majority of cases, it can be replaced by an electron transfer mediator. In this context, a mediator is a low molecular weight redox couple which shuttles electrons from the redox centre of the enzyme to the surface of the indicator electrode. During the catalytic cycle, the mediator reacts first with the reduced enzyme and then diffuses to the electrode surface where it undergoes rapid charge transfer.

Enzymes involved in the oxidation and reduction of biological molecules (oxidoreductases) often contain a flavin at their active site in conjuction with $NAD(P)^+$ as the redox active cofactor. Attempts have been made to exploit the direct electrochemistry between an enzymes' redox centre and a naked electrode, with the efficient recycling of reduced cofactor.

The essential element in making a successful biosensor utilising the biological cofactor $NAD(P)^+$, is the provision of a suitable electrocatalytic surface which can reoxidise coenzymes, both efficiently and in the correct biological form, i.e., a form that will be recognised by the enzyme. A large overvoltage is necessary to regenerate $NAD(P)^+$ from NAD(P)H at naked electrodes, though some fouling of the electrode surface has been found to

occur. Attempts to overcome these problems has led groups to develop suitably modified surfaces based on species such as catechols, hydroquinones and redox dyes.

It is our belief that the development of bifunctionalised flavin modified electrodes should allow fast oxidation of the redox cofactor $NAD(P)^+$. This is because the mode of regeneration mimics that to be found in biological systems, for example, within the enzyme glutathione reductase. The proposed sequence of reactions occurring at the electrode can be summarised as in figure 2.4.

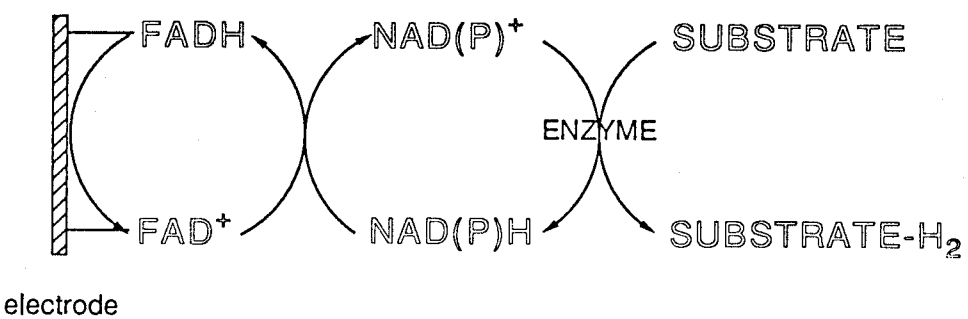


Figure 2.4

Co-planarity of the flavin nucleus and electrode surface may be achieved by binding of the flavin nucleus to the electrode surface at two or three positions. Key positions for such attachment would be N(3), C(7) or C(8) and N(10). With the two point mode of attachment at N(3) and C(7) or C(8), the flavin orientation at the electrode surface may be influenced by the surface concentration. At low flavin surface concentrations, the flavin would orientate itself with the expected co-planarity to the electrode surface. This would be due to the π-orbital interaction between the flavin aromatic ring and the electrode surface. At higher flavin surface concentrations the flavin may lie perpendicular to the electrode surface, probably with the C(6), N(5) and C(4) side of the isoalloxazine moiety in contact with the electrode surface, similar to the vertical orientation assigned to adsorbed nucleosides.[116,117] This suggestion is also supported by the large surface coverage obtained in this orientation, as the π-electrons of adjacent rings can overlap, leading to strong lateral

interactions within the flavin layer. Soriaga and Hubbard[118] have shown that the packing density, orientation, and mode of attachment of molecules adsorbed on smooth platinum electrodes in solution are dependent on the solute concentration.

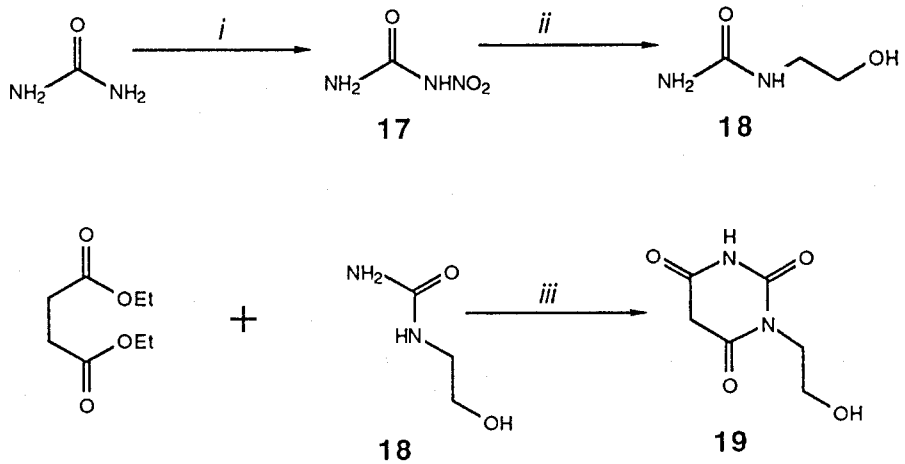
By attachment of the flavin nucleus to an electrode surface at three points, the number of possible orientations for the flavin nucleus would be reduced. The flavin nucleus would be prevented from orientating itself perpendicular to the electrode, due to the extra point of attachment at N(10). No concentration dependence would be observed as the adsorption of the flavin would be irreversibly bound to the electrode in a co-planar manner.

2.2 Synthesis of Flavin Precursors

The synthesis of riboflavin and other flavin analogues are well documented in the literature.[23,54,119-125] Of those where a side chain is attached, the attachment has taken place subsequent to flavin condensation.[125,128] There is no evidence to suggest that functionalisation prior to coupling cannot be achieved. This prior functionalisation would be advantageous as it would overcome the solubility problems that are encountered with flavins, which hamper all practical manipulations. Furthermore, independent modification of each side chain would be possible.

2.2.1 Barbituric acid derivatives

An N-substituted barbiturate (**19**) was synthesised by the procedure outlined, scheme 2.2. Nitrourea (**17**) was formed from urea by the method of Weakley,[126] which on treatment with hydroxylamine gave N-(2-hydroxyethyl) urea (**18**) in 47% overall yield. Coupling of this with diethylmalonate gave N-(2-hydroxyethyl)-barbituric acid (**19**) in 14% yield.

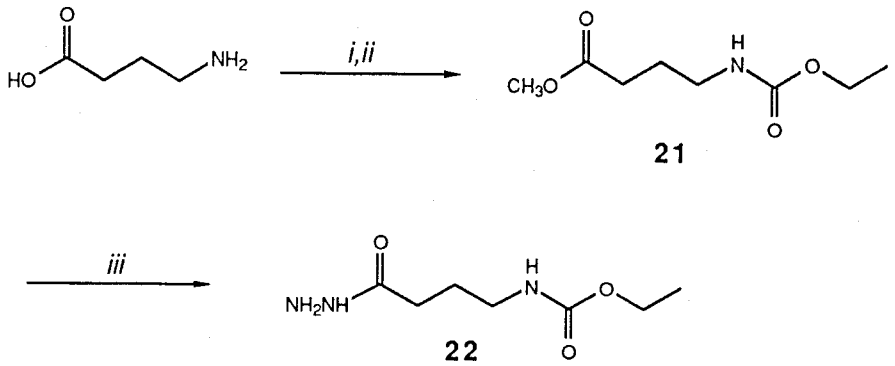


(i) HNO_3/H_2SO_4
(ii) H_2N⌒⌒OH
(iii) *EtONa, EtOH*

Scheme 2.2

However, all attempts to oxidise the barbiturate (**19**) to the corresponding alloxan derivative failed. The literature precedent suggests chromium trioxide to be the method of choice,[127,128] but in our hands the reaction did not prove satisfactory.

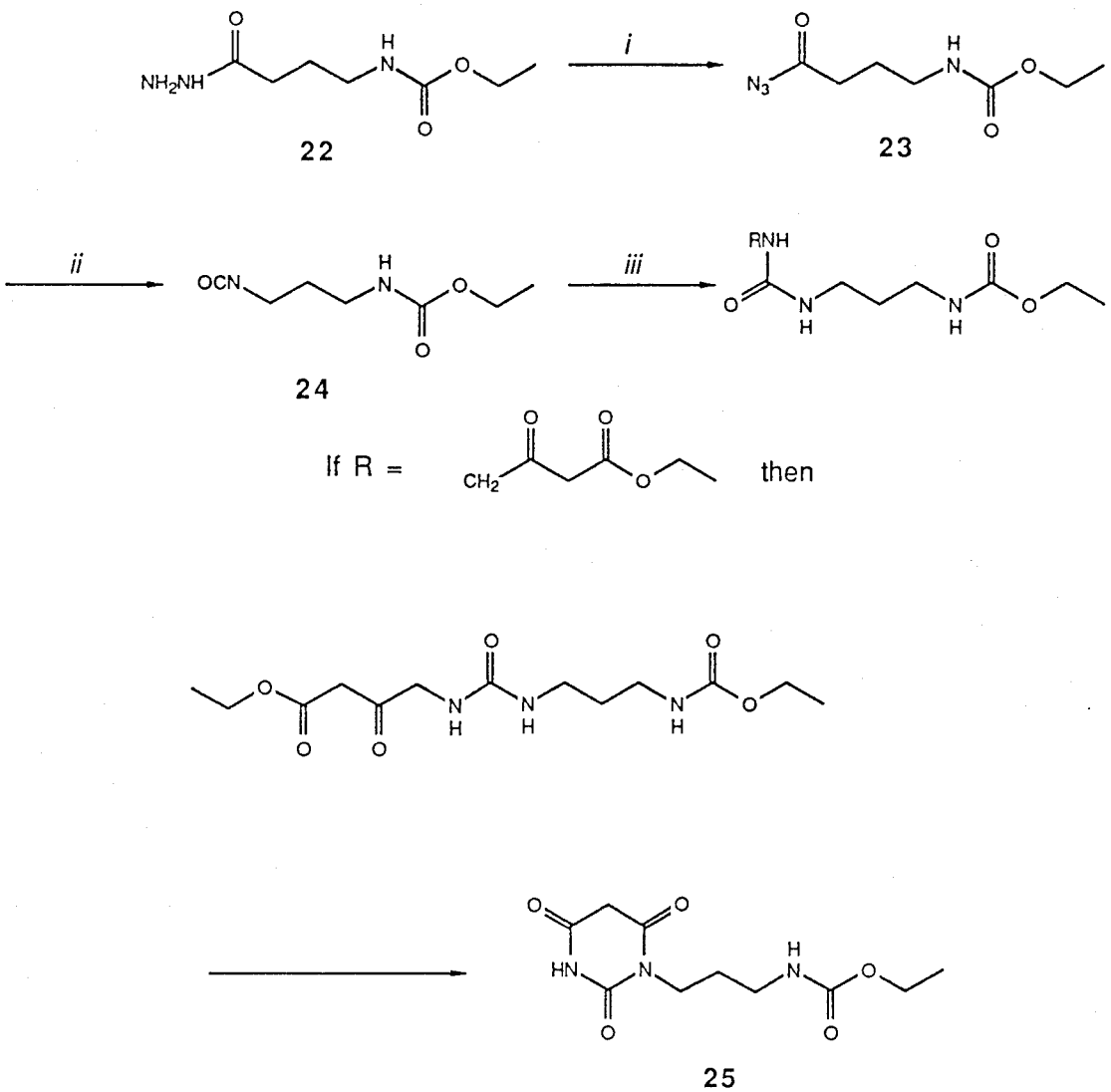
An alternative procedure, which would require the internal cyclisation of a suitably functionalised urea to give a mono-substituted barbiturate, looked to be more promising (scheme 2.3). However, this proved to be experimentally more difficult to achieve. γ-Aminobutyric acid (GABA) was esterified using thionyl chloride in methanol. Carbamate protection of the amino ester in THF was followed by formation of the acyl hydrazide (**22**), by refluxing the carbamate (**21**) with hydrazine hydrate in methanol for 1h. An overall yield of 33% was achieved for the three steps, scheme 2.3.



(i) $SOCl_2$, *MeOH*
(ii) C_2H_5OCOCl, Et_3N, *THF*
(iii) $NH_2NH_2 . H_2O$, *MeOH*

Scheme 2.3

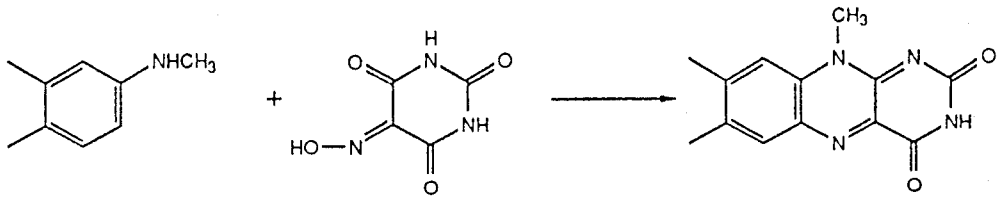
It was envisaged that treatment of the low melting acyl hydrazide (**22**) with nitrous acid would give the acyl azide (**23**). However all attempts towards the synthesis of this azide were unsuccessful. Scheme 2.4 shows the intended route. It was expected that the azide (**23**) would give the isocyanate (**24**), *via* a Curtius rearrangement, on heating. Treatment of this isocyanate with a disubstituted amine, would lead to an internal cyclisation to give the barbituric acid (**25**).



(i) HNO_2
(ii) Δ
(iii) RNH_2

Scheme 2.4

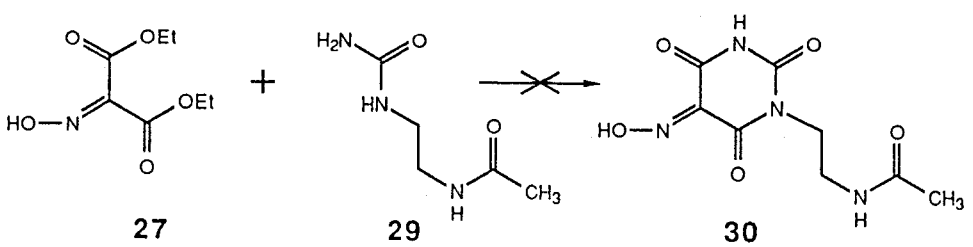
Other literature attempts towards the synthesis of various flavins have been successful.[57,129] In these methods viroluric acid (**26**), an oxime derivative of barbituric acid, has been employed. A generalised case of flavin synthesis by this method is shown in scheme 2.5.



26

Scheme 2.5

We planned to utilise this procedure to synthesize the disubstituted violurate (**30**) through the condensation of the N-mono-substituted urea (**29**) and diethyl-*iso*nitroso-malonate (**27**), scheme 2.6. However, the synthesis was unsuccessful and we were thus unable to attempt the condensation of the substituted violurate with an aromatic amine.

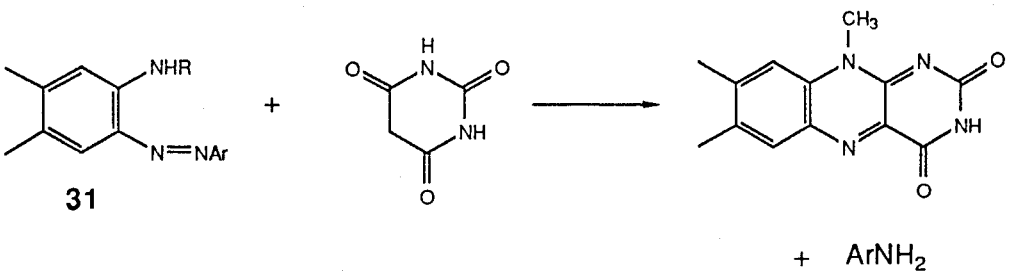


27 **29** **30**

Scheme 2.6

2.2.2 Substituted amines

In tandem with the substituted barbituric acid studies, the synthesis of the riboflavin nucleus was attempted where the future N(5) group of the flavin nucleus was attached to the amine component. The synthesis consisted of the reaction between an appropriate 2-aminoazo compound, and barbituric acid,[122,130] scheme 2.7.

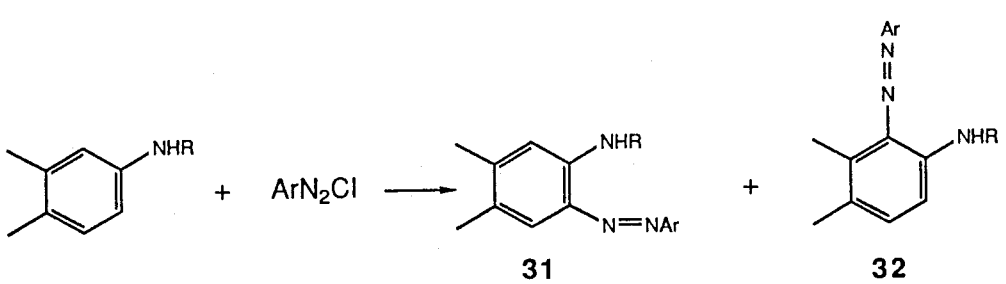


R = tetraacetyl-D-ribityl

Scheme 2.7

Tishler *et al.*[122] found that the 2-phenylenediamine required in the older, established procedures was difficult to prepare and was unstable in contrast to the corresponding 2-aminoazo compounds. Treatment of 1-(D-ribitylamino)-3,4-dimethylbenzene with benzenediazonium chloride gave 1-tetraacetyl-D-ribityl-2-phenylazo-4,5-dimethylbenzene (**31**). However the product was not pure 2-azo compound, but contained small amounts of the 6-azo compound (**32**), scheme 2.8.

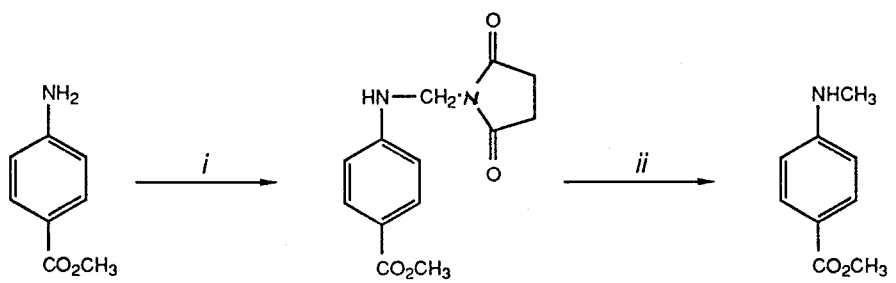
It is noteworthy that the 6-azo compounds do not react with barbituric acid under the same conditions in which the isomeric azo compounds form riboflavin, namely refluxing in a mixture of acetic acid and dioxane for 5h.



R = tetraacetyl-D-ribityl

Scheme 2.8

Instead of the 4,5 dimethylamine precusor, as shown in scheme 2.8, our synthetic strategy relied upon the presence of more suitable functionality at the 4-position. The 4-methyl group was replaced by an electron withdrawing carboxylic acid group. Accordingly, the 4-aminobenzoic acid was esterified using thionyl chloride in methanol. The amino group of the product was mono-methylated through treatment with succinimide and then borohydride,[131] to give 4-N-methylamino methyl benzoate (**35**), scheme 2.9. Purity of the methyl amino ester (**35**) was indicated by the good correlation of the melting point with the literature value.

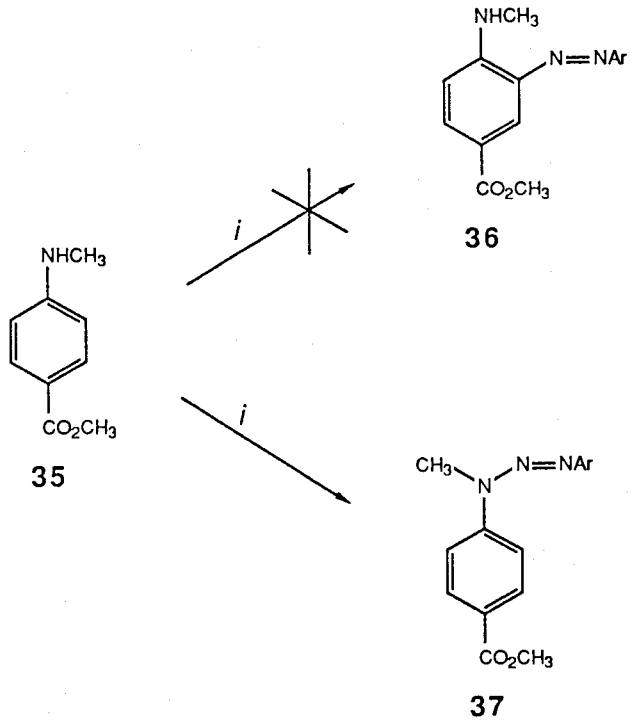


33 **34** **35**

(i) succinimide, HCHO, EtOH
(ii) $NaBH_4$/DMSO

Scheme 2.9

Treatment of the 4-N-methylamino benzoate (**35**) with diazotised aniline at 0°C did not give the expected azo compound (**36**). Apparently, deactivation of the aromatic ring by the electron withdrawing function, coupled to the accessibility of the nitrogen lone pair, led to diazotisation of the secondary amine to give the triazine (**37**), scheme 2.10. The high field proton nmr spectrum showed a shift, for the NCH_3 group from 2.88 to 3.90 ppm andfrom a doublet to a singlet for the triazine (**37**).



(i) Ar N_2^+

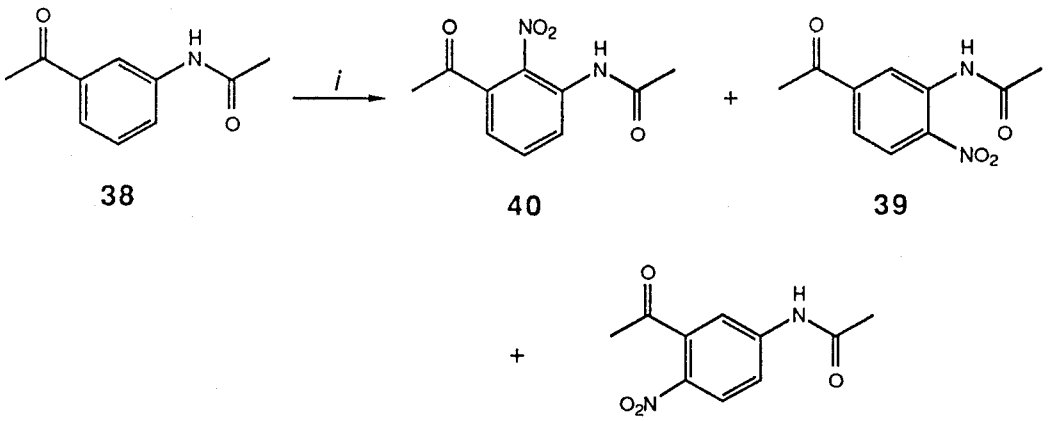
Scheme 2.10

2.3 Acetyl Flavins

In view of the poor results obtained for the synthesis of substituted barbiturates, we decided to concentrate on the synthesis of flavins from which we could then form the side chains necessary for bifunctionalisation.

2.3.1 8-Acetyl-10-methyl Flavins

Kaiser *et al.* in their important work towards the development of semi-synthetic enzymes synthesised a number of 6-,7- and 8-functionalised flavins.[71,75] The 7- and 8-acetyl-10- methyl isoalloxazines reported, appeared to be most suitable for our requirements. Our attempts to repeat the synthesis of the 8-acetyl flavin isomer were unsatisfactory. In particular, nitration of 3-acetamido-acetophenone (**38**) le d to purification of 4-nitro-3-acetamido-acetophenone (**39**) in a very low yield. It was found from nmr data, through coupling of the aromatic protons, that the major product was the 2-nitro isomer, scheme 2.11.



38 40 39

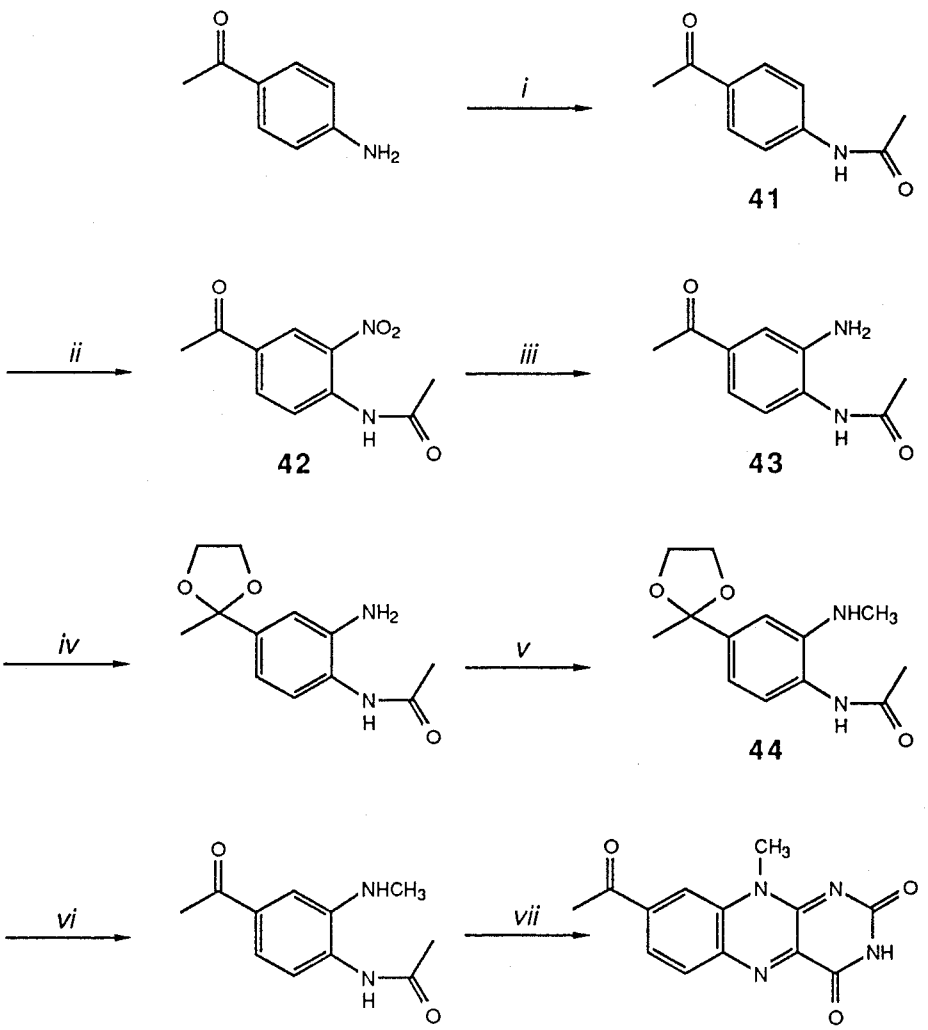
(i) HNO_3/Ac_2O

Scheme 2.11

This finding was in accordance with the observation that if a *meta* directing group is *meta* to an *ortho/para* directing group, then the incoming group goes *ortho* to the *meta*- directing, rather than *para*.[132] Kaiser in his paper[133] quotes that the method of Waters[134] was used. However, Waters, in agreement with our findings, states that the 4-nitro isomer (**39**) was a minor product of the nitration, with the 2-nitro isomer as the major product. No yields were reported for the 4-nitro isomer.[133,134]

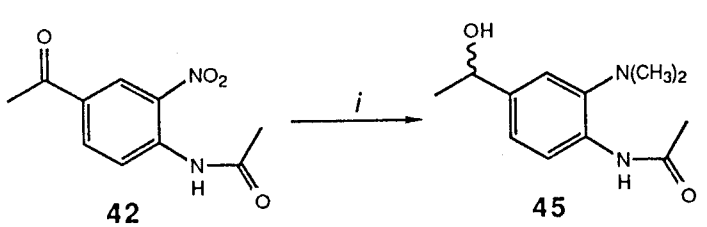
This observation made the synthesis of 8-acetyl-10- methyliso-alloxazine, by this route, uneconomical both in terms of yield and cost of starting materials. As the majority of flavin coenzymes are attached covalently to the active site of flavoenzymes are bonded through the 8-position, it was desirable to synthesise flavin derivatives which were functionalised through this position. The strategy we planned to pursue is detailed in scheme 2.12.

A number of problems were encountered using this method, such as the inability to protect the acetyl function of the 3-amino-4-acetamido-acetophenone (**43**) prior to formation of the mono-methyl amine (**44**) by treatment with acetic-formic acid and borohydride. The method of Emerson and Mohrman[135] enabled us to overcome this problem, by the formation of a monomethyl moiety from a nitro group. This procedure involved the reduction of the nitro acetamido-acetophenone (**42**) under a palladium catalysed hydrogen atmosphere, in the presence of formaldehyde, scheme 2.13.



(i) Ac_2O, (ii) HNO_3/H_2SO_4, (iii) H_2/Pd,C, (iv) OH⌒OH,H^+, (v) CH_3CO_2COH, $NaBH_4$/DMSO, (vi) HCl, (vii) alloxan, HCl, EtOH

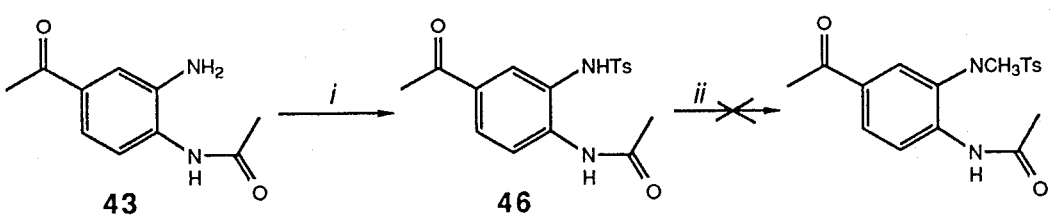
Scheme 2.12



(1) HCHO, H_2/Pd, C

Scheme 2.13

As can be seen from scheme 2.13, the method of Emerson and Mohrman led to the reduction of the acetyl function as well as the formation of a dimethyl amine group at the 3-position (**45**). Evidence for this comes from accurate mass spectral analysis and the relative integration for six protons at 2.65 ppm, in the nmr proton spectrum. We attempted to overcome the formation of the dimethyl species by utilising the method of Shinkai *et al.*[136] Mono-methylated amines were formed through the treatment of the amine to be methylated with tosyl chloride, sodium hydroxide and dimethylsulphate. Application of this procedure to the amine (**43**) did lead to the formation of the mono-tosyl derivative (**46**). nmr analysis in DMSO showed two NH peaks in the spectrum at 9.30 and 9.52, indicative of only mono-tosyl formation. However, this could not be methylated with dimethylsulphate, scheme 2.14.

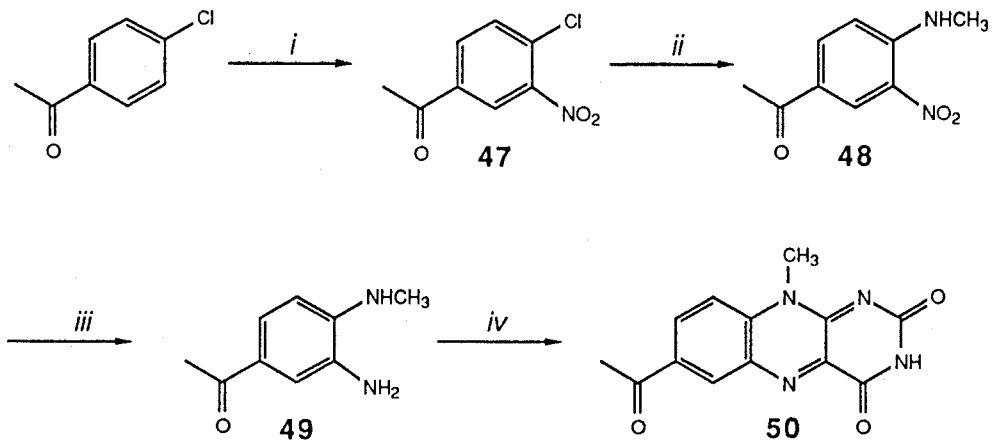


(i) TsCl, EtOAc, Et_3N
(ii) NaOH, $(CH_3)_2SO_4$

Scheme 2.14

2.3.2 7-Acetyl flavins

Parallel studies towards the synthesis of the isomeric 7-acetyl isoalloxazine were successful. This led to the curtailment of the 8-acetyl functionalization studies. The method of Levine and Kaiser[71] was followed for the formation of 7-acetyl isoalloxazine (**50**) from 4-chloro-aceto-phenone.The synthesis was achieved in four steps with a 44% overall yield. The synthetic route is detailed in scheme 2.15.

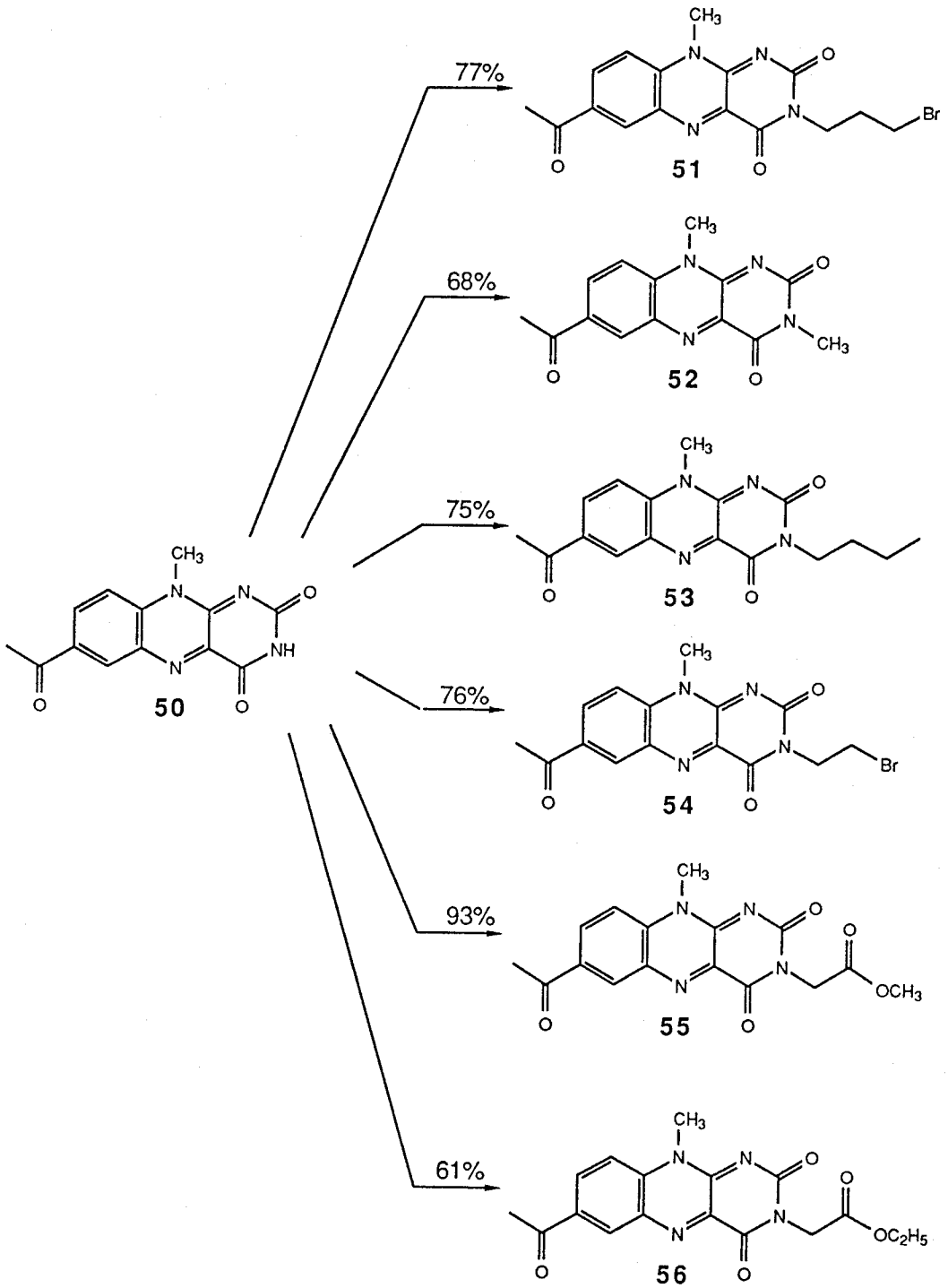


(i) HNO_3, H_2SO_4
(ii) CH_3NH_2/H_2O
(iii) H_2/Pd, *C*
(iv) EtOH, alloxan, HCl

Scheme 2.15

2.4.3 3- and 7-position functionalisation

The method of Kraus[137] was used initially to introduce functionality into the N-3 position of the 7-acetyl 10-methylisoalloxazine (**50**). The isoalloxazine was treated with anhydrous potassium carbonate and 1,3-dibromopropane and the 3-bromopropyl derivative (**51**) was isolated as yellow crystals in 44% yield after an aqueous wash and flash silica chromatography (CH_2Cl_2 / 5% MeOH). Higher yields were obtained with bases other than potassium carbonate, however the reactions with these bases were invariably more time consuming and elaborate. Treatment of the isoalloxazine with 1.1 equivalents of lithium di-*iso*propylamide at -50°C followed by 10 equivalents of 1,3-dibromopropane yielded 77% of the 3-alkylated product (**51**) after purification. A number of other 3-alkylated 7-acetyl isoalloxazines were synthesized (**52-56**) using this method with yields ranging from 61 to 93% to determine the utility of the method, scheme 2.16.



Scheme 2.16

Kaiser[71] in his methodology towards synthetic flavopapains prepared 7-acetylisoalloxazine for nucleophilic attack by forming the 7-bromo derivative through the action of bromine in glacial acetic acid. Evidence for the reaction was limited to an observed shift in the high field nmr spectrum. The spectrum showed new absorption peaks at δ 4.78 ($-CH_2Br$) a shift from 3.03, and 8.17, 8.93 and 9.27 (aromatic H) when compared to the parent compound; no yield was reported. In our hands, Kaiser's procedure formed the 7 α-bromo-10-methylisoalloxazine (**57**) in 67% yield. Purity, determined by nmr, was 95% by comparison of the bromomethyl peak at 4.78 and the parent acetyl peak at 3.01 ppm. Utilizing the method of McCormick[138] we were able to increase the yield to 84%. This method involved treating the 7-acetyl isoalloxazine with N-bromosuccinimide in trifluoroacetic acid, with a trace of benzoyl peroxide as a catalyst.

By applying this method of bromination to 7-acetyl-3-N-(3-bromo-propyl) -10-N-methylisoalloxazine (**58**) we were able to form a flavin species that was suitable for nucleophilic displacement. We achieved the synthesis of the dibrominated species (**58**) in an overall yield of 14% from 4-chloroacetophenone. Again there was a shift in the nmr spectrum. The 7-acetyl peak at 2.73 ppm shifted to 4.49 ppm on bromination. Further evidence was provided for the formation of the dibrominated species from elemental analysis and mass spectral data. To determine whether the dibrominated flavin would be suitable for attachment to modified electrode surfaces, we reacted the two mono-bromo flavins (**57**) and (**51**), and the di-brominated flavin (**58**) with thiophenol. The brominated species were treated with sodium thiophenolate in DMF at room temperature for 12h. Yields of less than 10% were found for the three thiol flavins (**59**), (**60**) and (**61**), scheme 2.17. Possible causes for the low recovery of products were lack of solubility, and light and air sensitivity of the products. Indications of thio ether formation were from high field nmr spectra. On formation of the thio ether from the 3(N)-(3-bromopropyl) side chain, the CH_2 signals at 3.50 and 2.35 ppm were shifted to 3.02 and 2.08 ppm respectively. There was a smaller shift for the 7-β-thio ether formation. A shift from 4.5 to 4.3 ppm was generally observed, however, this was compound dependent.

The coupling of flavins with thiol-activated polypyrroles[139-141] figure 2.5, and flavin modified gold electrodes with thiol linkages are discussed in chapter 3.

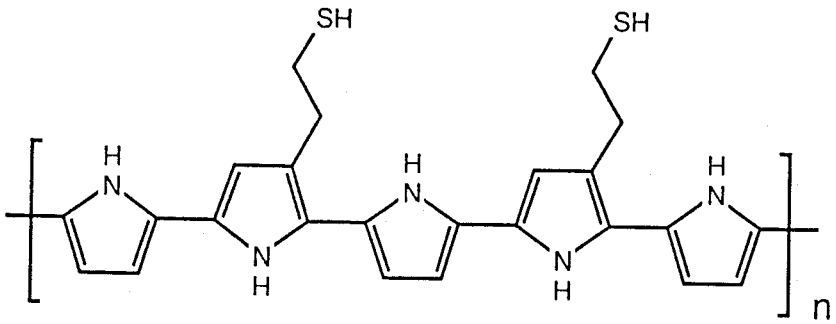
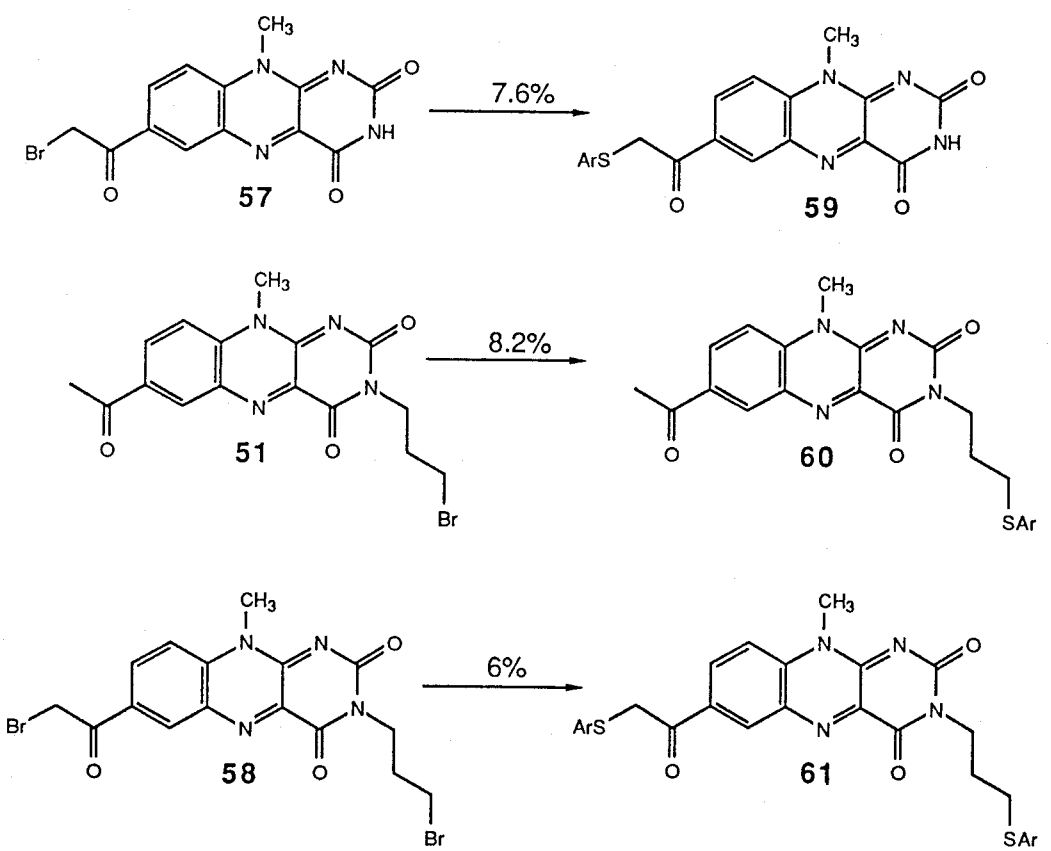


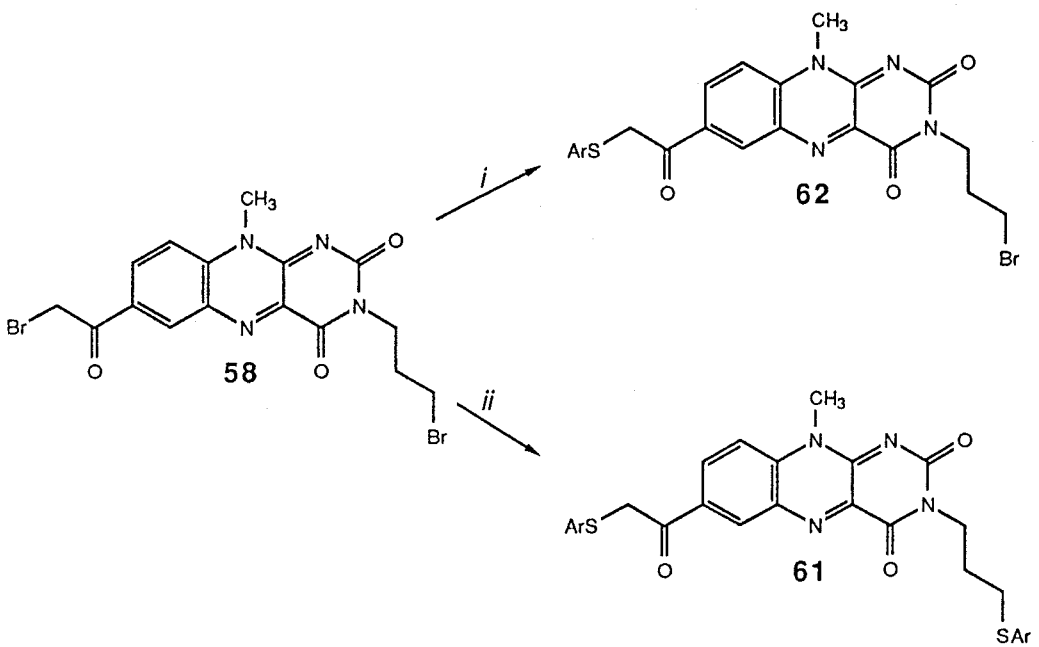
Figure 2.5 A thiol activated polypyrrole electrode surface



Scheme 2.17

Reaction of the brominated isoalloxazines with sodium thiophenolate

It is worth noting that treatment of the dibrominated flavin (**58**) with slightly more than one equivalent of sodium thiophenolate formed the mono thiophenoxy derivative (**62**) where the 7-position was the point of reaction, whilst an excess gave the dithiophenoxy derivative (**61**), scheme 2.18.



(i) 1.1 Ξ NaSAr, DMF
(ii) 2.2 Ξ NaSAr, DMF

Scheme 2.18

The low yields obtained indicated that the dibrominated flavins would not be suitable for attachment to thiol-activated polypyrrole electrodes under the conditions used. Attempts to improve the yields by using different conditions met with little success. One of the major problems, and this was echoed throughout the flavin work, was solubility. Flavins are well known to be highly insoluble in the majority of organic solvents, the exceptions being DMF, DMSO and trifluoroacetic acid. Priority was therefore given to synthesize more soluble flavins.

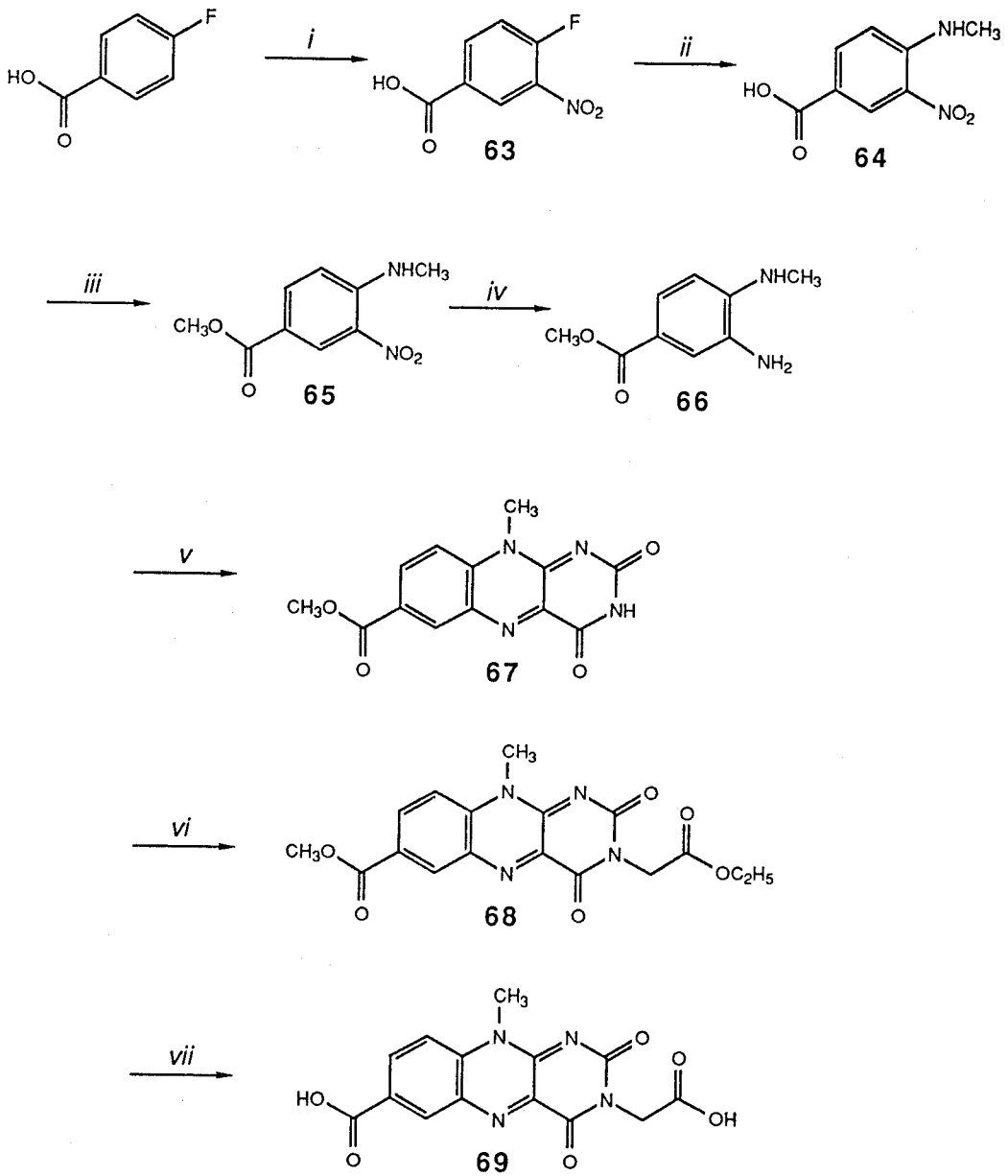
2.4 Diacid flavins

Previous work with the 7-acetyl flavins enabled us to alkylate the N(3) position with ethyl bromo acetate (**56**). This led us to attempt the synthesis of flavin species which had an ester function at the 7-position, and to form the ester at the flavin N(3) position from this. It was envisaged that by having similar functionality at the 3 and 7 positions this would enable us to carry the functionality further to give a bifunctionalised flavin species. It was also envisaged that if any solubility problems were encountered, the 10-(N) methyl group could then be replaced by groups that would help increase the solubility of the isoalloxazine.

2.4.1 10-Methylisoalloxazines

The synthetic strategy to formation of the 7-methylester-10(N)- methyl isoalloxazine (**67**) was similar to that for the 7-acetyl flavins. The five step synthesis, starting from 4-fluoro benzoic acid, is outlined in scheme 2.19. Refluxing of the 3-nitro 4-fluoro benzoic acid (**63**) with methylamine in ethanol for 3 h led to displacement of the fluoro group. The displacement reaction was important in that it was the key reaction to the formation of a number of N(10) functionalised flavins. Hydrogenation of the nitro ester (**65**) over a palladium catalysed hydrogen atmosphere led to the formation of the unstable diamine (**66**). This was immediately converted to the isoalloxazine (**67**) by treatment with a hot solution of alloxan in HCl, followed by refluxing for 10 minutes in the dark. On allowing the mixture to cool overnight at 4°C yellow crystals of the monoester flavin (**67**) formed. Formation of the diester (**68**) led to an intensely coloured, highly soluble flavin, which could be easily purified. Characteristic peaks in the nmr spectrum were 1.29, 4.23 and 4.01 ppm. These related to the ethyl and methyl ester groups respectively.

Formation of the diacid flavin (**69**) was achieved in a 15% yield from the starting material, 4-fluoro benzoic acid. The method of Takeda[142] led to the hydrolysis of the flavin diester (**68**). Because of the instability of the isoalloxazine in strong alkaline media,[143,144] the hydrolysis of the ethyl and methyl esters was performed in concentrated HCl at 80 - 90°C. On addition of ice-water, orange crystals began to form. These were collected and recrystallised from dilute acetic acid.



(i) HNO_3, H_2SO_4 (ii) CH_3NH_2, H_2O (iii) $SOCl_2$, MeOH (iv) H_2/Pd, C, MeOH (v) Alloxan, HCl, EtOH (vi) K_2CO_3, ethyl bromoacetate, DMF (vii) HCl

Scheme 2.19

2.4.2 Coupling methods

With the successful formation of the diacid flavin, elongation of the side chains was necessary. Amide bond formation was attempted, by reaction of the diacid with a number of amines. Attempts to condense the

flavin dicarboxylic acid (**69**) and ethylenediamine with the coupling agents dicyclohexylcarbodiimide, mixed anhydride, thionyl chloride and N,N'-disuccinimidyl carbonate were unsuccessful. However, condensation of the flavin diacid with dibenzylamine *via* the acid chloride (**70**) did form the flavin diamide (**71**) in low yield. The flavin diacid chloride was prepared from the diacid (**69**) by treatment with thionyl chloride. It was a very stable, isolatable derivative whose purity could be determined by infrared and ^{1}H nmr spectroscopies.

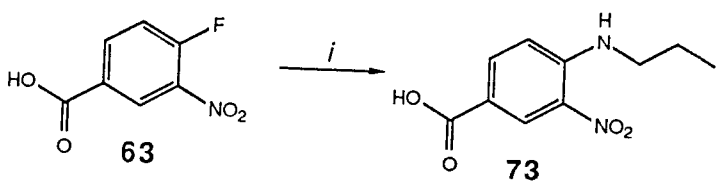
The flavin diacid chloride (**70**) and dibenzylamine were coupled to form the flavin-linked amide in DMF. Addition of a small amount of dry pyridine was effective in this case. The condensation reaction proceeded readily at room temperature and reached completion within 30 minutes. The diamide (**72**) was also formed under similar conditions from the diacid (**69**) and propylamine in 15% yield.

The use of N,N'-disuccinimidyl carbonate for the formation of active esters and their subsequent amides has been reported to give excellent yields for selected acid functionalised isoalloxazines.[145] Results from utilising this method with the diacid flavin (**69**) were not encouraging as low yields of products were obtained. Causes for these low yields were mainly attributable to solubility. Separation of the reactants and products was difficult using conventional techniques, such as column chromatography for this reason. The diamides (**71**) and (**72**) were slightly more soluble in chlorinated organic solvents than the parent diacid, thus allowing some degree of purification. The two amides (**71**) and (**72**) were clearly identifiable by high resolution nmr. The expected benzyl and propyl signals were seen in the respective spectra. The benzyl peaks at 7.16 - 7.35 ppm integrated correctly in relation to the rest of the spectra, whilst two sets of propyl signals were seen in the range 0.85 - 3.32 ppm. Futhermore, accurate mass spectral data gave support to these findings. This method of purification, however, was only suitable on a small scale (< 50 μM).

Our attention therefore turned to the synthesis of other flavins where the 10-methyl group was replaced by groups that would improve the solubility of the flavin nucleus in organic solvents.

2.4.3 10-N-Propylisoalloxazines

The synthetic scheme followed was similar to that for the 10-methyl-isoalloxazine (**50**), the only difference was that propylamine was used instead of methylamine to displace the 4-fluoro group, as detailed in scheme 2.20.

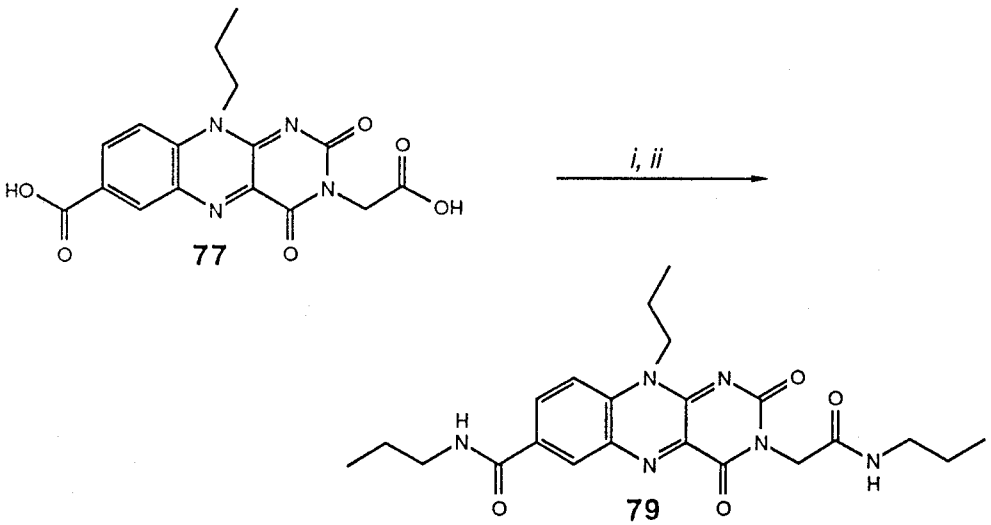


(i) propylamine, EtOH

Scheme 2.20

Formation of the respective diester (**76**) and diacid (**77**) was achieved as described for the method of 10-methyl isoalloxazine. Treatment of the diacid (**77**) with thionyl chloride at below 45°C did not form the propyl diacid chloride (**78**). No reaction was found to have occurred, as the diacid did not dissolve in the thionyl chloride. However, treatment of the propyl diacid (**77**) with isobutyl chloroformate and triethylamine in dry DMF did yield the product 3-(N-propyl)-N-acetamido-7-(N-propyl)-formamido-10-propyliso-alloxazine (**79**), scheme 2.21. The product was purified by column chromatography, but the recovered yield was low, 25%, due still mainly to the lack of solubility. The nmr spectra for this compound was similar to that for the 10-(N)-methyl-3,7-dipropylisoalloxazine (**72**), except that three sets of propyl signals could be seen in the range 0.85 - 4.65 ppm.

Further syntheses were therefore envisaged to overcome this major insolubility hurdle. One such example utilised, had an aromatic group at the N-10 position of the isoalloxazine nucleus.



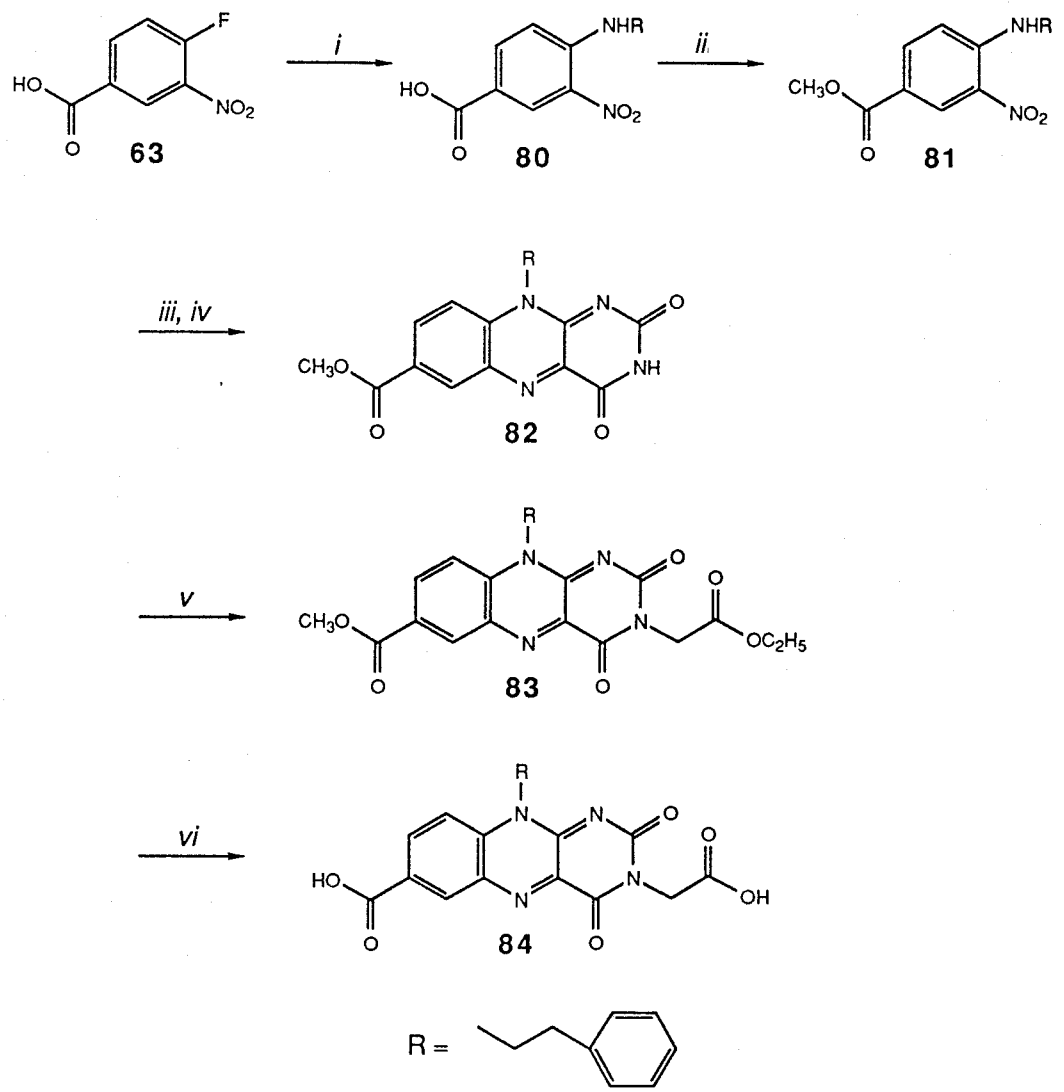
(i) DMF, Et_3N, isobutyl chloroformate
(ii) propylamine

Scheme 2.21

2.4.4 10-N-Phenethylisoalloxazines

The synthesis of a series of isoalloxazines, similar to the 10-methyl and 10-propyl isoalloxazines, but having a phenethyl group in the 10 (N) position are described within the synthetic scheme 2.22. This series of compounds were easily synthesised and readily purified. The solubility of the phenethylisoalloxazines was found to be more suitable when compared to the other isoalloxazine series, due to the introduction of the phenethyl N(10) functionality. High resolution mass spectral data confirmed this already well-characterised series of phenethylisoalloxazines.

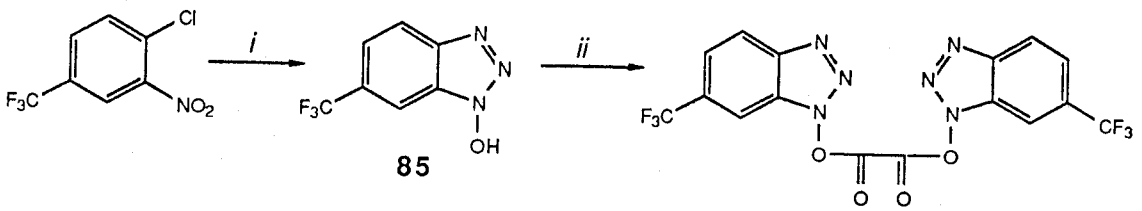
Coupling of the phenethyl diacid (**84**) with a number of amines was attempted. The most successful of the coupling agents was 1,1'-Bis[6-(trifluoromethyl) benzotriazolyl] oxalate (BTBO) (**86**).[146] BTBO was easily prepared from 1-hydroxy-6-(trifluoromethyl)benzotriazole (**85**) and oxalyl chloride in dry ether,[147] and the triazole was easily prepared from 4-chloro-3-nitro-trifluorotoluene and hydrazine hydrate in ethanol, scheme 2.23.



(i) phenethylamine, EtOH (ii) $SOCl_2$, MeOH (iii) H_2/Pd, C (iv) Alloxan, EtOH, HCl (v) K_2CO_3, ethyl-bromoacetate (vi) HCl, H_2O.

Scheme 2.22

The conversion of the flavin dicarboxylic acid (**84**) to a number of amides was achieved using BTBO by a one-pot procedure at room temperature in the presence of DMAP with the rapid liberation of carbon monoxide and carbon dioxide. The procedure consisted essentially of two reactions: formation of the activated ester from the flavin dicarboxylic acid in step 1 and subsequent aminolysis of the active ester in step 2, as shown in the generalised scheme 2.24.



85

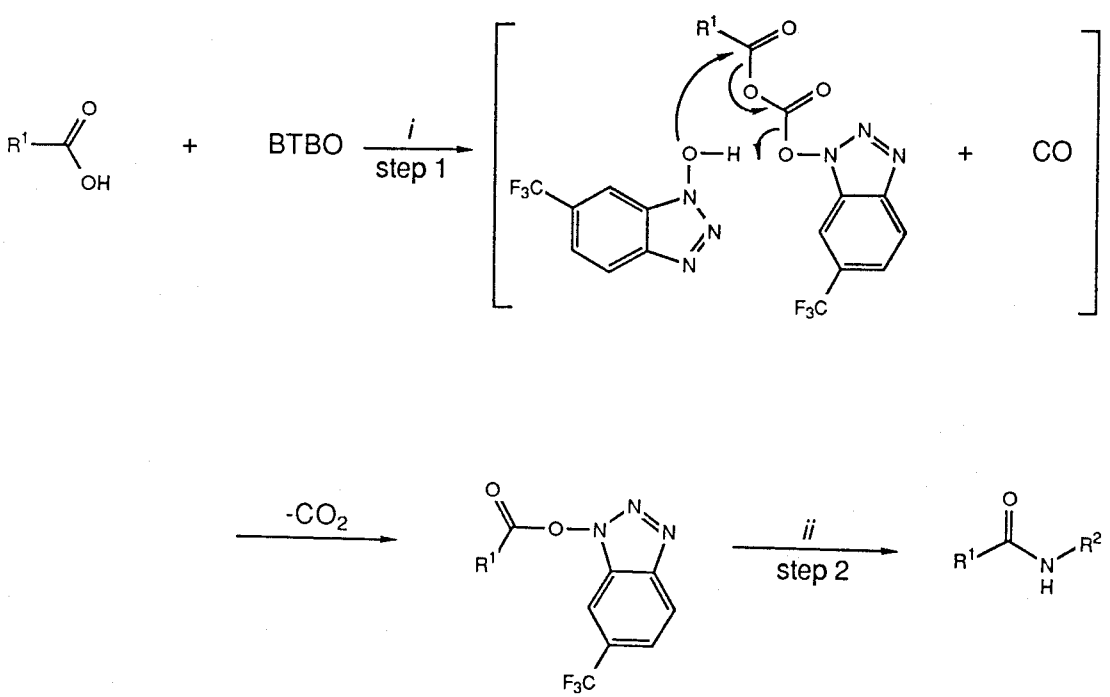
86

BTBO

(i) $NH_2NH_2 \cdot H_2O$, *EtOH*

(ii) $(COCl)_2$.

Scheme 2.23

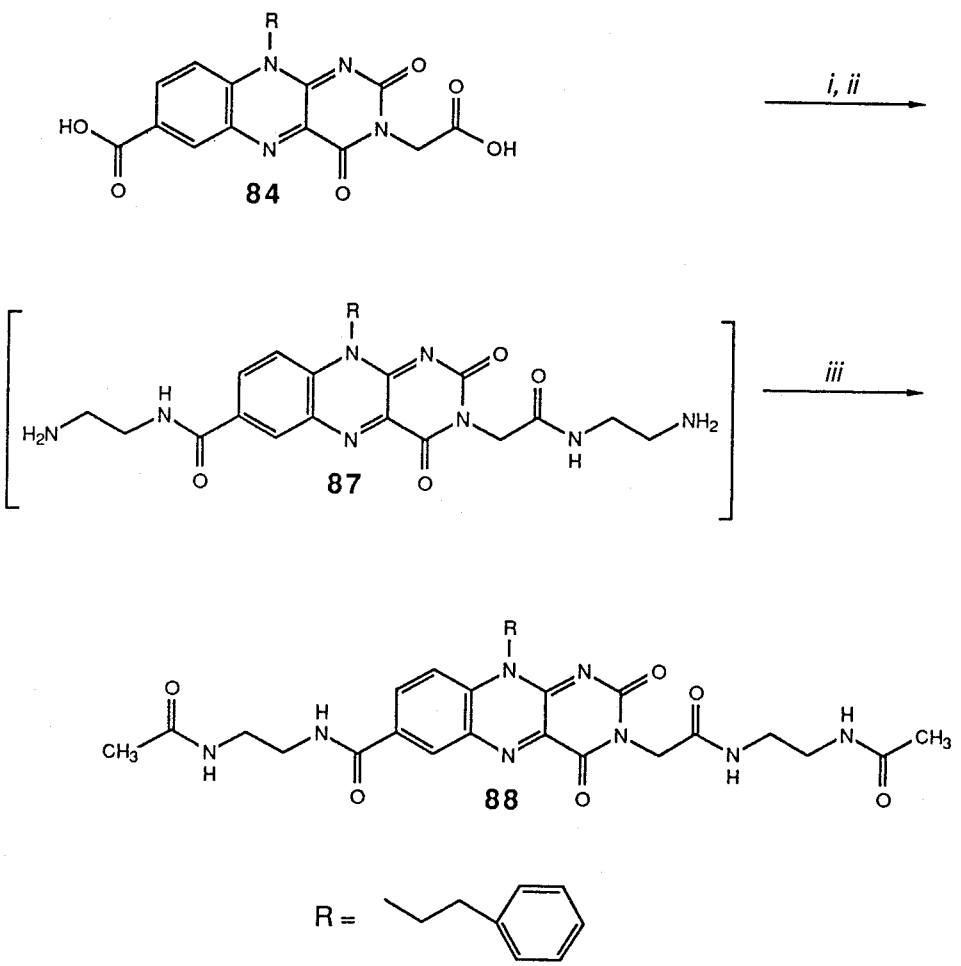


(i) pyridine

(ii) R^2-NH_2, Et_3N

Scheme 2.24

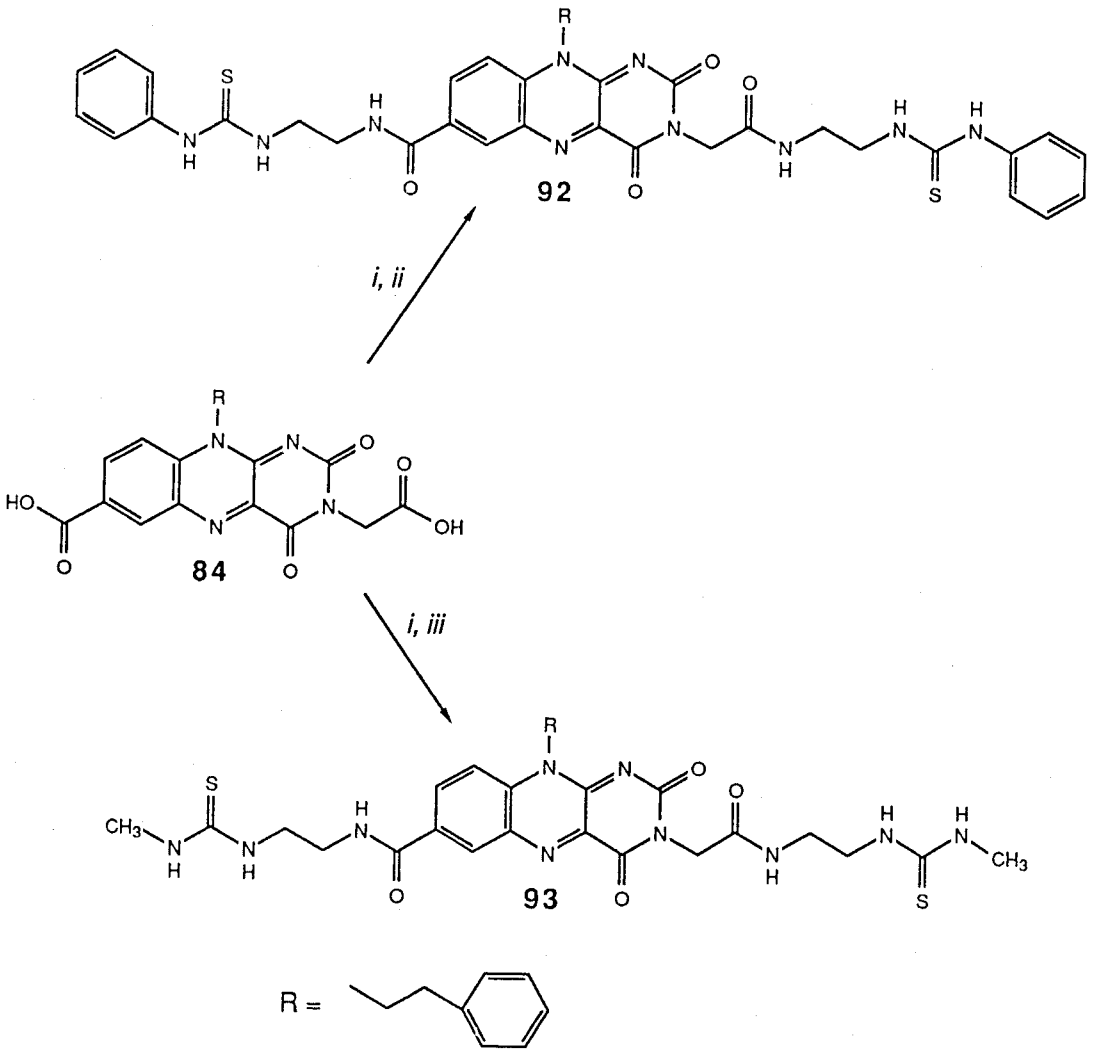
Amide formation was dictated by the suitability of the products to coupling with electrode surfaces. The reaction with functionalised polypyrroles was one aim, where the modified pyrrole envisaged would have a carboxylate 'side-arm' suitable for coupling with a flavin-diamine. This method of coupling led us to synthesize the diamide (**87**) from the diacid (**84**), BTBO (**86**) and ethylenediamine. The diamide (**87**) so formed was derivatised with acetic anhydride, due to the insolubility of the diamide, to give the diacetylated product (**88**). Data for the formation of this compound was poor, but high resolution mass spectral data did indicate its formation, as well as the formation of the diamide (**87**), scheme 2.25. Furthermore, high resolution mass spectral data was obtained for the free amine derivative (**87**).



(i) BTBO, pyridine
(ii) ethylenediamine, Et_3N
(iii) Ac_2O.

Scheme 2.25

Incorporation of thiols in the amide formation was another method by which the products would be suitable for reaction with electrode surfaces. Thiols are very reactive towards gold,[148] and it was because of this that we decided to form a number of flavin thiourea derivatives which could then be reacted with gold electrodes. Treatment of the diacid (**84**) with phenyl and methyl substituted ethylamino-thioureas (**90**) and (**91**) gave the corresponding flavin disubstituted thioureas (**92**) and (**93**), scheme 2.26. With a yield of 62%, the bisphenylthiourea derivative (**92**) was well characterised. Mixed solvents were necessary to aid solubility for nmr purposes. A peak in the ^{13}C spectrum at 182 ppm was indicative of C=S functionality, whilst in the proton spectrum a complex multiplet, integrating to 15 protons, was seen at 7.10 - 7.35 ppm. Low resolution mass spectral data clearly showed the mass ion of 776 relating to compound (**92**). The methylthiourea derivative proved more difficult to purify, however, high resolution mass spectral data did indicate its formation with the appearance of the mass ion at 651. The amino-thioureas were formed from ethylenediamine and phenyl and methyl isothiocyanide respectively.[149]



(i) BTBO, DMAP, DMF
*(ii) phenylthiourea (**90**)*
*(iii) methylthiourea (**91**)*

Scheme 2.26

2.5 Future Work

Future synthetic studies will aim to improve solubility and electron transfer rate. Improvement in solubility may be achieved by suitable substitution of groups at N(10) of the flavin nucleus, whilst improvements in electron transfer rate may be achieved through alteration of the side chain length and composition. Two variations to the flavin redox-centre could be implemented using the synthetic methodology detailed in schemes 2.22 and 2.26.

The chain length of the side arms could be varied using the diacid (**84**) as the starting material. Using a similar approach to that used in the successful synthesis of compound (**92**), the diacid would be treated with pre-fuctionalised 3-aminopropyl- and 4-aminobutyl-phenylthioureas in the presence of BTBO. Electrochemical characterisation of the products after attachment to gold, and comparison of the results with those for the 2-aminoethyl thiourea derivative *vide supra* would allow a flavin-electrode surface distance correlation with all characterisable parameters, e.g. reversibility and electron transfer rate.

A third side-chain could be introduced at N(10) through the use of a ω-protected α,ω-diaminoalkane instead of 2-phenethylamine at step *(i)* of scheme 2.22. The chain length in the trifunctionalised systems could also be varied. These compounds would allow the characterisation and comparison of the redox properties of two and three point attachment systems for given electrode-redox centre interfacial distances.

CHAPTER 3

Electrochemical Studies of Bifunctionalised Flavins

3.1 Cyclic Voltammetry Theory

3.1.1 Soluble Species
3.1.2 Surface Species

3.2 Flavin Electrochemistry at Metal Electrodes

3.2.1 Mercury Electrodes
3.2.2 Glassy Carbon Electrodes
3.2.3 Other Electrodes

3.3 General Experimental

3.3.1 Equipment
3.3.1.1 Electrodes
3.3.1.2 Cells and solutions
3.3.2 Results and discussion
3.3.2.1 Mercury electrodes
3.3.2.2 Carbon electrodes
3.3.2.3 Gold electrodes

3.4 Electron Transfer Rate Determination

3.5 Flavin Surface Coverage

3.6 Conclusions

3.7 Future Studies

Electrochemical Studies of Bifunctionalised Flavins

The flavin nucleus is an electroactive species and one of the most versatile prosthetic groups utilised by electron transfer proteins. The modification of electrodes with flavin offers significant potential for the development of an interface for electron transfer between proteins and electrodes. Of all the natural redox cofactors, only the flavin nucleotides have the ability to transfer one or two electrons and to promote the facile reduction of molecular oxygen. This makes them particularly important for study.

Immobilisation of flavin molecules, such as riboflavin derivatives on electrodes and their electrochemical behaviours have been well studied.[107,150,151] Although many studies have been reported wherein the flavoenzyme glucose oxidase has been immobilised on electrode surfaces, very few of these studies have included an investigation of the possibilities for direct electron transfer in the absence of mediators. With glucose oxidase, immobilised by adsorption on graphite electrodes, evidence for direct electron transfer has been claimed.[152-154] However, the voltammograms that showed direct electron transfer were attributed to oxidation-reduction of the flavin cofactor while still complexed within the apoenzyme, and not to flavin molecules that had become dissociated from the apoenzyme and adsorbed onto the electrode surface.

3.1 Cyclic Voltammetry Theory

Recent years have seen a dramatic increase in the application of electrochemical techniques to studies of the oxidation and reduction of many types of biological compounds.[82-87] Cyclic voltammetry (CV) is perhaps the most versatile electroanalytical technique. The technique allows the rapid observation of the properties of a system over a wide potential range. The resulting voltammogram is analogous to a conventional spectrum, in that it conveys information as a function of an energy scan. The versatility of cyclic voltammetry, combined with its ease of measurement, has resulted in the extensive use of CV in all areas of chemistry.

CV consists of cycling the potential of an electrode which is immersed in an electrolyte solution, and measuring the resulting current. The potential of this 'working electrode' is controlled versus a 'reference electrode', such as a saturated calomel electrode (SCE) or a silver/silver chloride electrode (Ag/AgCl). The controlling potential which is applied between the working and a counter electrode can be considered an excitation signal. The excitation signal for CV is a linear potential scan with a triangular waveform. This triangular potential excitation signal sweeps the potential between two potentials. A cyclic voltammogram is obtained by measuring the current at the working electrode during the potential scan. The current can be considered as the response to the potential excitation signal. The voltammogram is a display of current versus potential. Because the potential varies linearly with time, the horizontal axis can also be thought of as a time axis.

3.1.1 Soluble species

A typical CV for a soluble electroactive species is shown in figure 3.1

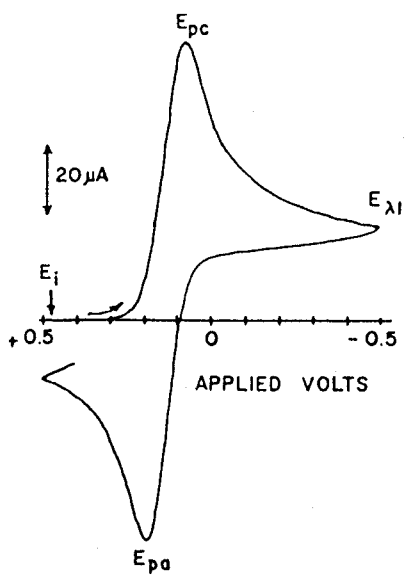


Figure 3.1

a. Typical cyclic voltammogram of 1 mM $K_3Fe(CN)_6$ at a platinum electrode in aqueous 0.1 M KCl solution. The scan rate was 100 mVs^{-1} and the reference electrode was Ag/AgCl in 0.1 M KCl solution.

The important parameters of a cyclic voltammogram are the magnitudes of the anodic and cathodic peak currents (i_{pa}) & (i_{pc}), and the anodic and cathodic peak potentials (E_{pa}) & (E_{pc}).

A redox couple present in solution where both species rapidly exchange electrons with the working electrode is termed an electrochemically reversible couple. The formal reduction potential ($E^{\circ'}$) for a reversible couple is centered between E_{pa} and E_{pc}.

$$E^{\circ'} = E_{1/2} = \frac{(E_{pa} + E_{pc})}{2} \qquad 3.1$$

The number of electrons transferred in the electrode reaction, for a reversible couple, can be determined from the separation between the peak potentials.

$$\Delta E_p = E_{pa} - E_{pc} \sim 0.059/n \qquad 3.2$$

The peak current for a reversible system is described by the Randles-Sevcik equation (3.3) for the forward sweep.[155]

$$i_p = (2.69 \times 10^5) n^{1/2} A D^{1/2} C v^{1/2} \qquad 3.3$$

where
- i_p = peak current (A).
- n = electron stoichiometry.
- A = electrode area (cm^2).
- D = diffusion coefficient ($cm^2 s^{-1}$).
- C = concentration (mol cm^{-3}).
- v = scan rate (Vs^{-1}).

Thus i_p increases linearly in relation to $v^{1/2}$ and is directly proportional to concentration. Electrochemical irreversibility is caused by the slow electron exchange of the redox species with the working electrode, which is characterised by a separation of peak potentials greater than that indicated by equation 3.2.[156]

3.1.2 Surface Species

Thus far an assumption has been made that all the reactants and processes have been fully soluble in solution and that the surface processes, such as reactant or product adsorbtion, need not be considered.

The essential difference between cyclic voltammograms of an adsorbed oxidised and reduced species and that of the oxidised and reduced species in free solution are: The peaks are sharp and symmetric; the current rises from essentially zero to a peak value and then falls again to zero; there is little or no peak separation. A typical voltammogram for an adsorbed species is shown in figure 3.2. The charges associated with anodic and cathodic processes are also equal. A symmetric peak arises because of the fixed amount of reactant; only the oxidised species on the surface at the start of the sweep can be reduced. The peak current is given by equation 3.4.

$$i_p = \frac{n^2F^2 \,.\, vA\Gamma_o}{4RT} \qquad 3.4$$

where Γ_o = surface excess concentration (mol cm^{-1}).

It is important to note that the peak current, and indeed the current at all points on the wave, is proportional to v, in contrast to the $v^{1/2}$ dependence observed for nernstian waves of diffusion species, equation 3.3.

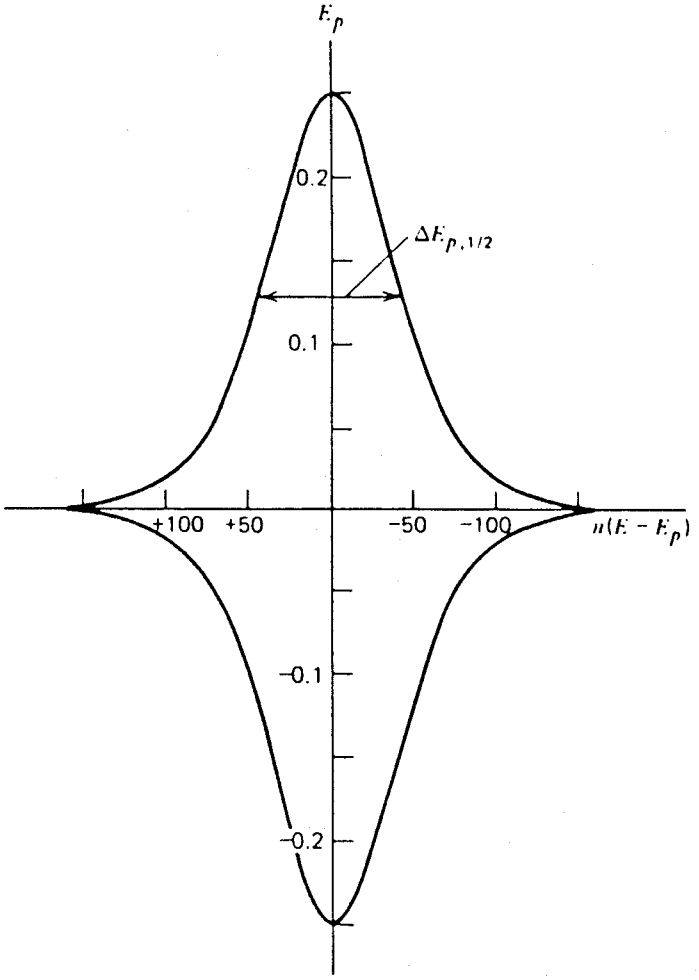


Figure 3.2
Current-potential curve for cyclic voltammetry for reduction of adsorbed O and sweep reversal.

3.2 Flavin Electrochemistry at Metal Electrodes

The flavin coenzymes occur in a variety of proteins and interface between organic oxidation and reduction reactions, which are usually two-electron processes, and one-electron transfer reactions. Metal containing proteins, such as the heme proteins, the cytochromes and the catalyses and peroxidases are all good examples of one-electron transfer processes.

All flavins share the isoalloxazine chromophore, which is the electroactive part of the molecule, see figure 1.2.[49] Therefore all compounds derived from flavin e.g. lumiflavin, riboflavin, FMN, and FAD, which vary only in the nature of the side group, are expected to have similar formal potentials to that of the isoalloxazine ring system.

In biological systems, the factors which govern the mechanism of FAD reduction and oxidation, as well as its redox potential, are related to the microenvironment of the cofactor. Thus the flavin-apoprotein interaction may modulate the redox potential of the coenzyme. Tight binding between the flavin coenzyme and apoprotein may shift the redox potential if the oxidised, the dihydro, and the semiquinone forms of the flavin coenzymes are bound differentially to apoproteins. The redox potential of a given flavoenzyme helps to determine which molecules the flavoenzyme can use as electron donors or acceptors In redox catalysis; therefore, variable redox potentials allow different functional niches for flavoproteins. The observed range of flavoenzyme redox potentials is from -465 mV to +149 mV.[157]

It is known that, in solution, the oxidised flavins have a planar structure, while the reduced flavin resembles a butterfly in conformation. Atoms N(1) to N(5) and N(10), and N(5) to C(9) and N(10) (figure 3.3) constitute two planes intersecting the N(5)-N(10) axis, with a dihedral angle of 21°.[158] Therefore, enforced flattening of the reduced flavin should increase its reducing power, and conversely, enhanced bending should increase the oxidising power. Selectivity for a one- or two-electron reaction could conceivably be assisted in such a manner.[159] This may occur in the protein and could perhaps also occur at the surface of an electrode.

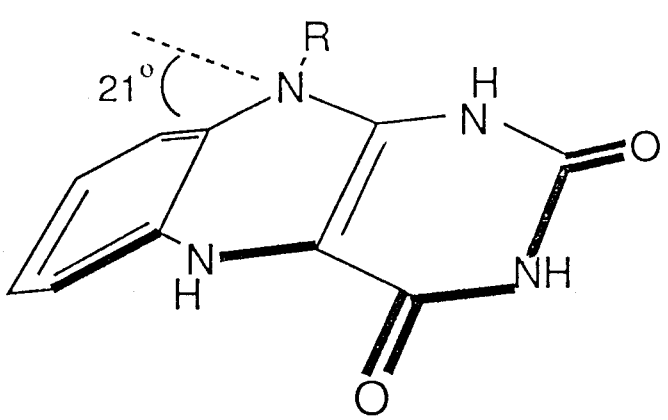


Figure 3.3

3.2.1 Mercury electrodes

The electrochemical behaviour of the flavins, particularly of riboflavin, have been studied at mercury electrodes through dc and ac polagraphic and chronopotentiometric methods.[160-163] It has been reported that in acidic solutions, both the oxidised and reduced forms of riboflavin are strongly adsorbed on a mercury surface.[159,160,164]

A study of the adsorbtion behaviour of FMN at mercury electrodes in acid solutions has also been reported.[165] Strong adsorbtion of both the oxidised and reduced forms of FMN were observed. Whilst a bilayer of the reduced form was presumed to exist, only a single layer of the oxidised form of FMN was found to adsorb at the mercury electrode.[165]

The work of Kakutani *et al.*[166] showed that the oxidised and the reduced forms of FMN are strongly adsorbed at a mercury electrode in buffered solutions of pH 4.9 and 6.9. Apparently the isoalloxazine ring system lies parallel to the electrode surface. A similar case has been found for FAD solutions, both the oxidised and reduced forms are adsorbed at pH 6.9.[167]

Detailed flavin orientation experiments by Birss and her group[168] on FAD in neutral solutions, at mercury electrodes, have been carried out. When a fresh mercury drop was exposed to FAD solution, FAD molecules adsorbed as a function of time. It was proposed that the isoalloxazine moiety was approximately parallel to the electrode surface and that the adenine groups were also on the mercury surface (figure 3.4a). As the surface concentration of the loose FAD structure of fig. 3.4a increased, a critical coverage was reached, leading to a change in orientation (figure 3.4b), where the isoalloxazine ring system orientates itself perpendicular to the mercury surface, with the C(6), N(5) and C(4) edge of the isoalloxazine nucleus in contact with the electrode surface. A parallel FAD orientation was re-established when the potential was extended positively to give a positively charged mercury electrode. When the potential was scanned negatively, the FAD layer reorientated itself again to the perpendicular orientation. If the potential cycling was continued for a few hours, the FAD adsorbtion remained in the parallel orientation, (figure 3.4c). However, due to the increased surface concentration, the adenine functionality was predicted to be above the flavin nucleus in a coplanar conformation.

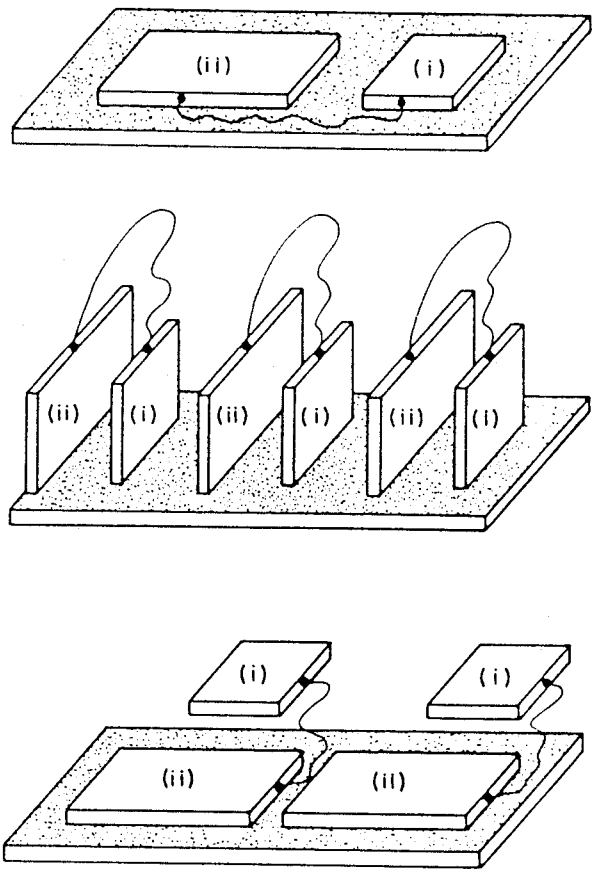


Figure.3.4

Scheme of the proposed orientation of adsorbed FAD at mercury electrodes, pH = 7. (i) depicts the adenine group, connected via the sugar-phosphato linkage to the isoalloxazine system (ii).

3.2.2 Glassy carbon electrodes

At glassy carbon (GC) electrodes, a single layer of FAD can be attached, while at porous graphite electrodes, up to an estimated 50-100 monolayers of FAD have been adsorbed.[169] It appears, however, that the roughness factor of these porous graphite electrodes was not taken into account when estimating the number of layers of adsorbed FAD. In all of the work at carbon electrodes, it was found that glucose oxidase activity in solution could be restored with the use of FAD coated electrodes.

Oxidation and reduction properities of FMN have been well studied and reviewed extensively.[170-172] In the case of strongly adsorbed FMN at graphite electrodes, a two-electron reduction-reoxidation wave was observed. This is due to two overlapping one-electron steps, indicating semiquinone formation.

3.2.3 Other electrodes

Flavin nucleotides have formed stable monolayers on other carbon and metal surfaces through the C(8) position of riboflavin. Formation of a mercaptopropylsilane riboflavin (MPS-flavin) coating, by Dufor *et al.*,[150] on a gold electrode led to a modified electrode which was stable to several hundred voltammetric scans. The linearity of the anodic and cathodic peak currents with sweep rates demonstrated the surface-bound nature of this flavin coenzyme. However, potential cycling of the gold-MPS-flavin electrode to -0.8 V caused the disappearance of the flavin redox waves.

On platinum electrodes hydrogen evolution interferes with the end of reduction for soluble FAD. The adsobtion was found to be time dependent, with a coverage much less than that on graphite. The weakly adsorbed cofactor was completely removed by washing.[174]

Hydrogen evolution interferes less with the flavin electrochemistry on gold electrodes. Adsorption is less than on Pt electrodes and any adsorbed material is easily removed.[150] No electrochemical activity has been found for adsorbed FMN molecules on gold electrodes.[151]

3.3 General experimental

3.3.1 Equipment

Standard three-electrode potentiostatic circuitry was employed, utilising a Hi-tek Instruments DT131 potentiostat with a Hi-tek Instruments RR1 wave form generator. I/E curves were usually plotted on a Bryans X/Y recorder 26000 A3, at slow sweep rates, and on a Gould digital storage scope OS4100 at sweep rates greater than 200 mVs^{-1}.

3.3.1.1 Electrodes

The working electrodes (WE) utilised in this work were; a hanging mercury drop electrode (HMDE); a carbon electrode and a gold electrode (Au) (Goodfellow metals 99.99%+). The EG & G PARC 303A HMDE was set on the medium drop size, producing a drop of 0.05 cm^2 average surface area. The Au electrode consisted of an Au wire with a spherical bead (surface area, 0.1 cm^2) placed in a glass capillary with the bead protruding from the capillary. The gold electrode was prepared for electrochemical studies by suspension in nitric acid for 2 h.[174] followed by thorough rinsing with tri-distilled water. Immediately prior to experimentation the electrode was annealed to red hot in a bunsen flame. Surface reaction was achieved by reacting a cleaned electrode surface with a solution of flavin (1.5 mM) in DMF. After one minute the electrode was removed from solution and washed in a stream of freshly distilled DMF, and then immersed in more fresh DMF for 30 minutes. Finally, the electrode was soaked in pH 7.0 aqueous buffer for one hour.

The glassy carbon electrode was of an in-house design, so designed that only a circular flat surface was available. It was polished with alumina paste on a polishing cloth and thoroughly rinsed with distilled water.

3.3.1.2 Cells and solution

All experiments were carried out in a polarographic cell, so constructed that the cell solution could be thoroughly deoxygenated by bubbling argon through the solution for 10 min. prior to electrochemical measurement, and the solution maintained under an atmosphere of argon during the experimental runs.

Aqueous experiments were carried out in pH 7.0 buffered (0.1 M potassium phosphate) solutions. 0.1 M KCl was used as the supporting electrolyte. All chemicals utilised were of reagent grade, All water was triply distilled and DMF was distilled from CaH under vacuum and stored over activated 4 Å molecular sieves. All experiments were carried out at room temperature and under subdued light to avoid flavin decomposition.

3.3.2 Results and Discussion

3.3.2.1 Mercury electrodes

Electrochemical studies were carried out on a variety of flavins at the HMDE in an attempt to determine the electrochemical characteristics of a number of synthesised flavins, and to compare these results with those of some well studied flavins. This would enable an early assesment of the binding properties of the new flavins to be made.

Experiments were carried out with the flavin derivatives at concentrations of 0.5 mM, in aqueous solution, over a scan range of -300 to -650 mV vs. Ag/AgCl. Typically, purge times and pulse delays of 30 seconds were used, with the scan rates ranging from 5 to 100 mVs^{-1}. A typical voltammogram for lumiflavin is shown in figure 3.5, where nine continuous scans are shown. The sharp peaks centred at -400 mV decreased with each cycle of potential, whilst the broader peaks at the more cathodic potential, gradually increased in size. On the first scan it can be seen that the adsorbed species (A & C) is predominant in terms of electrochemical activity. Although the peak separation was larger than expected, the peak current varies linearly with scan rate. After only a few scans this was significantly reduced, to make the solution species (B & D) the more redox active component. The effect of elapsed time between the formation of the mercury drop and the electrolysis experiment was similar to that observed by Hartley and Wilson[165] for the flavin FMN. They observed a sharp cathodic peak when the scan was begun immediately after formation of the mercury drop. With increasing equilibration times the prepeak was observed to merge with the main faradaic peaks and ultimately to disappear entirely. They concluded that the shift in the prepeak process resulting from equilibration of the electrode with the solution caused the electrolysis of the oxidised film in the potential region of reaction 3.5.

$$Ox_{soln} + 2e^- \rightleftharpoons Red_{soln} \qquad 3.5$$

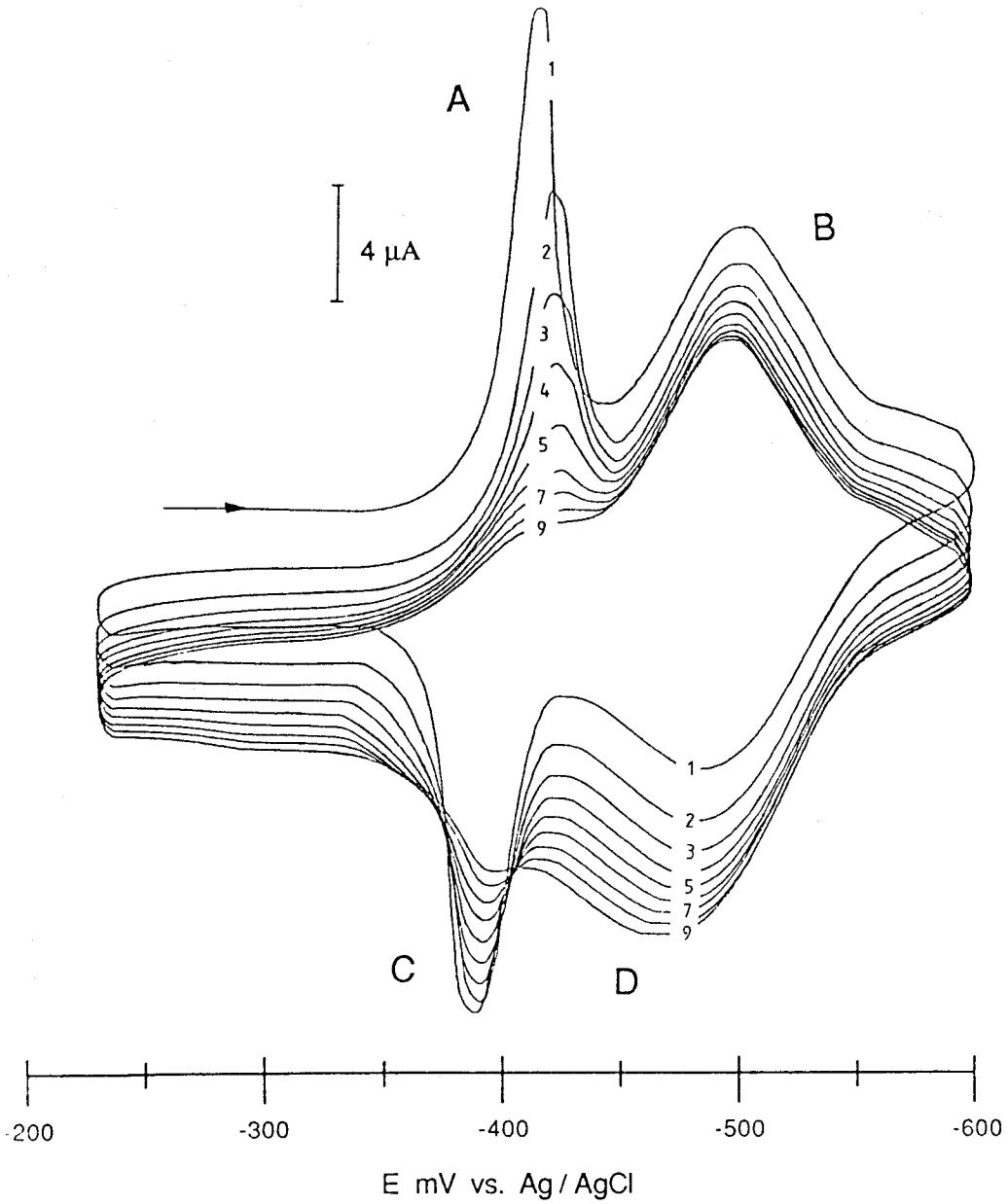


Figure 3.5
Cyclic voltammogram of lumiflavin at a HMDE, 0.1 M KH_2PO_4, pH 7.0, 100 mVs^{-1}

It was further concluded that the equilibrated film was reduced either at the same time, or following, the diffusion process. A possible reorientation of the flavin nucleus at the electrode surface could be the cause of the prepeak decay with the large equilibration times. It may be that the initial approach of the flavin to the mercury electrode occurs 'edge on' with the plane of the rings perpendicular to the electrode surface, forming a weakly bound unstable state which could slowly reorientate to form a stable film where the ring system is coplanar to the electrode surface.

Wopschall and Shain[175] have given a mathematical treatment to account for the observation of prepeaks. Their model predicts that for a reduced species which is more strongly adsorbed at an electrode than an oxidised species, a prepeak would be observed. The prepeak shows the electrode response for the oxidation of adsorbed flavin. The prepeak occurs at potentials more positive than the diffusion controlled process because of the ease of reduction of adsorbed flavin in comparison to the reduction of the solution species. However, no account was given for the observation that the prepeak decays with successive potential sweeps. A further example of this prepeak process can be seen in figure 3.6 for tetraacetylriboflavin, (TAR).

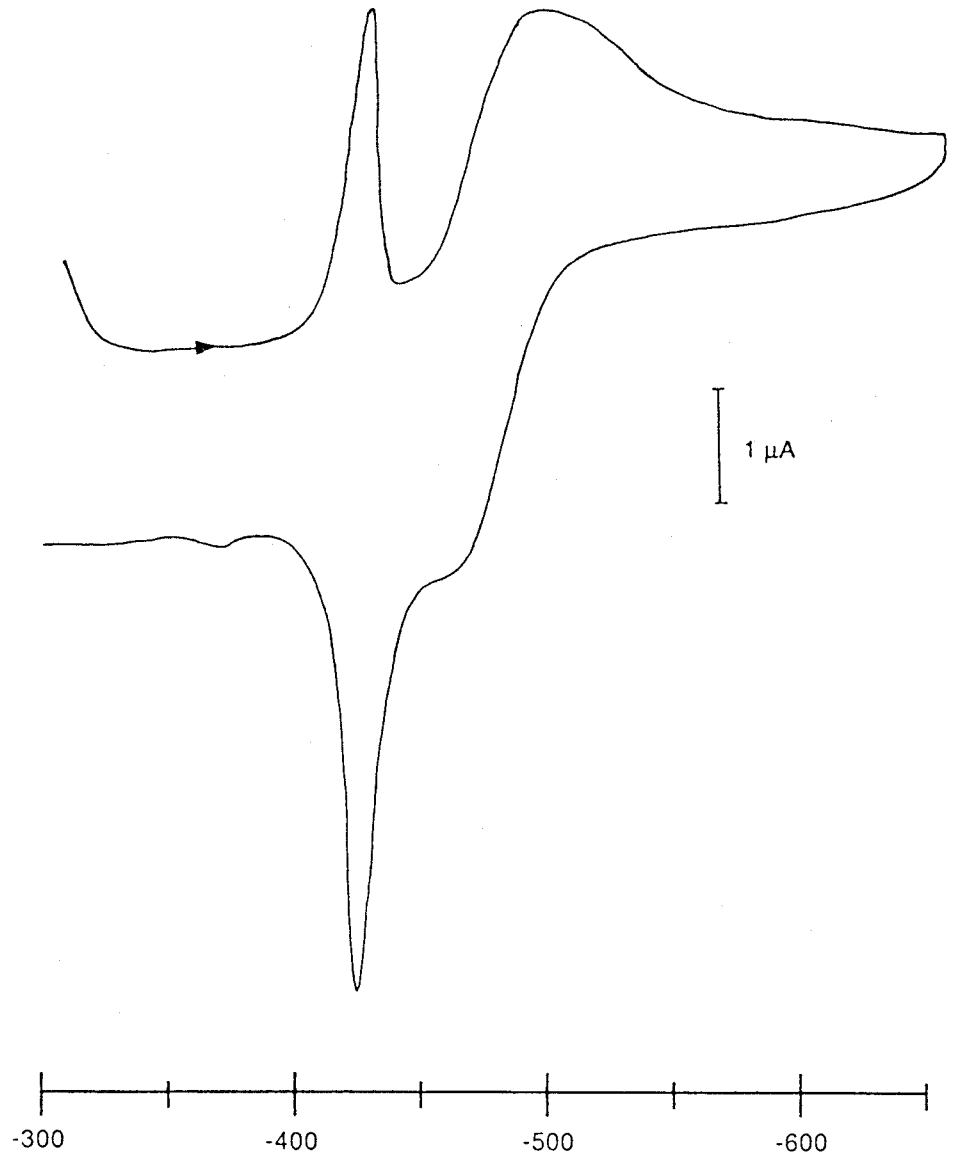


Figure 3.6

Cyclic voltammogram of TAR at a HMDE, showing pre-peaks, 0.1 M KH_2PO_4, 5 mVs^{-1}.

3.3.2.2 Carbon electrodes

Preliminary studies were carried out with flavins at carbon electrode interfaces to assess the suitability of the electrode surface for further studies. Coupling of a highly oxidised electrode with a flavin, similar in compostion to the diamino flavin (**87**), was the objective. However, this necessitated the determination of the adsorbtion characteristics of a non-oxidised electrode with flavins absent of suitable functionalisation.

Figure 3.7 shows a cyclic voltammogram of tetraacetylriboflavin at a glassy carbon electrode at 5 mVs^{-1}. The flavin (6.13 mM) was dissolved in dry DMF with tetrabutylammonium chloride (TBACl) (0.1 M) as the electrolyte. At 5 mVs^{-1} the peak separation was 100 mV, indicating a slow electrochemical response, whilst at higher sweep rates the behaviour becomes more irreversible. Figure 3.8 shows the relationship between sweep rate and peak cathodic current, i_{pc}, over the sweep range 5 to 100 mVs^{-1}.

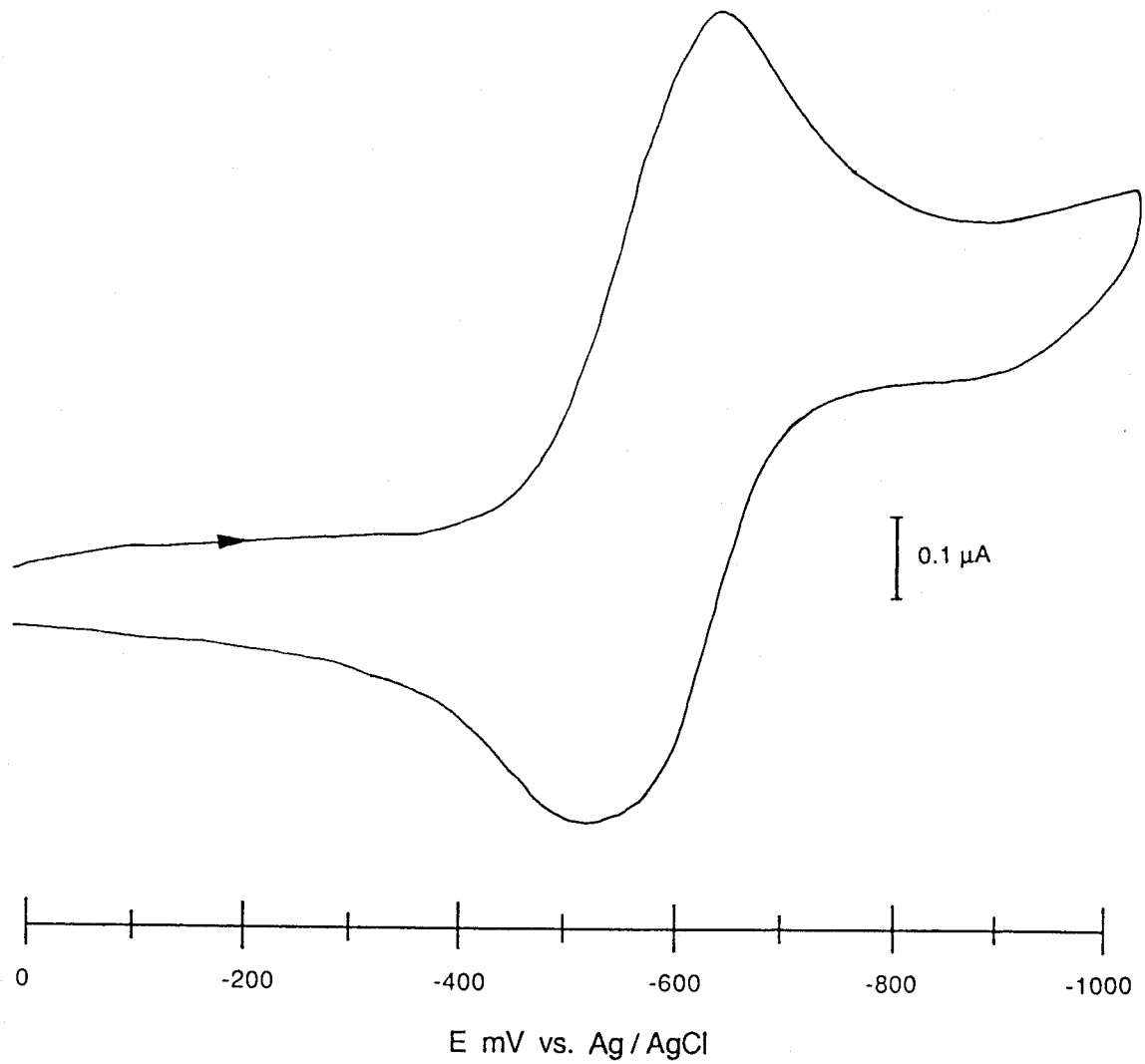


Figure 3.7

Cyclic voltammogram of a DMF solution of TAR at a carbon electrode, 5 mVs^{-1}.

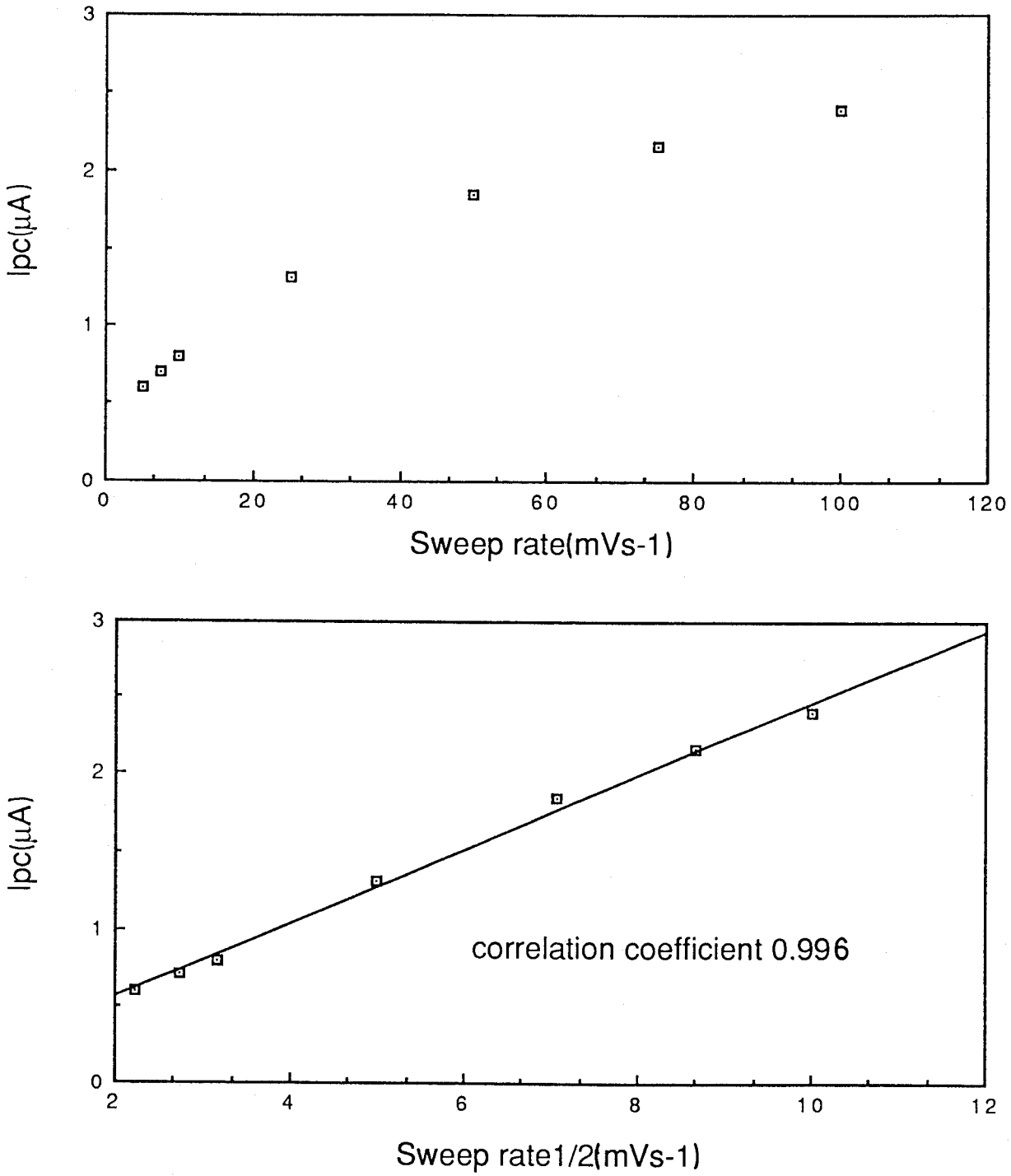


Figure 3.8

Variation of peak current with sweep rate and sweep rate$^{1/2}$, for TAR, at a glassy carbon electrode in DMF.

With reference to equation 3.3, the correlation coefficient for a linear plot, from regression analysis, of 0.996, for the plot of scan rate$^{1/2}$ vs. I_{pc} indicated that the flavin TAR was not adsorbed onto the electrode surface, but was a solution state species. Further evidence against a surface bound species came from the shape and separation of the anodic and cathodic peaks. For an adsorbed species, the peaks should be symmetrical, with the peak separation at a minimum. The cyclic voltammogram of TAR clearly does not exhibit the necessary symmetry or minimal peak separation associated with an adsorbed electrode species.

Results from two other flavins, lumiflavin and the phenethyldiester flavin (**83**) exhibited similar difussion controlled processes in DMF solutions to that of TAR. However, cathodic peak potentials and peak separations were significantly different to necessitate explanation. The essential results are summarised in table 3.1.

Flavin	scan rate mVs^{-1}	E_{pc} vs. Ag/AgCl	ΔE (mV)	$E_{1/2}$ (mV)
TAR	5	-621	108	-567
	50	-636	184	-544
	100	-640	191	-544
Lumiflavin	5	-730	155	-653
	50	-815	330	-650
	100	-848	398	-650
Phenethyl-	5	-399	59	-369
diester (**83**)	50	-415	97	-367
	100	-434	130	-368

Table 3.1

Two important points can be noticed from this data. First, that the half-wave potential of the phenethylester (**83**) occurs at a potential some 280 mV more positive than that for lumiflavin, and 200 mV more positive than that for TAR. Second, that the rate of electron transfer, determined

from the peak separation, at low sweep rates for the phenethyl flavin is almost reversible (ΔE = 59 mV). This is in contrast to lumifavin (ΔE = 155 mV) and to a lesser extent TAR (ΔE = 108 mV). Both these observations indicate that the orientation of the flavin nucleus at the electrode surface is more favourable for electron transfer in the case of the phenethyl flavin diester (**83**) than in the other two examples.

Futher studies with the phenethyl flavin nucleus, but with suitable 'side-arms', in conjunction with an oxidised carbon surface, should enable optimisation of this favourable electron transfer characteristic. However, it then would be expected that the peak current would exhibit a linear relationship with sweep rate.

3.3.2.3 Gold electrodes

The electrochemistry of flavins at gold electrodes has been poorly studied, mainly due to the poor flavin-Au interactions. FMN molecules do not adsorb at gold electrodes[151] and Gorton and Johansson[173] reported that though FAD was adsorbed, the material was easily removed. However, from detailed studies on the interaction of sulphur with gold,[176-179] attempts were made to attach flavins bifunctionalised with terminal sulphur linkages to gold electrodes.

From the synthesis of flavins incorporating sulphur, see 2.4.4 it can be seen that the essential thiol-gold interactions take place through substituted thio-ureas. Other thiol functionalisation may be used, and future studies will enable detailed assessments of a number of thiol linkages to be made. Thiourea derivatives were synthesised as the side groups in an attempt to aid solubility, a major problem encountered in flavin synthesis. The methodology utilised would also allow the 'side-chain' length to be altered simply by the use of various α,ω diaminoalkanes.

Prior to the attempted coupling of dithiourea flavins at gold electrodes, studies were carried out on flavin species where no thiol functionalisation had taken place. Lumiflavin (1.87 mM) in 0.1 M phosphate buffer and 0.1 M KCl at pH 7.0, under an atmosphere of argon, was studied by cyclic voltammetry over the potential range -200 to -700 mV vs. Ag/AgCl, with scan rates of 5 to 200 mVs^{-1}. Plots for scan rate and scan rate$^{1/2}$ vs. cathodic peak current are shown in figure 3.9.

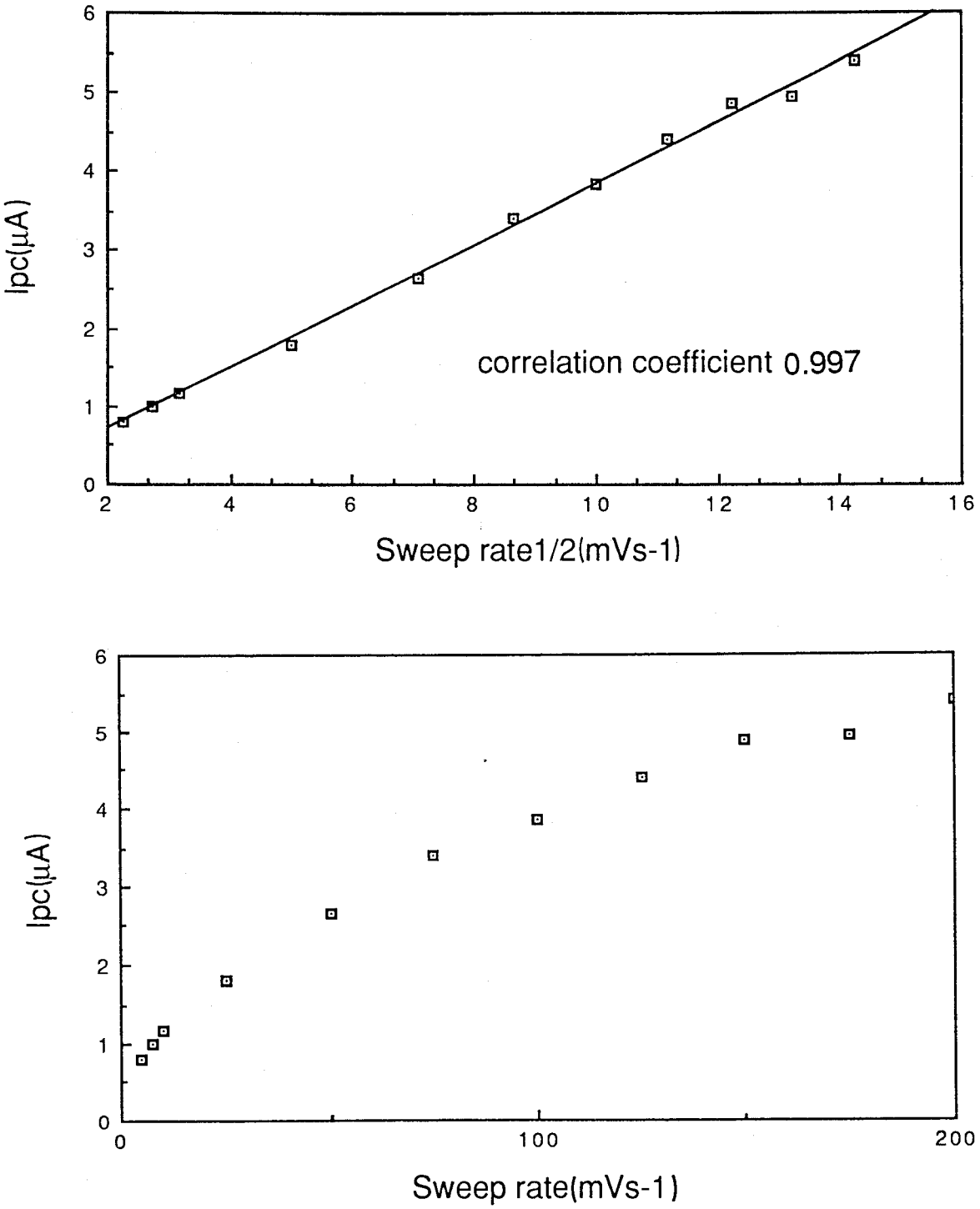


Figure 3.9

Plots of cathodic peak current depedance with sweep rate and sweep rate $^{1/2}$, for lumiflavin in 0.1 M KH_2PO_4, 0.1 M KCl, pH 7.0, at a gold electrode.

It can be seen that with a correlation coefficient of 0.997 for the regression fit to a straight line, the plot of I_{pc} vs. sweep rate indicates that the lumiflavin is a solution species and is in no way bound to the gold electrode surface. Further evidence to suggest that this deduction is correct can be judged from the results of the adsorbtion of lumiflavin onto a gold electrode from a solution of lumiflavin in DMF. A clean gold electrode was suspended in a solution of lumiflavin (1 mg/ml) in DMF for 2 mins. After this time the electrode was washed in a stream of DMF, soaked in fresh DMF for 10 minutes and finally washed with triply distilled water. A cyclic voltammogram of the lumiflavin adsorbed electrode is shown in figure 3.10. It can be seen that though there is an electrochemical response due to residual lumiflavin, this is effectively completely removed by leaching in approximately five minutes.

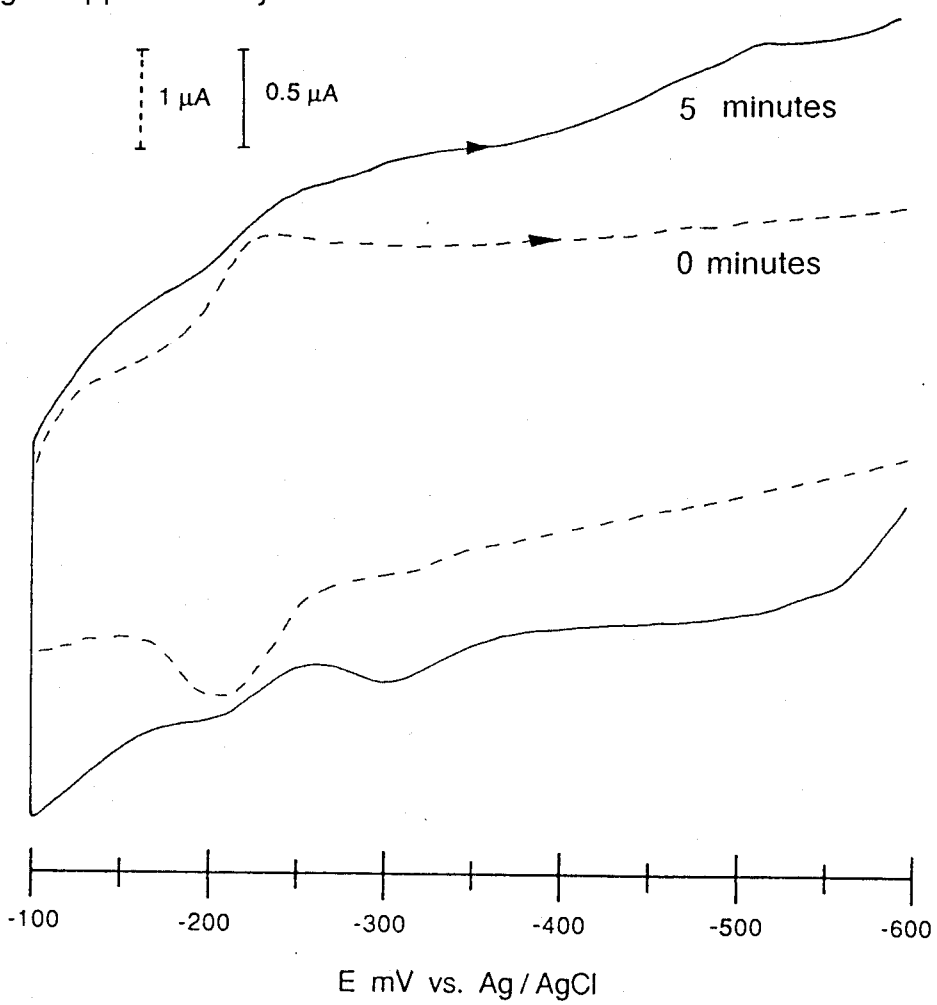


Figure 3.10

Electrochemical response of a lumiflavin adsorbed gold electrode with time, 0.1 M KH_2PO_4, 0.1 M KCl, pH 7.0, 100 mVs^{-1}.

Electrochemical treatment of gold electrodes with other non-thiol flavins, particularly the phenethyl flavin series, led to similar results and conclusions. All the tested flavins showed complete loss of electrochemical activity within 5 minutes of continuous potential cycling. If the sweep range was taken to the limits of the oxidation and reduction of water then the residual flavin was rapidly desorbed in one or two cycles. This indicated that the flavin was only weakly attached to the electrode.

Electrochemical studies with the flavin, bis-phenylthiourea 10(N)-phenethylisoalloxazine (**92**), led to results which were in contrast to those of the non-thiol activated flavins. The experimental procedure followed was as previously stated. The nitric acid cleaned gold electrode was immersed in a solution of the bisthiourea flavin (**92**) (1.29 mM) for 2 minutes, soaked for 10 minutes in freshly distilled DMF and finally washed with triply distilled water, in order to remove any residual non-adsorbed flavin. Cyclic voltammograms were run in phosphate buffer (0.1 M) with KCl (0.1 M) as the electrolyte. Sweep rates ranged from 5 mV to 400 V per second. A typical cyclic voltammogram is shown in figure 3.11.

It can be seen that the overall shape of the current peaks are symmetrical about a vertical axis. This is in contrast to that seen for the solution species of the non-thiol modified flavins, c.f. figure 3.7. With a mid-point potential of -414 mV vs. Ag/AgCl, the electron transfer rate was determined to be moderately fast (ΔE 40 mV), (for an adsorbed species the peak separation ΔE should be zero). Plots of sweep rate and sweep rate$^{1/2}$ vs. cathodic peak current are shown in figure 3.12. Good correlation to a straight line was found for the plot, sweep rate vs. i_{pc} (correlation coefficient 0.997), which is indicative of an adsorbed species.

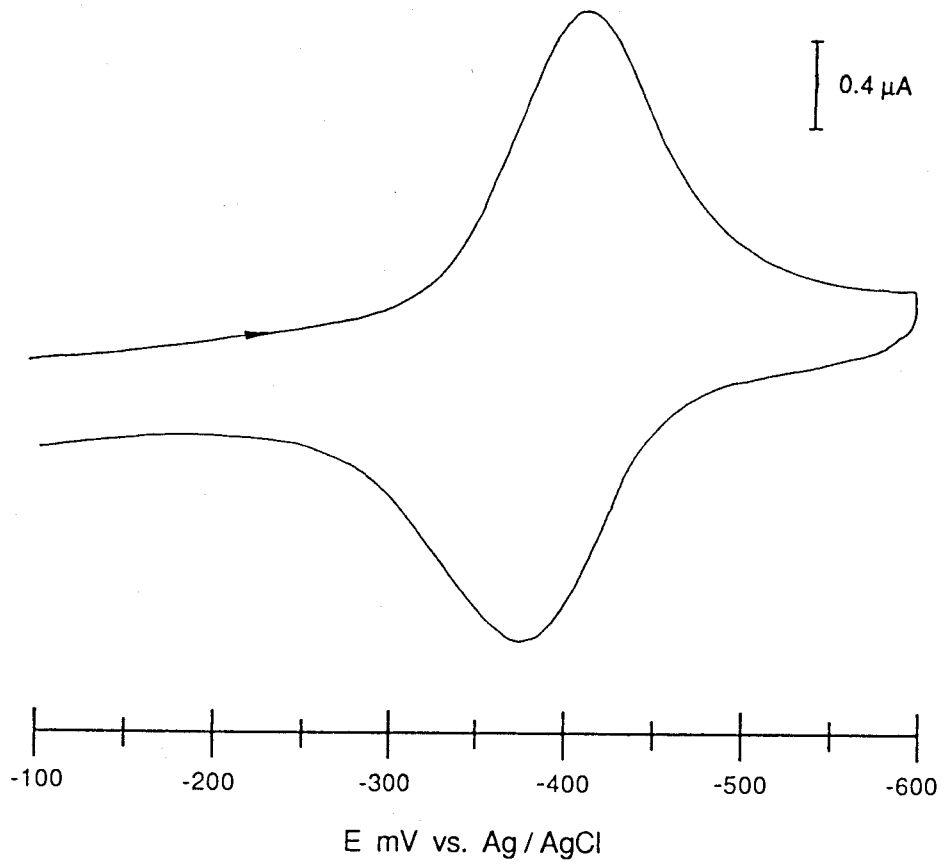
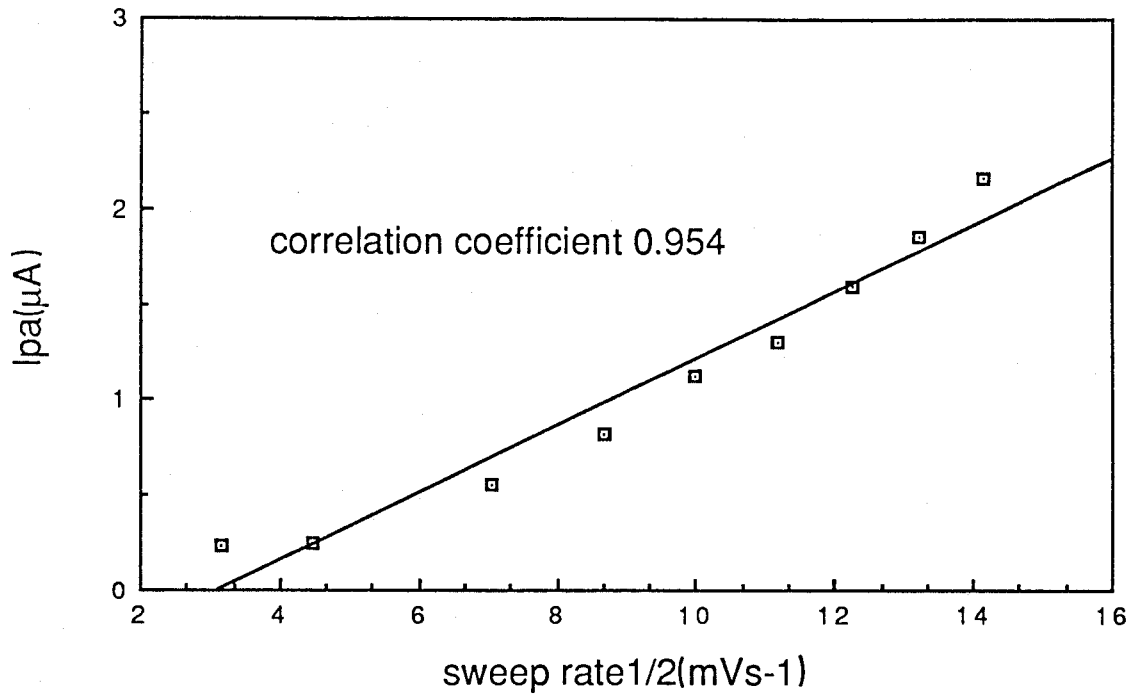


Figure 3.11

Cyclic voltammogram of bis-phenylthiourea-phenethylisoalloxazine adsorbed in a gold electrode, 0.1 M KH_2PO_4, 0.1 M KCl, pH 7.0, 50 mVs^{-1}.

Due to the proposed nature of binding of the flavin nucleus to the electrode surface, through the thiourea side arms, it may be that the mathematical treatment for adsorbed processes, in this case, is inaccurate. The mathematical treatment assumes that the electroactive part of the redox species is firmly located to the electrode. Molecular model calculations suggest that the flavin nucleus, with the 'side-arms' in their optim um positions, lies some 5 to 6 Å away from the electrode surface. The maximum distance that the flavin nucleus may be away from the electrode is 7 Å, based on molecular model measurements.



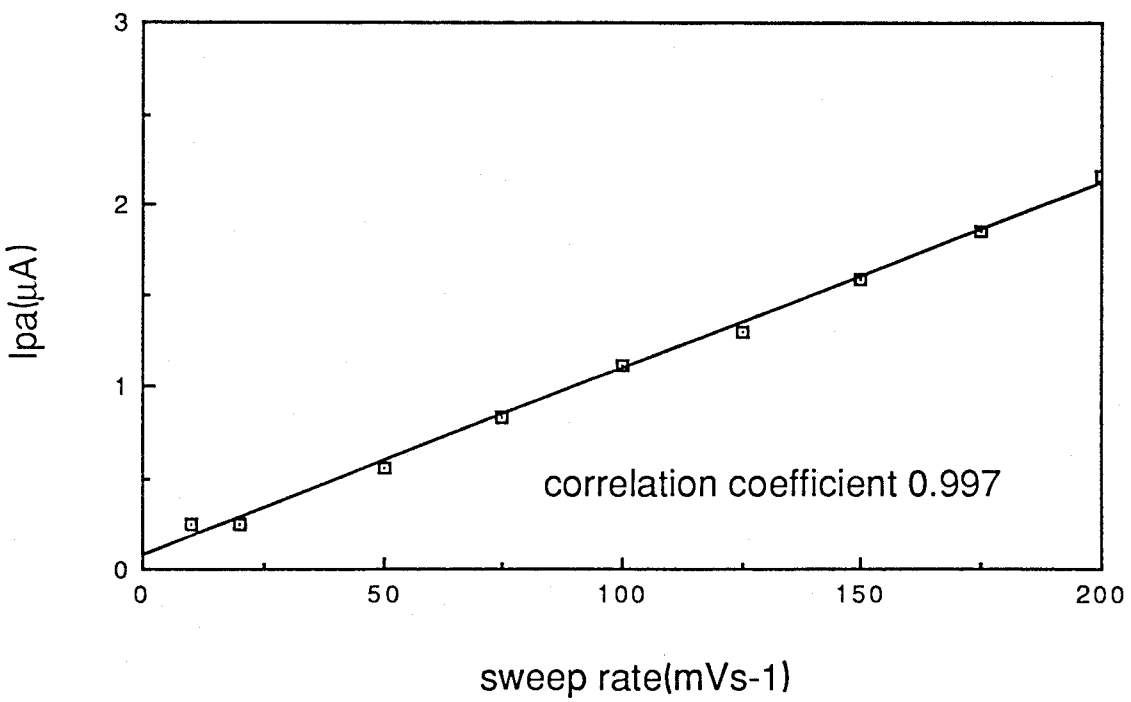


Figure 3.12

Peak current dependance of bis-phenylthioureaphenethylisoalloxazine with sweep rate and sweep rate$^{1/2}$, gold electrode, 0.1 M KH_2PO_4, 0.1 M KCl, pH 7.0

There is, therefore, sufficient solvent accessibility between the flavin and the electrode to give the flavin species some diffusional character. This may account for the comparitively low correlation of I_{pc} with sweep rate. Support for this can also be found from the half-height peak width, $\Delta E_{p\,1/2}$ which differs to that calculated for an ideal nernstian reaction. The peak width should equal 3.53RT/nF, which has been obtained for an ideal 'surface-wave'.[155,180] For the flavin system, n = 2 and this leads to a calculated value of 45 mV for $\Delta E_{p\,1/2}$. The average values for the bisthiourea flavin were 119 mV and 142 mV for $\Delta E_{pc\,1/2}$ and $\Delta E_{pa\,1/2}$ respectively. These findings indicate that the interactions among the adsorbed species must be taken into account when interpreting the voltammograms.

Continuous potential cycling for 30 minutes led to no visible reduction in the electrochemical response. Furthermore, extension of the potential range (-1100 to +300 mV vs. Ag/AgCl) to include the oxidation and reduction of the solvent lead to little or no reduction in peak height. This is in contrast to the work of Dufor[150] who observed that on cycling a mercaptopropylsilane-riboflavin modified gold electrode to -0.8 V vs. SCE, the disappearance of the flavin redox waves. This observation would indicate that the bis-thiourea flavin is firmly bound to the electrode surface.

3.4 Electron transfer rate determination

Electron transfer rates between a flavin nucleus and an electrode are considered to be one of the most important factors in the design and construction of electronic devices based on redox reactions. Several theoretical and experimental studies which deal quantitatively with redox electrode processes of electroactive species, fixed at the electrode surface in mono- or submono-layers, have been carried out.[166,181-186] Ueyama *et al.*[174] have formed flavin Langmuir-Blodgett monolayers on gold electrodes and studied the redox kinetics in order to determine the electron transfer rate. Hence, with the formation of suitably modified flavin electrode, a quantitative study of the behaviour of the adsorbed species with the surface of the electrode was of basic importance.

Cyclic voltammograms for the reduction and oxidation of the flavin bis-phenylthiourea phenethylisoalloxazine (**92**) were recorded at potential sweep rates from 0.005 to 400 Vs^{-1}, with no IR compensation, as the peak currents were in the microampere range. Typical spectra are shown in figure 3.13. The anodic and cathodic peak potential dependance with potential sweep rates are shown in figure 3.14. The difference between the anodic and cathodic peak potentials E_{pa} and E_{pc} was approximately 40 mV at slow sweep rates. This difference may be due to the conformational difference during the cathodic and anodic reactions. The oxidised flavins have a planar structure in solution, while the reduced flavin is distorted, with the flavin nucleus forming a butterfly configuration about the N(5) - N(10) axis, with a dihedral angle of 21°,[158] as shown in figure 3.3. The mean value of E_{pa} and E_{pc}, at $v < 0.2$ Vs^{-1}, -0.414 V was chosen as $E^{\circ'}$.

Daifuku *et al.*[186] showed that the α_a and α_c, the anodic and cathodic transfer coefficients, ($\alpha_a + \alpha_c = 1$), can be determined respectively from the slopes of the linear portions of E_{pa} and E_{pc} vs ln(v) plots for totally irreversible processes. Equation 3.5 for the anodic and cathodic processes predicts that the slopes of the linear plots of E_{pa} and E_{pc} vs. The value of ln(v) for totally irreversible processes are $RT/\alpha_a nF$ and $RT/\alpha_c nF$ respectively.

$$\Delta E_{pa\,1/2} = (RT/\alpha_a nF)\,[1.8217 - 1.0197\,(\Xi + \Delta\Xi^*) + 0.6216\exp\{0.4359\,(\Xi + \Delta\Xi^*) - 0.0183\,(\Xi + \Delta\Xi^*)^2\}\,] \qquad 3.5$$

where Ξ and $\Delta\Xi^*$ are interaction parameters. Comprehensive treatment of the mathematical derivations are given by Daifuku[186] and Laviron.[182,187]

The values of the the cathodic and anodic transfer coefficients α_a and α_c were determined to be 0.121 and 0.0726, respectively, from the values of the slopes in figure 3.14. By insertion of these, and the other known parameters into equation 3.5 and solving by an in-house computer programme, values of 2.1 and 3.12 were obtained for the anodic branch ($\Xi + \Delta\Xi^*$) and the cathodic branch ($\Xi - \Delta\Xi^*$) respectively.

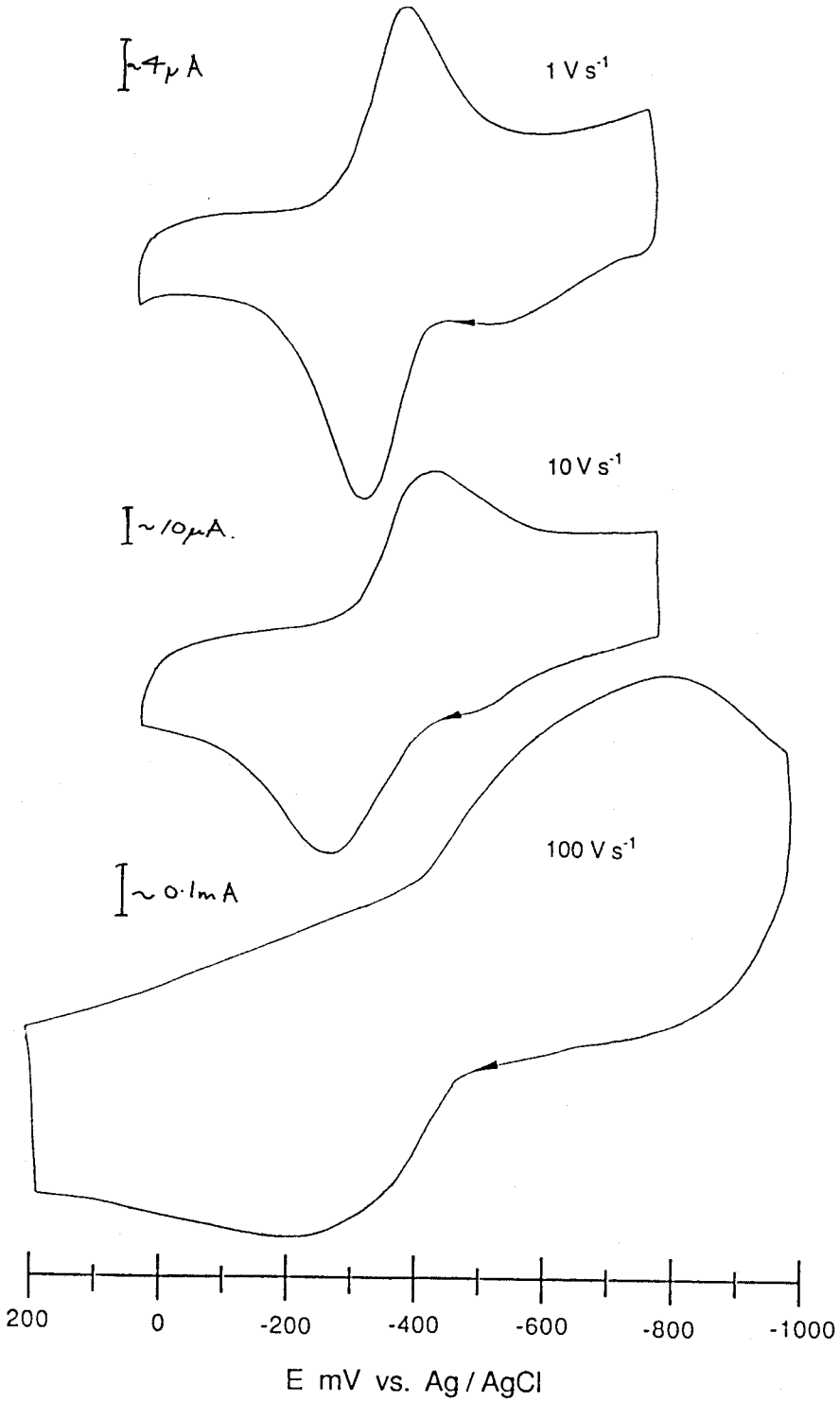


Figure 3.13

Observed peak potential dependence with sweep rate of bis-phenylthiourea-phenethylisoalloxazine, 0.1 KH_2PO_4, 0.1 M KCl, pH 7.0.

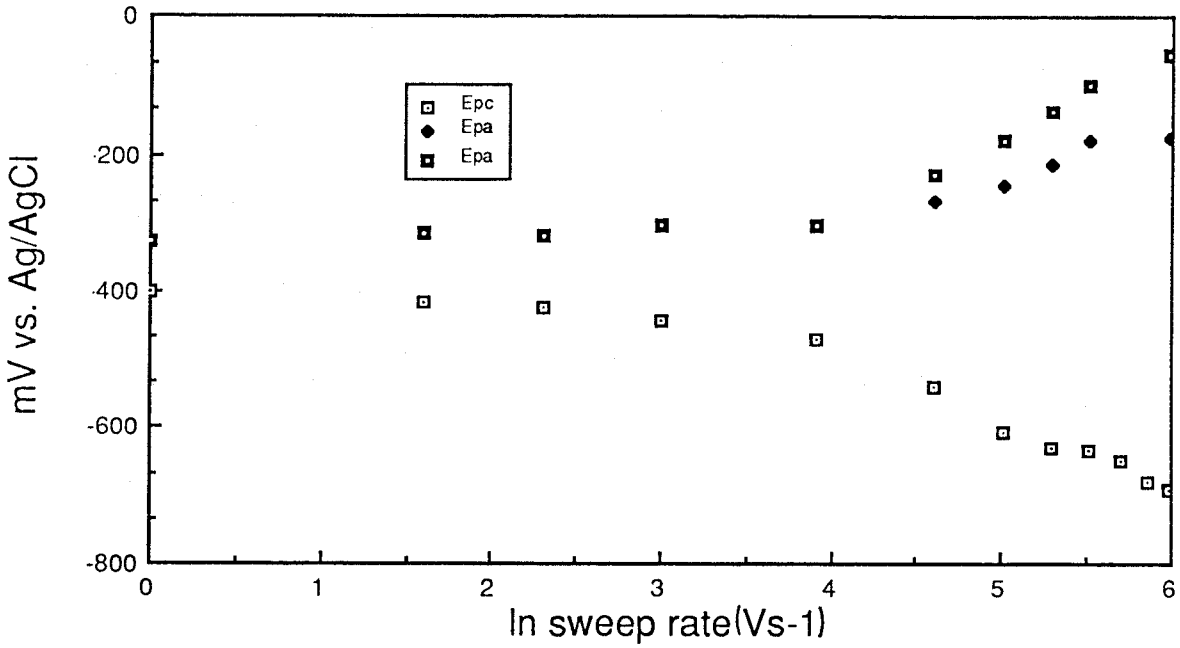


Figure 3.14

Plot of peak anodic and cathodic potential dependence with sweep rate for bis-phenylthioureaphenethylisoalloxazine, gold electrode, 0.1 M KH_2PO_4, 0.1 M KCl, pH 7.0.

Insertion of the value ($\Xi + \Delta\Xi^*$), in the anodic case, into equation 3.6 enabled the anodic formal rate constant, k°, for the surface redox-electrode to be determined,

$$E_{pa} = E^{\circ'} - (RT/\alpha_a nF)\,[\ln(2k^{\circ'}RT/\alpha_a nFv) + 0.0119 - 0.2358(\Xi + \Delta\Xi^*) + 0.0049(\Xi + \Delta\Xi^*)^2 - 0.00086(\Xi + \Delta\Xi^*)^3] \qquad 3.6$$

where E_{pa} is anodic peak potential at ln(v) = 0, for an irreversible process, determined by extrapolation of the linear portion of figure 3.14. Insertion of the known values into equation 3.6 gave:

$$-1.11 = -0.414 - \frac{8.314 \times 298}{0.0726 \times 2 \times 96484}\left[\frac{\ln 2k^{\circ'} \times 8.314 \times 298}{0.0726 \times 2 \times 96484 \times 1} + 0.0119 - 0.2358(3.12) + 0.0049(3.12)^2 - 0.00086(3.12)^3\right]$$

This gave a value for $k^{o'}$, the formal electrode rate constant of 2.92×10^2 s^{-1}. Similar treatment for the cathodic wave gave a value of 1.98×10^2 s^{-1}.

These values compare very favourably with the values determined by Ueyama *et al.*[174] for a flavin Langmuir-Blodgett monolayer. Ueyama and his group were only able to determine one formal rate constant, that for the cathodic process, as the anodic peaks were rather broad and would have led to large errors in determination of the anodic transfer coefficient. However, using the same method of calculation for the formal rate constant Ueyama obtained a value of 1.22 s^{-1} for the cathodic process.

With values for the rate constant >100 times larger than that for the flavin electrode of Ueyama, this led to the conclusion that the orientation of the flavin was more favourable to electron conduction in the case of the bis-thiourea flavin electrode.

3.5 Flavin Surface Coverage

It would be expected that different orientations of flavin at an electrode surface would affect the flavin surface coverage. Typically, flavins such as riboflavin, occupy an area of 150 $Å^2$, where the isoalloxazine moiety is parallel to the electrode surface,[162,163] this gives a surface coverage of 7 to 9×10^{-11} mol cm^{-2}.[166] The equation relating peak current to surface excess is given by:

$$i_p = n^2F^2/4RT.vA\Gamma_o \qquad 3.7$$

where v = sweep rate
Γ_o = surface excess (mol cm^{-2})
A = electrode surface area, 0.1 cm^2

A plot of i_{pa} and i_{pc} vs. sweep rate is shown in figure 3.15, where the slope is equivalent to $n^2F^2A\Gamma_o/4RT$. It can be seen that the anodic and cathodic peak currents, i_{pa} and i_{pc} are proportional to v at less than 0.2 Vs^{-1}, and that the proportionality factors are similar to those calculated from $n^2F^2A\Gamma_o/4RT$ for an ideal reversible wave.[155,180]

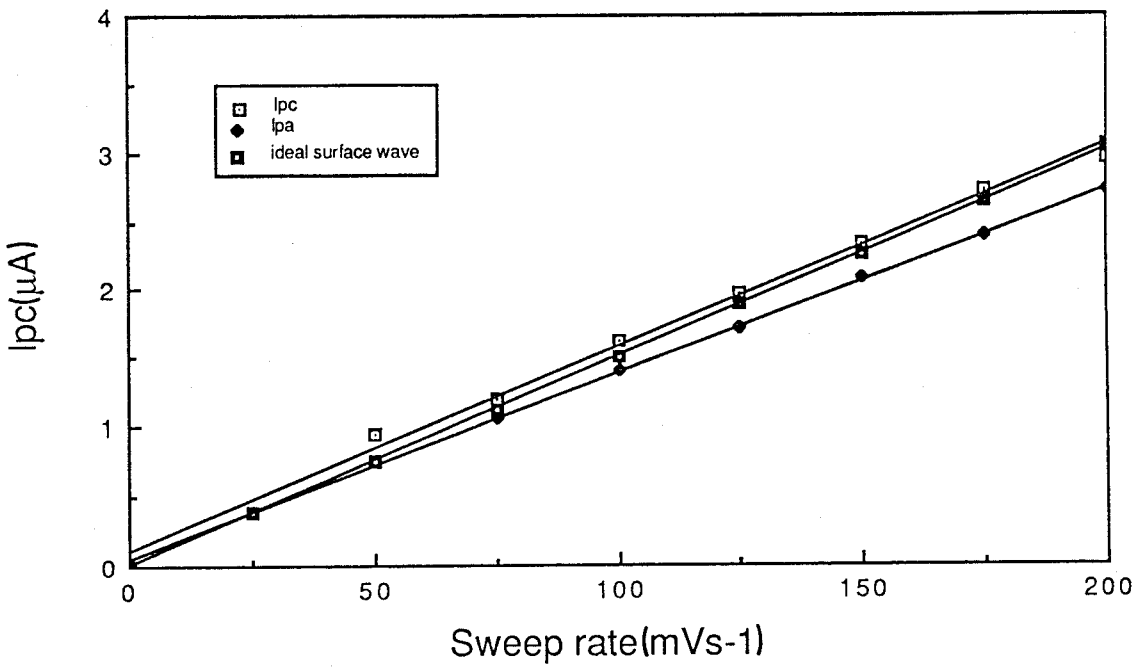


Figure 3.15

Variation of peak cathodic and anodic currents with sweep rate, compared to the calculated response for an ideal reversible surface wave

The averaged values of $\Delta E_{pa\,1/2}$ and $\Delta E_{pc\,1/2}$ were 142 and 119 mV respectively. These values are larger than 43.5 mV for an ideal wave[155] and it may therefore be expected that at higher sweep rates, >0.5 Vs^{-1}, the proportionality factors would be smaller than those calculated for an ideal reversible surface wave. This indicates that flavin-flavin interactions must be taken into account when interpreting the cyclic voltammograms. However, at slow sweep rates, <0.2 Vs^{-1}, the bis-thiourea flavin electrode has a similar response to that for an ideal reversible electrode.

It may be that at slow sweep rates <0.2 Vs^{-1} all flavin orientations undergo electron transfer, and at the higher sweep rates only the favourably orientated flavin molecules, those bifunctionally attached, undergo oxidation or reduction. Those molecules which are only attached at one site or have the flavin nucleus orientated in an unfavourable, perpendicular, orientation do not undergo rapid electron transfer. A representation of the flavin orientation is shown in figure 3.16.

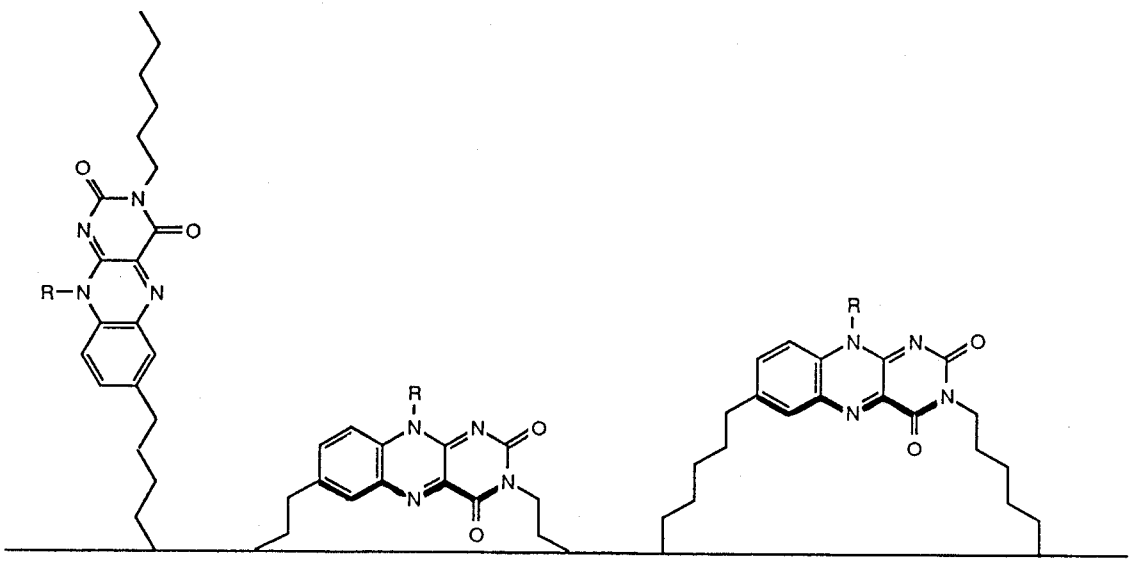


Figure 3.16

Possible orientations of the bifunctionalised flavins. All undergo electron transfer at slow sweep rates, however at higher sweep rates only the favourable, parallel face conformers undergo rapid electron transfer.

3.6 Conclusions

Transfer of an electron from one site to another in a molecule or between molecules is one of the most fundamental and ubiquitous processes in chemistry. Within the past few years rapid advances have occurred in many key areas, including electron transfer theory,[188,189] experiments on model reactions[190-192] and distance dependence of electron-transfer in biosystems.[193-196]

Electron transfer rates within and between molecules, as well as between electrodes, decay exponentially with the distance (d) between the involved centres,[197-199] i.e., $k \propto e^{-\beta d}$. In proteins, the electron-transfer rates drop by ~10^4 when the distance between an electron donor and an acceptor is increased from 8 to 17 Å.[193] However, it has been shown that significant electron transfer rates occur under physiological conditions across distances of 10 to 20 Å.[193,194] The distance between the bifunctionalised flavin and the electrode surface was calculated to be 5-6 Å. Fast reversible electrode kinetics were observed with this system, with some flavin-flavin interactions. At higher sweep rates the results indicated that the flavin electrode did not exhibit the fast reversible kinetics of an ideal reversible electrode. It was concluded that at slow sweep rates all the flavin molecules, including those at relatively large distances from the electrode, were redox active. As the sweep rate was increased only the flavin species close to, and favourably aligned with, the electrode underwent reduction. Furthermore, the flavin-flavin interactions must be slow and therefore at the faster sweep rates would appear as an irreversibility. Birss[168] has shown that the orientation of the flavin nucleus of FMN at a mecury electrode is dependent on the concentration of flavin in solution, where the attachment of the flavin to the electrode was through the adsorbtion interaction of the π-aromatic ring with the mercury. The concentration dependent orientations are shown in figure 3.4. The conformations available with the bifunctionalised bis-thiourea flavin system are fewer. This is because many rotometric conformations of the bound flavin are forbidden, due to the unfavourable Van der Waals interactions with gold. However, certain conformations which are not parallel to the gold electrode surface are allowed.

3.7 Future studies

Future objectives are to construct similar bifunctionalised flavin electrodes based on glassy carbon; through amine/carboxylic interactions; and through electrodes based on gold with other terminal thiol functionality. In order to understand the influence of the flavin-electrode distance and geometry relationship on the rate of electron transfer, flavins with side-arm linkages of different length will need to be synthesised, as will flavins which contain three linkage arms. With minor modifications the chemistry that has been developed should provide all of the necessary compounds.

Although the flavins described here can be precisely tailored to adsorb with the ring system a predetermined distance from the electrode surface, it is important that the orientation characteristics of the flavin are understood. Surface structure studies by near infrared spectroscopy and photoelectrospectroscopy should lead to a more accurate understanding of the nature of surface attachment and the orientation of the molecules at the electrode surface. The combination of these studies with further electron transfer experiments should lead to a fuller understanding of electron transfer and hence to the ability to design highly efficient electron transfer systems.

Experimental

Melting points were determined using a Kofler hot-stage or an electrothermal melting point apparatus. Melting points are uncorrected.

Elemental analyses were carried out at the microanalytical laboratory, University College, London.

Infrared spectra were recorded on a Perkin-Elmer 298 spectrometer, either as thin films between sodium chloride discs or as solutions In sodium chloride cells (thickness 0.1 mm). Adsorption maxima are given in wave numbers (cm^{-1}) relative to polystyrene standard.

^{1}H Nuclear magnetic resonance spectra were recorded at 60 MHz on a Hitachi-Perkin-Elmer R-24B spectrometer, at 90 MHz on a Jeol FX90Q, at 270 MHz on a Jeol JNM-GX270 and at 360 MHz on a Bruker AM360 instrument. All n.m.r. spectra are described as parts per million downfield shift from TMS and are reported consecutively as position (δ_H), relative integral multiplicity (s - singlet, d - doublet, t - triplet, q - quartet, m - multiplet, dd - double doublet and br - broad), coupling constant (*J* Hz) and assignment (numbering according to IUPAC nomenclature for the compound.[200]

^{13}C Nuclear magnetic resonance spectra were recorded at 67.9 MHz on a Jeol JNM-GX270 and at 90.56 MHz on a Bruker AM360 instrument.

Mass spectra and accurate mass measurements were recorded at Southampton University on a Kratos MS 30, or a VG 70 250 SE spectrometer, or by the SERC service at Swansea using a VG ZAB E spectrometer. Major fragments are given as percentages of the base peak density (100%). Fast atom bombardment (FAB) spectra were recorded using either glycerol or 3-nitro benzyl alcohol as matrix.

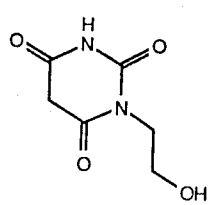
Flash chromatography was performed according to the procedure of Still and co-workers[201], using Macherey-Nagel Kieselgel 60 (230 - 400 mesh) silica gel.

Analytical thin layer chromatography was carried out on 0.25 mm precoated silica gel plates (MN SIL G/UV_{254}) and compounds were visualised by UV fluorescence, iodine vapour, phosphomolybdic acid in ethanol, aqueous potassium permanganate or ninhydrin.

Solvents were dried and purified according to the methods of Perrin, Armarego and Perrin[202].

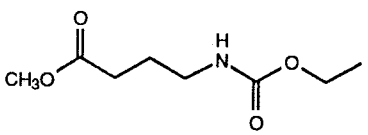
Nitrourea (**17**). - In a 3-necked, 500 ml flask, urea (20 g, 0.33 mol) was dissolved in previously cooled sulphuric acid (142 g, specific gravity 1.84) at 0 - 3°C. Fuming nitric acid (22.8 g, 42.3 ml) was added over 2½ hours, maintaining the temperature at 0 - 3°C. The resulting mixture was poured onto ice (172 g), filtered, washed with water and dried to give the desired product (21.48 g, 62%). A small sample was recrystallised from propyl alcohol, m.p. 156 - 158°C (lit.,[203, 204] 150 - 164°C); $\nu_{max.}$ (nujol) 3440w (-$CONH_2$), 3250w (CONH), 1750m (-CONH) and 1620b cm^{-1} (N-NO_2).

N-(2-hydroxethyl)-urea (**18**). - Nitrourea (**17**) (10 g, 95 mmol) was added slowly to a stirred solution of aminoethanol (4.88 g, 80 mmol) in water (6 ml), the temperature being maintained below 3°C. After allowing the mixture to stir at room temperature overnight, the water was removed *in vacuo*. The residual oil solidified on standing in the freezer to give white crystals. (6.2 g, 75%). A small sample was recrystallised from dioxan, m.p. 92 - 93°C (lit.,[205], 94-95°C) (Found: C, 34.7; H, 7.8; N, 27.0. $C_3H_8N_2O_2$ requires C, 34.6; H, 7.8; N, 26.9%); $\nu_{max.}$ (nujol) 3440m (CONH), 3300m (CONH), 1650s (CONHCO) and 1550m cm^{-1} (CONH); δ_H (60 MHz, D_2O) 3.15 (2H, t, *J* 6 Hz, CH_2O), and 3.48 (2H, t, *J* 6 Hz, CH_2N).

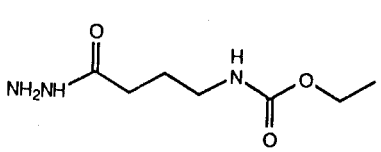


N-(2-Hydroxyethyl)-barbituric acid (**19**). - Sodium metal (0.58 g, 25.2 mmol) was added carefully to dry ethanol (14 ml) and diethyl malonate (2 g, 12.4 mmol), followed by a solution of N-(2-hydroxyethyl)-urea (**18**) (1.24 g, 12.4 mmol) in hot ethanol (14 ml). The mixture was refluxed for 7h at 110°C, during this time a white solid formed. Hot water (11 ml, 50°C) was added and the mixture acidified with hydrochloric acid (s.g. 1.18). The solvent was removed *in vacuo* and the residue treated with hot ethanol (20 ml). After filtration of the insoluble material, the solvent was removed *in vacuo* to give a yellow oil, which on triturating with ether gave a yellow solid (1.33 g, 65%), m.p. 115 - 120°C; $\nu_{max.}$ (nujol) 3380b (NH), 1700s cm^{-1} (CONHCO); δ_H (60 MHz, D_2O), 3.56 (2H, t, *J* 4 Hz, CH_2O) and 3.75 (2H, t, *J* 4 Hz, CH_2N).

4-Aminomethyl butyrate hydrochloride (**20**). - To a mixture of 4-aminobutyric acid (500 mg, 5 mmol) and methanol (25 ml) was added thionyl chloride (0.47 ml, 7.5 mmol) at -5°C over 15 mins. The mixture was allowed to warm slowly to room temperature and then refluxed for 1h. After removal of the solvent *in vacuo*, the solid was crystallised from methanol/ether to give white crystals (0.41 g, 70%), m.p. 118 - 120°C (lit.,[206] 117 - 118°C); $\nu_{max.}$ (nujol) 2000b (NH, amino salt), 1730m (CO_2-) and 1600m cm^{-1} (-NH_3^+); δ_H (360 MHz, D_2O) 1.99 (2H, m, C<u>H</u>$_2$CH$_2$NH$_3$Cl), 2.55 (2H, t, *J* 7.2 Hz, C<u>H</u>$_2$NH$_3$Cl), 3.07 (2H, t, *J* 7.7 Hz, CH_2CO) and 3.74 (3H, s, CH_3O).



<u>4-N-Ethylcarbamate-methyl butyrate</u> (**21**). - Triethylamine (1.2 ml, 8.6 mmol) was added at 0°C over 30 mins, to a mixture of the butyrate hydrochloride (**20**) (500 mg, 4.3 mmol), ethylchloroformate (0.49 ml, 5.2 mmol), THF (2 ml) and water (1 ml). After stirring overnight, the volatile solvents were removed *in vacuo* and the residue neutralised (pH 6-7) with saturated sodium bicarbonate, The aqueous phase was extracted with ether (3 x 10 ml), and the ether phase washed with water (3 x 10 ml), dried (Na_2SO_4) and evaporated to afford a colourless oil (0.47 g, 58%), $\nu_{max.}$ (neat) 3350w (CONH), 1720s (CO_2-) and 1700s cm^{-1} (CONH); δ_H (360 MHz, $CDCl_3$) 1.23 (3H, t, *J* 7.1 Hz, CH_2C<u>H</u>$_3$), 1.85 (2H, m, CH_2C<u>H</u>$_2$CH$_2$), 2.39 (2H, t, *J* 7.4 Hz, CH_2O), 3.20 (2H, q, *J* 6.5 Hz, C<u>H</u>$_2$NH), 3.68 (3H, s, CH_3CO), 4.10 (2H, q, *J* 7.1 Hz, C<u>H</u>$_2$CH$_3$) and 5.45 (1H, sb NH).

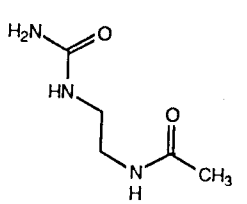


<u>3-Hydrazido-ethyl carbamate</u> (**22**). - A mixture of the carbamate (**21**) (500 mg, 2.6 mmol) and hydrazine hydrate (0.15 ml, 3.2 mmol) were refluxed in methanol (10 ml) for 1h after which time the methanol was removed under vacuum to give a white solid. The product was crystallised from ethyl acetate/petrol (410 mg, 82%), m.p. 41 - 43°C; ν_{max} (nujol) 3310s (NH),

1700s (NCO_2-), 1640s (NH_2) and 1560m cm^{-1} (CONH); δ_H (60 MHz, $CDCl_3$) 1.85 (2H, m, $CH_2\underline{CH_2}CH_2$), 2.20 (2H, t, *J* 6 Hz, CH_2CO), 3.15 (2H, q, *J* 6 Hz, $C\underline{H_2}NH$), 3.85 (2H, sb, $NHN\underline{H_2}$), 4.05 (2H, q, *J* 7 Hz, $C\underline{H_2}CH_3$), 5.45 (1H, sb, $NHCO_2$) and 8.00 (1H, sb, $N\underline{H}NH_2$).

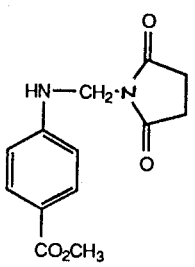
Diethyl-*iso*nitroso-malonate (**27**). - Sodium nitrite (9.5 g, 135 mmol) in water (20 ml) was added to a mixture of diethyl malonate (8 g, 50 mmol) in glacial acetic acid (8.6 ml) over 6h. The lower, aqueous layer was extracted with ether (3 x 10 ml), the two organic fractions combined, dried ($MgSO_4$) and the solvent removed *in vacuo*. The product was distilled to give a colourless oil (7 g, 74%), b.p. 176 - 178°C @ 13 mm Hg (lit.,[207] 172°C @ 12 mm Hg); ν_{max} (thin film) 3360s (OH), 3000s (CH), 1740m (CO_2-) and 1635w cm^{-1} (C=N-); δ_H (60 MHz, $CDCl_3$) 1.34 (6H, t, *J* 7 Hz, $CH_2C\underline{H_3}$), 4.28 (2H, q, *J* 7 Hz, $C\underline{H_2}CH_3$) and 4.31 (2H, q, *J* $C\underline{H_2}CH_3$).

3-Aminoethyl urea (**28**). - To a stirred solution of nitrourea (**17**) (5.25 g, 50 mmol) in water (3 ml) was added ethylenediamine (3.7 ml, 55 mmol) over 10 mins. The temperature was maintained at 55 - 60°C until the liberation of gas had ceased, the temperature was then increased to 90 - 100°C for 15 mins. The water was removed *in vacuo* and the crude material crystallised from ethanol, filtered, washed with ether to give the product **28** (3.81 g, 74%), m.p. 190-192°C (lit.,[208] HCl 139 - 140°C); $\nu_{max.}$ (nujol) 3420m (NH), 3350m (NH), 1655m (NCON) and 1601m cm^{-1} (NH_2); δ_H (60 MHz, DMSO-d_6) 2.81 (4H, dd, *J* 3 & 6 Hz, $(CH_2)_2$), 3.12 (2H, s, NH_2), 5.25 (2H, s, $CONH_2$) and 5.75 (1H, s, CONH).



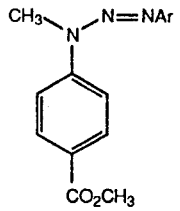
3-N-Acetylethyl urea (**29**). - A mixture of the mono-substituted urea (**28**) (0.75 g, 7.3 mmol) and acetic anhydride (2.4 ml, 18 mmol) were refluxed for 10 - 15 mins. After evaporation, the residual oil was crystallised from ethanol to give a white solid, which was filtered, washed with ether and dried (0.86 g, 81%), m.p. >300°C; $\nu_{max.}$ (nujol) 3300m (NH), 3150w (NH) and 1700s cm^{-1} (NCON); δ_H (60 MHz, DMSO-d_6) 1.89 (3H, s, CH_3CO), 2.80 (4H, dd, *J* 3 & 6 Hz, $(CH_2)_2$), 3.95 (1H, s, $CH_3CON\underline{H}$), 5.25 (2H, s, $CONH_2$) and 5.75 (1H, s, CONH).

4-Amino methylbenzoate (**33**). - 4-Aminobenzoic acid (6.55 g, 50 mmol) was placed in a two-necked flask with dry methanol (150 ml) at -5°C. Thionyl chloride (5.5 ml, 75 mmol) was added over 30 min. keeping the temperature below -5°C. The mixture was basified with potassium hydroxide solution (2.5 M) until the pH was just alkaline. White crystals, formed on cooling overnight, were filtered, washed with water and dried (7 g, 93%). A small sample was crystallised from aqueous ethanol, m.p. 110 - 111°C (lit.,[209] 112°C ($MeOH_{aq}$)); $\nu_{max.}$ (nujol) 3420w (NH), 3340w (NH), 1680m (NH_2) and 1600 cm^{-1} (Ar-H); δ_H (60 MHz, $CDCl_3$), 3.88 (3H, s, $COOCH_3$), 6.67 (2H, d, *J* 8 Hz, Ar-H) and 7.89 (2H, d, *J* 8 Hz, Ar-H).



4-(N-Methylsuccinimide)-amino-methyl benzoate (**34**). - A mixture of 4-amino-methyl benzoate (**33**) (5.25 g, 35 mmol), succinimide (4.17 g, 42 mmol) and 37% aqueous formaldehyde (3.18 ml) in ethanol (56 ml) were refluxed for 4h in a three necked flask. On cooling, white crystals formed, which were filtered and dried (4.48 g, 48%), m.p. 192 - 194°C; $\nu_{max.}$ (nujol) 3350w (NH), 1710s (CONHCO) and 1610m cm^{-1} (Ar-H); δ_H (60 MHz, DMSO-d_6) 2.54 (4H, s, $(CH_2)_2$), 3.64 (3H, s, CH_3), 4.70 (2H, d, *J* 7 Hz, NCH_2), 6.72 (2H, d, *J* 9 Hz, Ar-H), 7.15 (1H, t, *J* 6 Hz, NH) and 7.56 (2H, d, *J* 9 Hz, Ar-H).

4-N-Methylamino-methyl benzoate (**35**). - A warm solution of the benzoate (**34**) in dry DMSO (32 ml) was placed in a 250 ml flask. Sodium borohydride (0.57 g, 15 mmol) was added over a 5 - 10 min. period, followed by heating on a steam bath for 10 - 15 mins. The clear solution was poured onto cold water (100 ml), extracted with diethyl ether (3 x 50 ml), dried ($MgSO_4$) and the solvent removed *in vacuo* to give white crystals (1.56 g, 70%). A small sample was recrystallised from aqueous ethanol, m.p. 90 - 92°C (lit.,[210] 95.5°C); $\nu_{max.}$ (nujol) 3400m (NH), 1690m (CO_2CH_3) and 1610m cm^{-1} (Ar-H); δ_H (60 MHz, $CDCl_3$) 2.88 (3H, s, NCH_3), 3.85 (3H, s, $COOCH_3$), 6.55 (2H, d, *J* 8 Hz, Ar-H) and 7.89 (2H, d, *J* 8Hz, Ar-H).



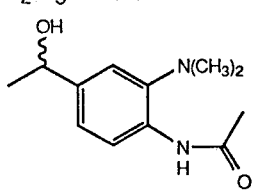
4-N-Methylamino-N-phenyltriazine-methyl benzoate (**37**). - A solution of aniline (1.45 g, 16 mmol) in a mixture of hydrochloric acid (s.g. 1.18, 4.3 ml) and water (10 ml) was cooled to 0°C. Sodium nitrite (1.08 g, 1.56 mmol) was added at 3°C or below and then maintained at 0°C for 30 min. A suspension of the ester (**35**) (2 g, 13 mmol) in water (25 ml) was treated with hydrochloric acid (s.g. 1.18, 4.1 ml) and anhydrous sodium acetate (4.1 g, 50 mmol) and then cooled to -5°C. The solution of diazotised aniline was added to the suspension of the methyl benzoate at 0°C and stirred for a further 2h at that temperature. After warming to 20°C, the stirred solution was treated with a solution of anhydrous sodium acetate (3.98 g, 47.4 mmol) in water (32 ml) whilst maintaining the pH (~3) and temperature (17 - 20°C) constant. The crude triazine was filtered, washed with water and dried (2.02 g, 56%). A small sample was recrystallised from aqueous methanol to give orange crystals, m.p. 103 - 105°C (Found: C, 67.0; H, 5.6; N 15.6. $C_{15}H_{15}N_3O_2$ requires C, 66.9; H, 5.6; N, 15.6%); $\nu_{max.}$ (nujol) 1720s (CO_2CH_3) and 1610m cm^{-1} (Ar); δ_H (360 MHz, $CDCl_3$) 3.65 (3H, s, CO_2CH_3), 3.90 (3H, s, NCH_3), 7.25 (1H, t, *J* 10 Hz, Ar-H), 7.44 (2H, t, *J* 10 Hz, Ar-H), 7.50 (2H, d, *J* 12 Hz, Ar-H), 7.59 (2H, d, *J* 10 Hz, Ar-H) and 8.05 (2H, d, *J* 10 Hz, Ar-H); δ_C (90.56 MHz, $CDCl_3$) 31.60 ($CO_2\underline{C}H_3$), 51.98 (NCH_3), 115.67 (Ar-<u>C</u>), 121.78 (Ar-<u>C</u>), 124.72 ($\underline{C}CO_2CH_3$), 127.51 (Ar-<u>C</u>), 129.17 (Ar-<u>C</u>), 131.12 (Ar-<u>C</u>), 148.90 ($\underline{C}NCH_3$) and 150.08 (Ar<u>C</u>N).

4-Acetamido-acetophenone (**41**). - 4-Aminoacetophenone (3.3 g, 24 mmol) was dissolved in acetic anhydride (10 ml, 100 mmol) and allowed to stand for 3h at room temperature. The off-white solution was then poured onto crushed ice (100 ml) and allowed to stand for 5 min. The precipitated solid was filtered, washed with water and recrystallised from aqueous ethanol (3.7 g, 92%), m.p. 170-172°C (lit.,[211] 174 - 175°C); $\nu_{max.}$ (nujol) 3350w (CONH), 1685m (CONH), 1610m (Ar-H) and 1530m cm^{-1} (CONH).

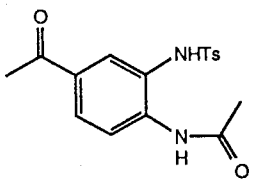
3-Nitro-4-acetamidoacetophenone (**42**). - 4-Acetamido-acetophenone (**41**) (3 g, 16.9 mmol) was added over 30 min. to well-stirred nitric acid (s.g. 1.49, 15 ml) at 0°C. The mixture was allowed to stand for 15 min. and then poured onto ice (50 ml). After filtration and washing with water, the residue was recrystallised from ethanol to afford yellow crystals (2.1 g, 56%),

m.p. 134 - 136°C (lit.,[212] 137°C), $\nu_{max.}$ (nujol) 3420w (CONH), 1685m (Aryl CO), 1590m (Ar) and 1550 cm^{-1} (NO_2); δ_H (60 MHz, $CDCl_3$), 2.28 (3H, s, $NHCOC\underline{H}_3$), 2,57 (3H, s, $ArCOCH_3$), 8.04 (1H, dd *J* 3 & 9 Hz, 6-H), 8.61 (1H, d, *J* 3 Hz, 5-H) and 8.76 (1H, d, *J* 9 Hz, 2-H).

3-Amino-4-acetamidoacetophenone (**43**). - 3-Nitro-4-acetamidoaceto phenone (**42**) (500 mg, 2.25 mmol) in ethylacetate/methanol (1:1, 25 ml) was stirred for 24 h. under an atmosphere of hydrogen with palladium on charcoal (50 mg) as the catalyst. After removal of the catalyst by filtration, the solvent was removed *in vacuo*. Suction chromatography (CH_2Cl_2/EtOAc 5:1) yielded **43** (390 mg, 91%), (Found: M^+, 192.0876. $C_{10}H_{12}N_2O_2$ requires *M*, 192.0898); $\nu_{max.}$ (nujol) 3420w (CONH), 3380w (NH), 1680m (Aryl-CO), 1650m (NH) and 1590m cm^{-1} (Ar); δ_H (360 MHz, DMSO-d_6) 2.13 (3H, s, $C\underline{H}_3CONH$), 2.52 (3H, s, CH_3CO), 5.25 (2H, bs, $-NH_2$), 7.24 (1H, dd, *J* 2.4 & 7.7 Hz, 6-H), 7.38 (1H, d, *J* 2.5 Hz, 5-H) and 7.55 (1H, d, *J* 8.1 Hz, 2-H); *m/z* 192 (M^+, 59%), 174 (20, $(M\text{-}H_2O)^+$), 149 (13, $(M\text{-}C_2H_3O)^+$) and 135 (100, $(M\text{-}C_2H_3NO)^+$).



2-Acetamido-5-(1-hydroxyethyl)-NN-dimethylaniline (**45**). - 3-Nitro-4-acetamido-acetophenone (**42**) (50 mg, 0.23 mmol) in dry ethanol (3 ml) was hydrogenated over palladium/charcoal (10%, 5 mg) in the presence of formaldehyde (50 µl, 0.66 mmol). After stirring for 3 h. the mixture was filtered through celite, and the solvent volume reduced *in vacuo*. This remaining mixture was subjected to suction chromatography ($CHCl_3$/EtOAc 5:1, silica gel) to yield a white powder (48 mg, 89%), (Found: M^+, 222.1397. $C_{12}H_{18}N_2O_2$ requires *M*, 222.1368); $\nu_{max.}$ ($CHCl_3$) 3600w (NH), 3350w (CONH), 1685m (CONH), 1610w (ArH) and 1520m cm^{-1} (CONH); δ_H (90MHz, $CDCl_3$) 1.48 (3H, d, *J* 6 Hz, CH_3CAr), 2.19 (3H, s, $NHCOC\underline{H}_3$), 2.65 (6H, s, $N(CH_3)_2$), 4.83 (1H, q, *J* 6 Hz, $CH_3C\underline{H}COH$), 7.05 (1H, dd, *J* 8.3 & 1.6 Hz, 6-H), 7.21 (1H, d, *J* 1.3 Hz, 5-H), 8.25 (1H, d, *J* 8.6 Hz, 2-H) and 8.38 (1H, bs, NH); *m/z* 222 (M^+, 100%), 204 (33, $(M\text{-}H_2O)^+$), 179 (97, $(M\text{-}C_2H_3O)^+$), 179 (97, $M\text{-}C_2H_6N)^+$), 165 (32, $(M\text{-}C_2H_3NO)^+$).

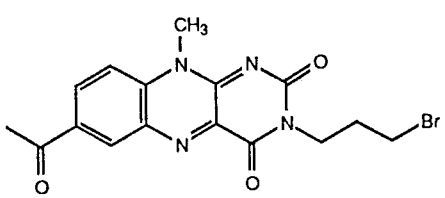


3-(N-Toluene sulphonyl)-amino-4-acetamidoacetophenone (**46**). - Toluene sulphonyl chloride (324 mg, 1.7 mmol) in chloroform (4 ml) was added carefully to 3-amino-4-acetamidoacetophenone (**43**) (300 mg, 1.5 mmol) in water (20 ml). Pyridine (2 drops) was added and the mixture stirred for 30 mins, extracted with dichloromethane (3 x 20 ml), dried (Na_2SO_4) and the solvent removed *in vacuo*. Suction chromatography (silica gel, CH_2Cl_2/EtOAc 5:1) yielded the mono-tosyl derivative (**46**) (462 mg, 85.5%) and the di-tosyl derivative (31 mg, 9%) as a minor product, (Found: M^+, 346.0995. $C_{17}H_{18}SN_2O_4$ requires *M*, 346.0987); $\nu_{max.}$ (nujol) 3420w (CONH), 1780m (CONH), 1335s (SO_2-) and 1160 cm-^{1} (SO_2-); δ_H (90 MHz, DMSO-d_6) 1.97 (3H, s, $ArCH_3$), 2.33 (3H, s, CH_3CONH), 2.45 (3H, s, $COCH_3$), 7.50 (7H, m, Ar-H), 9.30 (1H, bs, NH) and 9.52 (1H, s, NH); *m/z* 346 (M^+, 12%), 313 (24, (M-CH_5O)$^+$), 191 (24, (M-$C_7H_7SO_2$)$^+$) and 149 (100, (M-$C_9H_{10}SO_3$)$^+$).

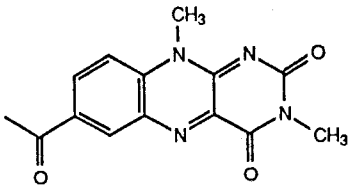
4-Chloro-3-nitro-acetophenone (**47**). - 4-Chloro-acetophenone (4.4 ml, 34 mmol) was added dropwise over 15 min. to a stirred solution of sulphuric acid (s.g. 1.84, 40 ml) and nitric acid (s.g. 1.42, 20 ml) cooled to -5°C. After stirring for 30 mins, the mixture was poured over ice water (250 ml). The resulting crystals were collected, washed with several portions of ice-water and recrystallised from methanol (5.5 g, 81%), m.p. 100 - 102°C (lit.,[213] 99 - 100°C); $\nu_{max.}$ (nujol) 1680m (Aryl-CO), 1590m (Ar) and 1550w cm^{-1} (NO_2); δ_H (60 MHz, $CDCl_3$), 2.59 (3H, s, $COCH_3$), 7.48 (1H, d, *J* 6.6 Hz, 5-H), 7.93 (1H, dd, *J* 2.4 & 7 Hz, 6-H) and 8.23 (1H, d, *J* 2.4 Hz, 2-H).

4-N-Methylamino-3-nitro-acetophenone (**48**). - An aqueous solution of methylamine (25%, 7 g, 57 mmol) was added to a stirred solution of 4-chloro-3-nitro-acetophenone (**47**) (2 g, 10 mmol) in ethanol (15 ml). After refluxing for 3h, ammonium hydroxide was added and the turbid solution was allowed to stand at 4°C overnight. The product which precipitated was collected and recrystallised from methanol to give orange needles (1.52 g, 78%), m.p. 174 - 174°C (lit.,[214] 167 - 169°C) (Found: C, 55.7; H, 5.3; N, 14.4; M$^+$, 194.0691. $C_9H_{10}N_2O_3$ requires C, 55.7; H, 5.2; N, 14.4%; *M*, 194.0691); δ_H (90 MHz, $CDCl_3$) 2.56 (3H, s, $COCH_3$), 3.10 (3H, d, *J* 6 Hz, $NHCH_3$), 6.90 (1H, d, *J* 10 Hz, 5-H), 8.07 (1H, dd, *J* 2.5 & 10 Hz, 6-H) and 8.78 (1H, d, *J* 2.6 Hz, Ar-H).

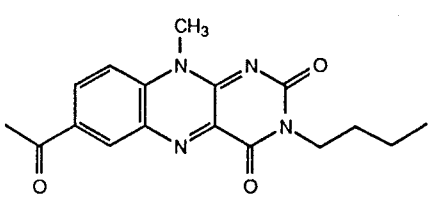
7-Acetyl-10-N-methylisoalloxazine (**50**). - 4-N-methylamino-3-nitro-acetophenone (**48**) (0.5 g, 2.58 mmol) was dissolved in ethyl acetate (60 ml) and catalytically hydrogenated over palladium on charcoal (10%, 50 mg). When the reduction was complete, as judged by tlc, the solution was filtered through a plug of celite and evaporated to dryness. The residue was dissolved in absolute ethanol (100 ml). Alloxan monohydrate (415 mg, 2.59 mmol) in hydrochloric acid (s.g. 1.18, 5 ml) was added and the solution refluxed for 15 min. The product crystallised upon standing at 4°C, and was washed with ether and vacuum sublimed (485 mg, 69%), m.p. 294°C dec. (lit.,[71] 292 - 295°C dec.) (Found: C, 57.4; H, 3.7; N, 20.8%; M^+, 270.0762. $C_{13}H_{10}N_4O_3$ requires C, 57.8; H, 3.7; N, 20.7%; *M*, 270.0752); $\lambda_{max.}$ (Phosphate buffer, 50 mM, pH 7.0) 426 (ϵ 11 600 $M^{-1}cm^{-1}$), 344 (5 250), and 285 nm (34 000); $\nu_{max.}$ (nujol) 1730m, 1600m and 1570s cm^{-1}; δ_H (360 MHz, CF_3CO_2D) 3.01 (3H, s, CH_3), 4.66 (3H, s, NCH_3), 8.49 (1H, d, *J* 7 Hz, 9-H), 9.03 (1H, d, *J* 10 Hz, 8-H) and 9.23 (1H, bs, 6-H); δ_C (67.9 MHz, CF_3CO_2D), 27.75 ($C_{7\beta}$), 39.14 (NCH_3), 120-147 (Aromatics), 153.07 (C_2) and 161.37 (C_4).



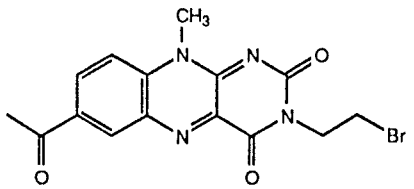
3-N-(3-Bromopropyl)-7-acetyl-10-N-methylisoalloxazine (**51**). - 7-Acetyl-10-N-methylisoalloxazine (50 mg, 0.18 mmol) and potassium carbonate (43 mg, 0.31 mmol) in DMF (5 ml) were heated on an oil bath to 60 - 70°C. 1,3-dibromopropane (209 µl, 1.8 mmol) was added to the mixture and the temperature maintained at 60 - 70°C for 3h. Water (2 ml) was added and the mixture extracted with dichloromethane (3 x 10 ml), washed with water (4 x 10 ml), dried (Na_2SO_4) and the solvent removed *in vacuo*. Crystallisation from DMF/ether gave yellow crystals (31 mg, 44%), m.p. 210°C dec. (Found: C, 48.9; H, 4.2; Br, 20.0; N, 13.5%; $(M+H)^+$, 391.0413. $C_{16}H_{15}BrN_4O_3$ requires C, 49.1; H, 3.9; Br, 20.4; N, 14.3%; (*M+H*), 391.0408); $\lambda_{max.}$ (DMF) 433 (ϵ 8 800 $M^{-1}cm^{-1}$), and 289 nm (35 800); δ_H (270 MHz, $CDCl_3$) 2.33 (2H, quintet, *J* 7 Hz, $CH_2\underline{CH_2}CH_2Br$), 2.73 (3H, s, CCH_3), 3.48 (2H, t, *J* 6.9 Hz, $CH_2CH_2\underline{CH_2}Br$), 4.15 (3H, s, NCH_3), 4.27 (2H, t, *J* 6.9 Hz, $\underline{CH_2}CH_2CH_2Br$), 7.74 (1H, d, *J* 9 Hz, 9-H), 8.50 (1H, dd, *J* 2.2 & 9 Hz, 8-H) and 8.84 (1H, d, *J* 2 Hz, 6-H); δ_C (67.9 MHz, $CDCl_3$) 26.71 ($C_{7\beta}$), 30.34 ($-CH_2Br$), 31.13 ($-\underline{CH_2}CH_2Br$), 32.50 (NCH_3), 41.35 (NCH_2), 115.89 (C_9), 134.21 (C_8), 134.61 (C_6), 134.78 (C_{5a}), 136.38 (C_{9a}), 149.84 (C_{10a}), 155.17 (C_2), 159.24 (C_4) and 195.57 ($C_{7\alpha}$).



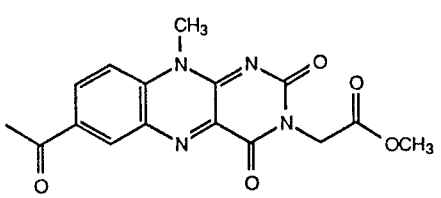
3-N-Methyl-7-acetyl-10-N-methylisoalloxazine (**52**). - *n*-Butyl lithium (0.21 ml, 0.21 mmol) was added to a solution of di-*iso*propylamine (0.19 ml, 1.4 mmol) in dry THF (3 ml) at 0°C. After stirring at 0°C for 30 min, the mixture was added to a solution of 7-acetyl-10-N-methylisoalloxazine (**50**) (50 mg, 0.18 mmol) in freshly distilled DMF (10 ml) at -50°C. After a further 30 min at -50°C, methyl iodide (112 µl, 1.8 mmol) was added and the mixture allowed to warm slowly to room temperature for 1h. The organic solvents were removed *in vacuo* and the residue extracted with dichloromethane (3 x 10 ml), washed with water (3 x 10 ml) and dried (Na_2SO_4). After removing the solvent *in vacuo*, the product (**52**) was recrystallised from trifluoroacetic acid/diethyl ether to give yellow crystals (35 mg, 68%), m.p. 240°C dec. (Found: $(M+H)^+$, 285.0981. $C_{14}H_{12}N_4O_3$ requires $(M+H)$, 285.0988); δ_H (270 MHz, $CDCl_3$) 2.73 (3H, s, CH_3CO-), 3.54 (3H, s, -$CONCH_3$), 4.15 (3H, s, N-CH_3), 7.73 (1H, d, *J* 8.8 Hz, 9-H), 8.50 (1H, dd, *J* 2 & 9 Hz, 8-H) and 8.88 (1H, d, *J* 2 Hz, 6-H); δ_C (67.9 MHz, $CDCl_3$) 26.79 (s, $C_{7\beta}$), 29.16 (s, N(3)-CH_3), 32.55 (N-CH_3), 115.94 (C_9), 134.30 to 135.04 (aromatics), 136.48 (C_{9a}), 149.82 (C_{10a}), 155.76 (C_2), 159.49 (C_4) and 195.71 ($C_{7\alpha}$).



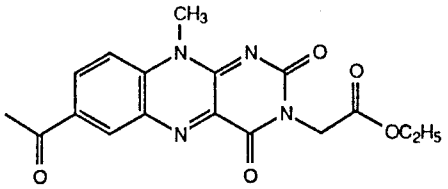
3-N-Butyl-7-acetyl-10-N-methylisoalloxazine (**53**). - Procedure as for (**52**), using 1-bromobutane (193 µl, 1.8 mmol) stirring at room temperature for 1h. Purification by suction chromatography (silica gel, CH_2Cl_2/5% MeOH) gave yellow crystals (44 mg, 75%), m.p. 205 - 207°C (Found: $(M+H)^+$, 327.1448. $C_{17}H_{18}N_4O_3$ requires $(M+H)$, 327.1457); δ_H (270 MHz, $CDCl_3$) 0.93 (3H, t, *J* 7.5 Hz, -$CH_2CH_2CH_2CH_3$), 1.37 (2H, sextet, *J* 7.5 Hz, -CH_2CH_3), 1.66 (2H, pentet, *J* 7.5 Hz, -$CH_2CH_2CH_3$), 2.69 (3H, s, CH_3CO-), 4.05 (2H, t, *J* 7.5 Hz, NCH_2CH_2-), 4.11 (3H, s, N-CH_3), 7.71 (1H, d, *J* 9 Hz, 9-H), 8.44 (1H, dd, *J* 1.7 & 9 Hz, 8-H) and 8.79 (1H, d, *J* 1.7 Hz, 6-H); δ_C (67.9 MHz, $CDCl_3$) 13.98 (-CH_2CH_3), 20.34 (-CH_2CH_3), 26.69 (-$COCH_3$), 29.92 (NCH_2CH_2-), 32.36 (N-CH_3), 42.41 (N-CH_2CH_2-), 115.80 (C_9), 134.16 to 134.98 (aromatics), 136.41 (C_{9a}), 138.24 (C_7), 149.74 (C_{10a}), 155.33 (C_2), 159.10 (C_4) and 195.63 ($C_{7\alpha}$).



3-N-(2-Bromoethyl)-7-acetyl-10-N-methylisoalloxazine (**54**). - Procedure as for (**52**), but using 1,2 dibromoethane (155 µl, 1.8 mmol) and stirring at room temperature over 3h. The product (**54**) was purified by suction chromatography (silica gel, CH_2Cl_2/5% MeOH) to give yellow crystals (51 mg, 76%), m.p. 220°C dec. (Found: $(M+H)^+$, 377.0256. $C_{15}H_{13}BrN_4O_3$ requires $(M+H)$, 377.0249); δ_H (270 MHz, $CDCl_3$) 2.72 (3H, s, CH_3CO-), 3.65 (2H, t, *J* 6.9 Hz, -CH_2Br), 4.16 (3H, s, N-CH_3), 4.52 (2H, t, *J* 6.9 Hz, N-CH_2-), 7.74 (1H, d, *J* 9 Hz, 9-H), 8.50 (1H, dd, *J* 1.9 & 9 Hz, 8-H), 8.84 (1H, d, *J* 1.9 Hz, 6-H); δ_C (67.9 MHz, $CDCl_3$) 26.69 ($CO\underline{C}H_3$), 27.73 (-CH_2Br), 32.57 (N-CH_2-), 115.96 (C_9), 134.19 to 135.05 (aromatics), 136.36 (C_{9a}), 137.96 (C_7), 149.91 (C_{10a}), 154.82 (C_2), 159.82 (C_4), 195.54 ($C_{7\alpha}$).

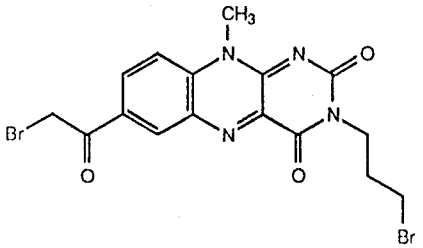


3-N-(Methylacetate)-7-acetyl-10-N-methylisoalloxazine (**55**). - Procedure as for (**52**) but using methyl bromoacetate (170 µl, 1.8 mmol). The product (**55**) was crystallised from dichloromethane/ diethyl ether to give yellow crystals (57 mg, 93%), m.p. 252°C dec. (Found: $(M+H)^+$, 343.1057. $C_{16}H_{14}N_4O_5$ requires M, 343.1043); $\lambda_{max.}$ (DMF) 290 (ϵ 15 230 $M^{-1}cm^{-1}$) and 435 nm (3 550); δ_H (270 MHz, $CDCl_3$) 2.73 (3H, s, -$COCH_3$), 3.78 (3H, s, -$COOCH_3$), 4.17 (3H, s, N-CH_3), 4.87 (2H, s, N-CH_2-), 7.75 (1H, d, *J* 9 Hz, 9-H), 8.51 (1H, d, *J* 1.9 & 9 Hz, 8-H) and 8.85 (1H, d, *J* 1.9 Hz, 6-H); δ_C (67.9 MHz, $CDCl_3$) 26.72 ($C_{7\beta}$), 32.65 (N-CH_3), 42.93 (N-CH_2), 52.78 (-$COO\underline{C}H_3$), 115.99 (C_9), 134.23 to 136.42 (aromatics), 150.13 (C_{10a}), 154.72 (C_2), 158.94 (C_4), 168.17 ($\underline{C}OOCH_3$) and 195.57 ($C_{7\alpha}$).

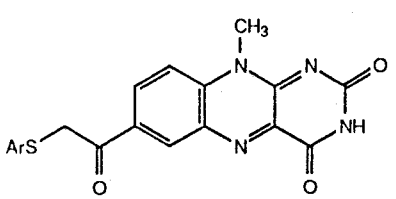


3-N-(Ethylacetate)-7-acetyl-10-N-methylisoalloxazine (**56**). - Procedure as for (**52**) but with ethyl bromoacetate (199 µl, 1.8 mmol). The product (**56**) was crystallised from dichloromethane/diethyl ether to give yellow crystals (39 mg, 61%), m.p. 228 - 230°C (Found: $(M+H)^+$, 357.1207. $C_{17}H_{16}N_4O_5$ requires $(M+H)$ 357.1199); $\lambda_{max.}$ (DMF) 274 (ϵ 10 600 $M^{-1}cm^{-1}$) and 450 nm (2 970); δ_H (270 MHz, $CDCl_3$) 1.27 (3H, t, *J* 7.1 Hz, $-CH_2\underline{C}H_3$), 2.70 (3H, s, CH_3CO-), 4.15 (3H, s, N-CH_3), 4.20 (2H, quartet, *J* 7.1 Hz, $-\underline{C}H_2CH_3$), 4.81 (2H, s, N-CH_2), 7.75 (1H, d, *J* 9 Hz, 9-H), 8.48 (1H, dd, *J* 2.1 & 9 Hz, 8-H) and 8.81 (1H, d, *J* 1.9 Hz, 6-H); δ_C (67.9 MHz, $CDCl_3$) 14.23 ($-CH_2\underline{C}H_3$), 26.72 ($C_{7\beta}$), 32.66 (s, N-CH_3), 42.69 (s, N-CH_2-), 61.76 (s, $-\underline{C}H_2CH_3$), 116.13 (s, C_9), 134.07 to 136.42 (aromatics), 137.96 (s, C_{9a}), 150.07 (s, C_{10a}), 154.70 (s, C_2), 158.91 (s, C_4), 167.68 (s, $-\underline{C}OOC_2H_5$) and 195.61 (s, $C_{7\alpha}$); *m/z* 356 (M^+, 30%), 283 (100), 227 (10) and 77 (3).

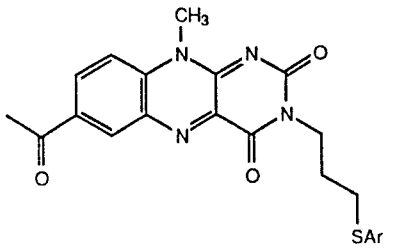
7α-Bromoacetyl-10-N-methylisoalloxazine (**57**). - A mixture of the flavin (**50**) (50 mg, 0.18 mmol), N-bromosuccinimide (36 mg, 0.2 mmol) and a trace of benzoyl peroxide were stirred at 50°C for 2h in trifluoroacetic acid (15 ml). The succinimide was then precipitated by the addition of water (10 ml) and filtered. The filtrate was reduced in volume under vacuum and the residue crystallised from trifluoroacetic acid/ether (53 mg, 84%), m.p. 195°C dec. (Found: $(M+H)^+$, 348.9928. $C_{13}H_9BrN_4O_3$ requires $(M+H)$, 348.9936); $\nu_{max.}$ (nujol) 1725m, 1600m, 1572s and 700s cm^{-1}; δ_H (270 MHz CF_3CO_2D) 4.78 (2H, s, CH_2Br), 4.67 (3H, s, NCH_3), 8.17 (1H, bs, 9-H), 8.93 (1H, bs, 8-H) and 9.27 (1H, bs, 6-H); δ_C (67.9 MHz, CF_3CO_2D) 30.25 (CH_2Br), 38.50 (CH_3), 120.90 (C_9), 135.59 (C_7), 137.48 (C_8), 137.74 (C_{4a}), 141.03 (C_{5a}), 142.14 (C_{9a}), 148.06 (C_{10a}), 153.76 (C_2), 161.61 (C_2) and 195.86 ($C_{7\alpha}$); *m/z* 270 (3.5%, $(M-Br)^+$), 115 (100), 81.9 (81) and 79.9 (83).



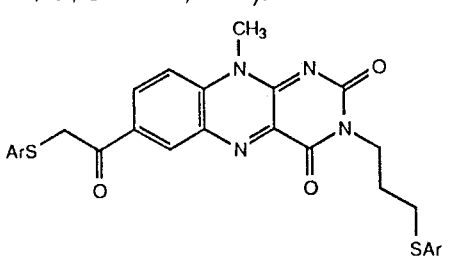
7-β-Bromoacetyl-3-N-(3-bromopropyl)-10-N-methylisoalloxazine (**58**). - N-Bromosuccinimide (50 mg, 0.28 mmol) and benzoyl peroxide (catalytic) were added to the 3-N-alkylated flavin (**51**) (100 mg, 0.26 mmol) in trifluoroacetic acid (20 ml). The mixture was heated at 60 - 70°C for 4h. The solvent was then removed under vacuum and the residue dissolved in dichloromethane (20 ml), washed with water (3 x 5 ml), dried (Na_2SO_4) and vacuum evaporated. The residual oil was subjected to column chromatography (CH_2Cl_2/5% MeOH) to yield the pure product (85 mg, 71%), m.p. 185°C dec. (Found: C, 41.0; H, 3.3; N 11.6%; $(M+H)^+$, 470.9501. $C_{16}H_{14}Br_2N_4O_3$ requires C, 40.9; H, 3.0; N, 11.9%; $(M+H)$, 470.9491); $\nu_{max.}$ (nujol) 1725m, 1600m, 1570s and 700s cm^{-1}; δ_H (270 MHz, $CDCl_3$) 2.33 (2H, quintet, *J* 7 Hz, $CH_2C\underline{H}_2CH_2Br$), 3.48 (2H, t, *J* 7 Hz, $CH_2CH_2C\underline{H}_2Br$), 4.15 (3H, s, NCH_3), 4.25 (2H, t, *J* 7 Hz, $C\underline{H}_2CH_2CH_2Br$), 4.49 (2H, s, $BrCH_2CO$), 7.76 (1H, d, *J* 9 Hz, 9-H), 8.52 (1H, dd, *J* 1.95 & 9 Hz, 8-H) and 8.89 (1H, d, *J* 1.95 Hz, 6-H); δ_C (67.9 MHz, CF_3CO_2D) 30.08 (CH_2Br), 30.43 ($C_{7\beta}$), 31.62 ($\underline{C}H_2CH_2Br$), 38.89 (NCH_3), 45.28 (NCH_2), 120-145 (aromatics), 151.84 (C_2), 161.06 (C_4) and 195.4 ($C_{7\alpha}$).



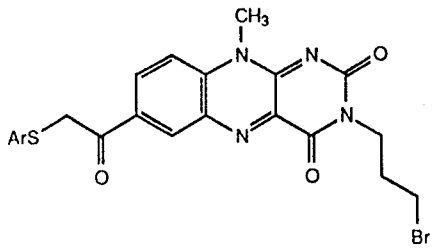
7-(Thio phenoxy)-acetyl-10-N-methylisoalloxazine (**59**). - Freshly distilled thiophenol (72 µl, 0.7 mmol) was added to the brominated flavin (**57**) (50 mg, 0.14 mmol) in DMSO (1 ml), protected from light and air. Triethylamine (40 µl, 0.29 mmol) was added over 10 min. and the mixture left stirring overnight. After addition of water (5 ml), the mixture was extracted with dichloromethane (3 x 10 ml), washed with water (3 x 5 ml), dried (Na_2SO_4) and vacuum evaporated. The product (**59**) was crystallised from DMF/ether (4 mg, 7.6%), δ_H (270 MHz, DMSO-d_6), 3.97 (3H, s, NCH_3), 4.80 (2H, s, SCH_2), 7.30 (5H, m, Ar-H), 8.01 (1H, d, *J* 9 Hz, 9-H), 8.36 (1H, dd, *J* 1.8 & 8.9 Hz, 8-H) and 8.85 (1H, d, *J* 1.9 Hz, 6-H).



3-N-(3-Thiolphenoxy)-7-acetyl-10-N-methylisoalloxazine (**60**). - Thiophenol (446 µl, 4.34 mmol) was added to a stirred solution of sodium methoxide (100 mg, 4.34 mmol, Na in MeOH, 10 ml). After stirring for 10 mins, part of the mixture (0.32 ml, 141 µmol) was added to 3N-3-bromopropyl-7-acetyl -10-N-methylisoalloxazine (**51**) (50 mg, 128 µmol) in dry DMF (10 ml), and the mixture stirred for 2h at room temperature. Water (10 ml) was added and the mixture extracted with dichloromethane (3 x 10 ml). The organic layer was separated and washed with water (3 x 10 ml), dried (Na_2SO_4) and the solvent removed *in vacuo.* Column chromatography (silica gel, CH_2Cl_2/5% MeOH) gave the 3-thio alkylated derivative (4.4 mg, 8.2%), δ_H (270 MHz, $CDCl_3$) 2.10 (2H, quintet, *J* 7.2 Hz, -$CH_2\underline{CH_2}CH_2S$), 2.72 (3H, s, CH_3CO-), 3.02 (2H, t, *J* 7.2 Hz, -$CH_2CH_2\underline{CH_2}S$), 4.15 (3H, s, N-$CH_3$), 4.25 (2H, t, *J* 7.1 Hz, -$\underline{CH_2}CH_2CH_2S$), 7.30 (5H, m, aromatics), 7.74 (1H, d, *J* 8.5 Hz, 9-H), 8.42 (1H, dd, *J* 2 & 9 Hz, 8-H) and 8.87 (1H, d, *J* 2 Hz, 6-H).



3-N-(3-Thiolphenoxy)-7-(thiolphenoxy)acetoxy-10-N-methylisoalloxazine (**61**). - Sodium metal (100 mg, 4.3 mmol) was added to dry methanol (11 ml) over 5 min. After the sodium had reacted, thiophenol (0.49 ml, 4.78 mmol) was added, to form sodium thiophenolate. The methanolic solution of sodium thiophenolate (511 µl, 0.22 mmol) was added to 3-N-(3-bromopropyl)-7β-bromoacetyl-10-N-methylisoalloxazine (**58**) (50 mg, 0.11 mmol) in dry DMF (6 ml) and the mixture stirred for 4h. Water (10 ml) was added and the mixture extracted with dichloromethane (3 x 10 ml), the organic extracts washed with water (3 x 5 ml), dried (Na_2SO_4), and the solvent removed *in vacuo*, to yield a solid. The product (**61**) was obtained by flash chromatography (silica gel, CH_2Cl_2/5% MeOH), as a yellow solid (3.4 mg, 6%), δ_H (270 MHz, $CDCl_3$), 2.08 (2H, quintet, *J* 7 Hz, -$CH_2\underline{CH_2}CH_2S$), 3.02 (2H, t, *J* 7 Hz, -$CH_2CH_2\underline{CH_2}S$), 4.14 (3H, s, N-$CH_3$), 4.27 (2H, t, J 7 Hz, -$\underline{CH_2}CH_2CH_2S$), 4.31 (2H, s, -$SCH_2CO$), 7.35 (10H, m, C_6H_5-S), 7.75 (1H, d, *J* 9 Hz, 9-H), 8.45 (1H, dd, *J* 2 & 9 Hz, 8-H) and 8.87 (1H, d, *J* 2 Hz, 6-H).



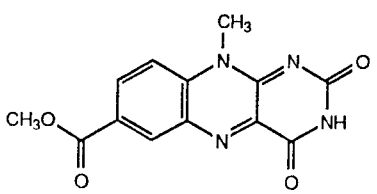
3N-(3-Bromopropyl)-7(thiophenoxy)acetoxy-10-N-methylisoalloxazine (**62**). - Thiophenol (446 µl, 4.34 mmol) was added to a stirred solution of sodium methoxide (100 mg, 4.34 mmol, Na in MeOH 10 ml), and stirred for a further 10 min. An aliquot (53 µl, 32.1 µmol) was added to the dibromoflavin (**58**) (10 mg, 21 µmol) in methanol (2.5 ml) and the mixture stirred at room temperature for 2 h. The mixture was extracted with dichloromethane (3 x 10 ml) after addition of water (5 ml), washed with water (3 x 5ml), dried (Na_2SO_4) and the solvent removed *in vacuo*. Column chromatography (CH_2Cl_2/5% MeOH) gave the 7-thioalkylated derivative (0.7 mg, 6.7 %), δ_H (270 MHz, $CDCl_3$), 2.35 (2H, quintet, *J* 7 Hz, $CH_2\underline{CH_2}CH_2Br$), 3.50 (2H, t, *J* 7 Hz, $CH_2CH_2\underline{CH_2}Br$), 4.15 (3H, S, N-$CH_3$), 4.27 (2H, t, *J* 7 Hz, $\underline{CH_2}CH_2CH_2Br$), 4.32 (2H, s, -$SCH_2CO$), 7.34 (5H, m, aromatics), 7.72 (1H, d, *J* 9Hz, 9-H), 8.46 (1H, dd, *J* 1.95 & 9 Hz, 8-H) and 8.86 (1H, d, *J* 2 Hz, 6-H).

3-Nitro-4-fluorobenzoic acid (**63**). - Sulphuric acid (s.g. 1.8, 21 ml) was added slowly, with stirring, to nitric acid (s.g. 1.42, 15 ml) at 0°C. Portions of 4-fluorobenzoic acid (in total 12 g, 85.7 mmol) were added to the nitrating mixture avoiding a rapid rise in temperature. The solution was allowed to warm slowly to room temperature and then heated to 150°C for 1h. The reaction mixture was cooled and poured slowly into a slurry of ice/water (300 ml). The precipitated solid was collected by filtration and recrystallised from water (11.94 g, 74%), m.p. 120 - 121°C (lit.,[215] 121.5°C), $\nu_{max.}$ (nujol) 3300-2400b (CO_2H), 1620s (Ar-H), 1540s (NO_2) and 1350 cm^{-1} (NO_2); δ_H (360 MHz, 10% NaOD), 7.07 (1H, dd, *J* 2.4 & 9 Hz, 6-H), 7.92 (1H, ddd, *J* 2, 6 & 9 Hz, 5-H) and 8.19 (1H, dd, *J* 2 & 6 Hz, 2-H); *m/z* 185 (M^+, 100%).

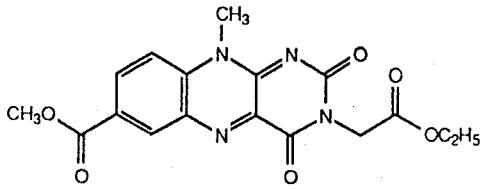
3-Nitro-4-N-methylaminobenzoic acid (**64**). - 3-Nitro-4-fluorobenzoic acid (**63**) (2.0 g, 10.8 mmol) and methylamine (0.74 ml, 21.6 mmol) were dissolved in ethanol (20 ml) and the orange mixture heated to reflux for 3h. This mixture was then acidified with glacial acetic acid (10 ml), and the resultant precipitate filtered and recrystallised from ethanol (1.17 g, 81%), m.p. 300 - 302°C (lit.,[216] 303 - 305°C), (Found: C, 48.5; H, 4.0; N, 14.7; M^+, 196.0482. $C_8H_8N_2O_4$ requires C, 49.0; H, 4.1; N, 14.3%; *M*, 196.0484); $\nu_{max.}$ (nujol) 3420-3400s (NH), 1690s (CO_2H) and 1590w cm^{-1} (NO_2); δ_H (360 MHz,

DMSO-d_6) 3.05 (3H, d, *J* 7 Hz, CH_3), 3.14 (1H, bs, NH), 7.10 (1H, d, *J* 9 Hz, 5-H), 8.01 (1H, dd, *J* 2 & 8.9 Hz, 6-H), 8.61 (1H, d, *J* 2 Hz, 2-H) and 8.66 (1H, s, COOH).

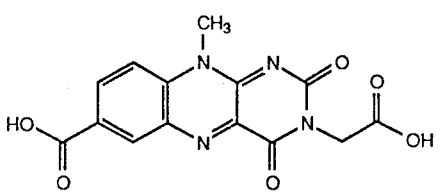
3-Nitro-4-N-methylamino methylbenzoate (**65**). - 3-Nitro-4-N-methyl-aminobenzoic acid (**64**) (1 g, 5 mmol) in dry methanol (130 ml) was cooled to -5°C. Thionyl chloride (0.4 ml, 5.5 mmol) was added at -5°C over 1h. The resultant mixture was heated on a water bath until the solution became clear (4h). Yellow crystals formed on standing at 4°C overnight, these were filtered, washed with ether and dried (1.05, 78%), m.p. 141 - 143°C (lit.,[217] 145°C) (Found: C, 51.3; H, 4.8; N, 13.4; M^+, 210.0623. $C_9H_{10}N_2O_4$ requires C, 51.4; H, 4.8; N, 13.3%; *M*, 210.0641); $\nu_{max.}$ (nujol) 3390s (NH), 1720s (CO_2H) and 1570w cm^{-1} (NO_2); δ_H (360 MHz, $CDCl_3$) 3.15 (3H, d, *J* 5 Hz, $NHC\underline{H}_3$), 3.95 (3H, s, $COOCH_3$), 6.85 (1H, d, *J* 9 Hz, 5-H), 8.10 (1H, dd, *J* 2 & 9 Hz, 6-H), 8.35 (1H, bs, NH) and 8.85 (1H, d, *J* 2 Hz, 2-H).



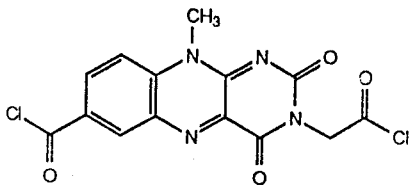
<u>7-Methoxycarbonyl-10-N-methylisoalloxazine.</u> (**67**). - 3-Nitro-4-N-methyl-aminobenzoate (**65**) in methanol (25 ml) and water (5 ml) was hydrogenated at atmospheric pressure over palladium on charcoal (5%, 50 mg) for 4h. The product was filtered through celite, washed with methanol and the solvent removed *in vacuo*. To this was added a mixture of alloxan monohydrate (0.17 g, 1.1 mmol) and boric acid (70 mg, 1.1 mmol) in glacial acetic acid (5 ml), which had been thoroughly degassed. The reaction mixture was degassed again and heated to reflux for 5 - 10 min. After stirring for ~12h at room temperature, the solution gave a green solid, which was collected by filtration and washed with water and ether (0.21 g, 67%), m.p. 308 - 310°C (Found: $(M+H)^+$, 287.0770. $C_{13}H_{10}N_4O_4$ requires (*M+H*), 287.0780); $\lambda_{max.}$ (EtOH) 223 (ϵ 16 000 $M^{-1}cm^{-1}$), 275 (17 900), 335 (2 300), and 425 nm (4 600); $\nu_{max.}$ (nujol) 1750m (amide), 1600m (Ar) and 1570s cm^{-1} (N-H); δ_H (360 MHz, DMSO-d_6) 3.58 (3H, s, NCH_3), 4.24 (3H, s, $COOCH_3$), 8.23 (1H, d, *J* 9 Hz, 9-H), 8.61 (1H, dd, *J* 2 & 9 Hz, 8-H), 8.75 (1H, d *J* 2 Hz, 6-H) and 11.82 (1H, s, NH); δ_C (89.55 MHz, DMSO-d_6) 32.03 (N-CH_3), 52.50 ($COOCH_3$), 117.10 (C_9), 126.67 (C_{4a}), 128.54 (C_8), 130.69 (C_6), 132.40 (C_{7a}), 133.84 (C_{9a}), 136.36 (C_{10a}), 151.25 (C_2), 159.19 (C_4) and 165.01 ($C_{7\alpha}$).



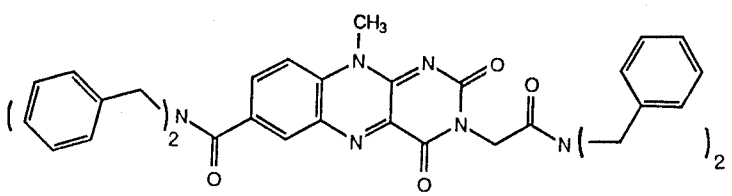
3-N-Ethylaceto-7-methoxycarbonyl-10-N-methylisoalloxazine. (68). - Procedure as for 52, using 7-(methylester)-10-N-methylisoalloxazine (67) (51 mg, 0.18 mmol) and ethyl bromoacetate (199 µl, 1.8 mmol). Crystallised from dichloromethane/diethyl ether (45 mg, 67%), m.p. >250°C (Found: C, 54.1; H, 4.4; N, 14.8. $C_{17}H_{16}N_4O_6.½H_2O$ requires C, 53.6; H, 4.5; N, 14.7%); δ_H (270 MHz, $CDCl_3$) 1.29 (3H, t, *J* 7.1 Hz, $-CH_2\underline{C}H_3$), 4.01 (3H, s, CH_3O), 4.16 (3H, s, $N-CH_3$), 4.23 (2H, q, *J* 7.1 Hz, $-\underline{C}H_2CH_3$), 4.85 (2H, s, $N-CH_2-$), 7.72 (1H, d, *J* 9 Hz, 9-H), 8.52 (1H, dd, *J* 1.9 & 8.9 Hz, 8-H) and 8.96 (1H, d, *J* 1.9 Hz, 6-H); δ_C (67.9 MHz, $CDCl_3$) 14.36 ($CH_2\underline{C}H_3$), 32.66 ($N-CH_3$), 43.15 ($N-CH_2-$), 53.15 ($-COO\underline{C}H_3$), 61.97 ($-\underline{C}H_2CH_3$), 115.73 (C_9), 135.15 to 136.27 (aromatics), 138.03 (C_{9a}), 150.04 (C_{10a}), 154.78 (C_2), 158.89 (C_4), 165.06 ($C_{7\alpha}$), 167.68 ($-\underline{C}OOC_2H_5$).



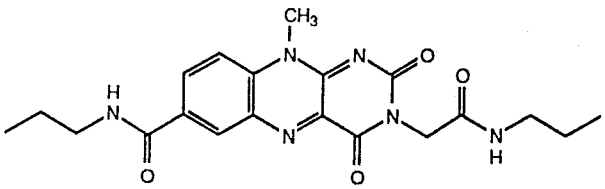
3-N-Carboxymethyl-7-carboxy-10-N-methylisoalloxazine. (69). - Compound 68 (63 mg, 0.134 mmol) was dissolved in concentrated hydrochloric acid (s.g. 1.18, 0.44 ml) and the resultant mixture stirred at 80 - 90°C for 45 min. The reaction mixture was cooled, and ice-water (2 ml) was added to the solution. Yellow crystals that precipitated were collected by suction filtration and washed with water. Recrystallisation from 2 N acetic acid gave fine orange needles (31 mg, 70%), m.p. >300°C (Found: C, 47.1; H, 3.7; N, 15.1; $(M+H)^+$, 331.0666. $C_{14}H_{10}N_4O_6.1½H_2O$ requires C, 47.1; H, 3.7; N, 15.6%; (*M+H*), 331.0679); $\nu_{max.}$ (nujol) 3420m, 1740m, 1670m, 1600m; δ_H (270 MHz, CF_3COOD) 4.68 (3H, s, $N-CH_3$), 5.20 (2H, s, $N-CH_2-$), 8.53 (1H, d, *J* 9Hz, 9-H), 9.06 (1H, dd, *J* 1.9 & 8.9 Hz, 8-H), 9.36 (1H, d, *J* 1.9 Hz, 6-H); δ_C (67.9 MHz, CF_3COOD) 38.12 ($N-CH_3$), 44.51 ($N-CH_2$), 119.82 (C_9), 134.20 to 136.40 (aromatics), 137.97 (C_8), 141.75 (c_6), 145.34 (C_{10a}), 151.21 (C_2), 159.90 (C_4), 170.19 ($C_{7\alpha}$), 173.40 ($C_{3\beta}$); *m/z* 331 (89%).



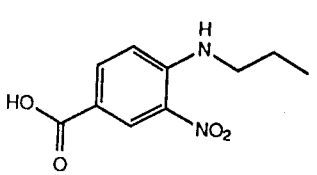
3-N-Carboxymethyl-7-carboxy-10-N-methylisoalloxazine-diacid chloride. (**70**). - Compound **69** (50 mg, 0.15 mmol) was added to thionyl chloride (1.0 ml) and the suspension stirred at 45°C for 2h. After this time, the solid had dissolved and the solution was stirred for a further 20 mins. Excess thionyl chloride was removed by rotary evaporation at <45°C (above 45°C the product decomposed to an unknown green substance). Remaining traces of the thionyl chloride were removed by repeated evaporation of a benzene solution. The yield was almost quantitative, as calculated for the hydrochloride salt. The crude acid chloride was used without further purification, $\nu_{max.}$ (nujol) 1790w, 1760w, 1730w, 1650w and 1590m cm^{-1}; δ_H (270 MHz, DMSO-d_6) 4.03 (3H, s, N-CH_3), 4.60 (2H, s, N-CH_2-), 8.09 (1H, d, *J* 9Hz, 9-H), 8.40 (1H, dd, *J* 1.9 & 9 Hz, 8-H), 8.56 (1H, d, *J* 1.9 Hz, 6-H).



3-(N,N-Dibenzyl)-N'-acetamido-7-(N,N-dibenzyl)-formamido-10-N-methyl-isoalloxazine (**71**). - A solution of dibenzylamine (57 µl, 0.31 mmol) in DMF (2 ml) containing pyridine (96 µl, 1.2 mmol) was added to the compound **70** (55 mg, 0.15 mmol), and stirred under an argon atmosphere at room temperature. After 2h, the reaction mixture was added to water (10 ml) and extracted with dichloromethane (2 x 20 ml). The organic phase was washed with water (4 x 10 ml), dried (Na_2SO_4) and concentrated by rotary evaporation. The crude product was purified by chromatography on silica gel (CH_2Cl_2/5% MeOH) to give a dark yellow oil which could not be crystallised (12 mg, 12%), m.p. 178 - 180°C; (Found: C, 69.8; H, 5.6; N, 11.1; $(M+H)^+$, 689.2924. $C_{42}H_{36}N_6O_4.2H_2O$ requires C, 69.6; H, 5.6; N, 11.5%; (*M+H*), 689.2876); δ_H (270 MHz, $CDCl_3$) 4.03 (3H, s, N-CH_3), 4.20-4.80 (8H, m, N-CH_2Ar), 4.97 (2H, s, N-CH_2), 7.16-7.35 (20H, m, Ar-H), 7.60 (1H, d, *J* 9 Hz, 9-H), 7.95 (1H, dd, *J* 1.9 & 8.9 Hz, 8-H), 8.34 (1H, d, *J* 1.9 Hz, 6-H); δ_C (67.9 MHz, $CDCl_3$) 32.36 (N-CH_3), 43.26 ($C_{3\alpha}$), 48.01 (CH_2), 48.72 (CH_2), 49.40 (CH_2), 50.24 (C_2), 116.16 (C_9), 126.92 - 129.22 (benzyl aromatics), 134.38 - 136.75 (aromatics), 138.19 (C_{9a}), 149.82 (C_{10a}), 155.81 (C_2), 159.30 (C_4), 166.61 ($C_{7\alpha}$), 169.58 ($C_{3\beta}$).

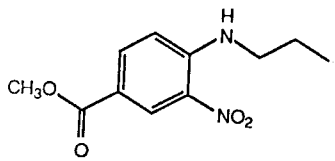


3-(N,n-Propyl)-N'acetamido-7-(N,n-propyl)-formamido-10-N-methyl-isoalloxazine (**72**). - 3-N-Acetyl chloride-7-formyl chloride-10-N-methylisoalloxazine (**70**) (55 mg, 0.15 mmol) dissolved in DMF (2 ml) was stirred under an atmosphere of argon. Dry pyridine (96 µl, 1.2 mmol) was added to this solution and this mixture stirred at room temperature for 3h. The reaction was quenched with water (10 ml) and extracted with dichloromethane (3 x 10 ml). The organic phase was washed with water (5 x 10 ml), dried (Na_2SO_4) and vacuum evaporated. The product was purified by column chromatography (CH_2Cl_2/10% MeOH) to yield a yellow solid (9 mg, 15%), m.p. 153 - 156°C, (Found: $(M+H)^+$, 413.1927. $C_{20}H_{24}N_6O_4$ requires $(M+H)$ 413.1937); δ_H (270 MHz, DMSO-d_6) 0.85 (3H, t, *J* 7.3 Hz, -$CH_2C\underline{H}_3$), 0.95 (3H, t, *J* 7.3 Hz, -$CH_2C\underline{H}_3$), 1.42 (2H, m, *J* 7.3 Hz, -$C\underline{H}_2CH_3$), 1.62 (2H, m, *J* 7.3 Hz, -$C\underline{H}_2CH_3$), 3.05 (2H, q, *J* 7.3 Hz, -$C\underline{H}_2CH_2CH_3$), 3.32 (2H, q, *J* 7.3 Hz, -$C\underline{H}_2CH_2CH_3$), 3.36 (3H, s, N-CH_3), 4.05 (2H, s, N-CH_2CO-), 8.04 (1H, tb, -NH), 8.08 (1H, d, *J* 8.9 Hz, 9-H), 8.40 (1H, dd, *J* 8.9 & 1.9 Hz, 8-H), 8.73 (1H, d, *J* 1.9 Hz, 6-H), 8.85 (1H, tb, 7-NH); δ_C (67.9 MHz, DMSO-d_6) 11.35 ($CH_2\underline{C}H_3$), 11.52 ($CH_2\underline{C}H_3$), 22.29 ($\underline{C}H_2CH_3$), 22.34 ($\underline{C}H_2CH_3$), 32.06 (N-CH_3), 41.23 ($\underline{C}H_2CH_2CH_3$), 41.26 ($\underline{C}H_2CH_2CH_3$), 43.73 (N-CH_2-), 116.89 (C_9), 130.01 (C_6), 131.63 (C_7), 133.58 (C_8), 134.21 (C_{4a}), 134.87 (C_{5a}), 138.50 (C_{9a}), 149.86 (C_{10a}), 154.50 (C_2), 159.08 (C_4), 164.06 ($C_{7\alpha}$), 166.38 ($C_{3\beta}$).

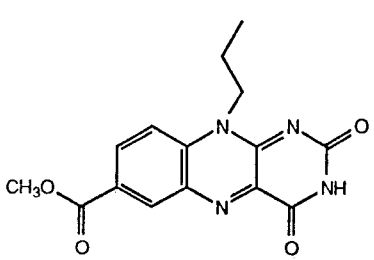


4-N-Propylamino-3-nitrobenzoic acid (**73**). - Freshly distilled propylamine (1.34 ml, 8.9 mmol) was added to 4-fluoro-3-nitrobenzoic acid (**63**) (1.5 g, 8.1 mmol). The mixture was heated to reflux for 2 h. The resultant orange solution was acidified (HCl, 2N) to give a yellow precipitate. Yellow crystals were obtained after filtration and recrystallisation from methanol (1.51 g, 83%), m.p. 207 - 209°C (Found: C, 53.6; H, 5.4, N, 12.6. $C_{10}H_{12}N_2O_4$ requires C, 53.5; H, 5.4; N, 12.5%); δ_H (270 MHz, DMSO-d_6) 0.95 (3H, t, *J* 7.3 Hz, $CH_2C\underline{H}_3$), 1.65 (2H, m, *J* 7.1 Hz, $C\underline{H}_2CH_3$), 3.65 (2H, m, *J* 7.9 Hz, NCH_2),

7.09 (1H, d, *J* 9.1 Hz, 5-H), 7.94 (1H, dd, *J* 1.9 & 9.1 Hz, 6-H), 8.49 (1H, t, *J* 5.4 Hz, NH) and 8.60 (1H, d, *J* 2.1 Hz, 2-H); δ_C (67.9 MHz, DMSO-d_6) 11.15 ($\underline{C}H_3CH_2$), 21.49 (NCH_2), 44.05 ($CH_3\underline{C}H_2$), 114.51-147.27 (aromatics) and 165.89 (C=O).

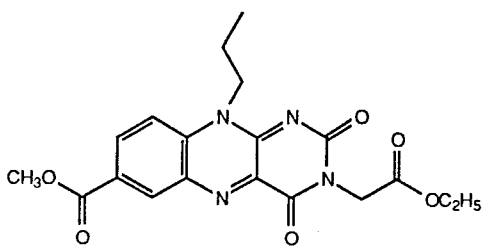


4-N-Propylamino-3-nitro-methylbenzoate (**74**). - A solution of 4-N-propyl-3-nitro-benzoic acid (**73**) (1 g, 4.46 mmol) in dry MeOH (50 ml) was cooled to -5°C and whilst maintaining this temperature, thionyl chloride (1.63 ml, 22 mmol) was added over 15 min. After heating to reflux (1½ hr) the excess thionyl chloride and methanol were removed *in vacuo*. Bright yellow crystals were obtained by recrystallising the residue from methanol (707 mg, 66%), m.p. 62 - 64°C (Found: C, 55.4; H, 5.8; N, 11.7. $C_{11}H_{14}N_2O_4$ requires: C, 55.5; H, 5.9; N, 11.8%); δ_H (270 MHz, $CDCl_3$), 1.08 (3H, t, *J* 7.4 Hz, $CH_2\underline{C}H_3$), 1.79 (2H, m, *J* 6.9 Hz, $CH_3\underline{C}H_2$), 3.34 (2H, m, *J* 7.6 Hz, N-CH_2), 3.90 (3H, s, CH_3), 6.87 (1H, d, *J* 9.1 Hz, 5-H), 8.04 (1H, dd, *J* 9.1 & 2.2 Hz, 6-H), 8.37 (1H, bs, N-H) and 8.88 (1H, d, *J* 2.1 Hz, 2-H); δ_C (67.9 MHz, $CDCl_3$), 11.74 ($\underline{C}H_3CH_2$), 22.40 (N-$\underline{C}H_2$), 45.21 ($CH_3\underline{C}H_2$), 52.09 ($\underline{C}H_3O$), 113.75-148.07 (aromatics) and 165.91 (C=O).

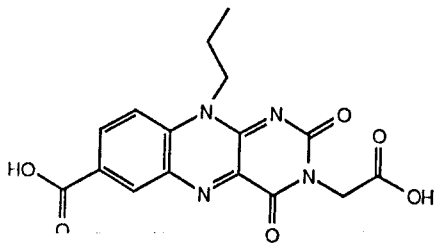


7-Methoxycarbonyl-10-N-*n*-propylisoalloxazine. (**75**). - 4-N-proplyamino-3-nitro-methylbenzoate (**74**) (5.94 g, 24.9 mmol) in methanol (250 ml) was hydrogenated over palladium on charcoal (10%, 500 mg) at room temperature, until tlc analysis showed complete conversion (~6h). The resultant mixture was filtered through a celite plug, washed with methanol and evaporated *in vacuo*. The diamine thus obtained was dissolved in absolute ethanol (500 ml). Alloxan monohydrate (4.04 g, 30 mmol) dissolved in hot concentrated hydrochloric acid (47 ml) was added and the solution refluxed in the dark for 15 min. The product which crystallised at 4°C was collected and washed with ether. Recrystallisation from acetic acid (2N)

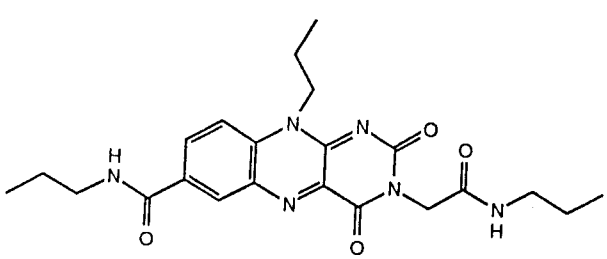
gave fine lime-coloured crystals (5.32 g, 68%), m.p. > 300°C (Found: C, 55.7; H, 4.2; N, 17.6. $C_{15}H_{14}N_4O_4.½H_2O$ requires C, 55.7; H, 4.6; N, 17.3%); $\nu_{max.}$ (nujol) 1730m, 1605m and 1570m cm^{-1}; δ_H (270 MHz, DMSO-d_6), 1.08 (3H, t, *J* 7.3 Hz, $CH_2C\underline{H}_3$), 1.79 (2H, m, *J* 7.1 Hz, $C\underline{H}_2CH_3$), 3.97 (3H, s, CH_3), 4.55 (2H, t, *J* 7.8 Hz, NCH_2), 8.12 (1H, d, *J* 9 Hz, 9-H), 8.33 (1H, dd, *J* 1.9 & 9.1 Hz, 8-H) and 8.53 (1H, d, *J* 1.9 Hz, 6-H); δ_C (67.9 MHz, DMSO-d_6) 10.92 ($\underline{C}H_3CH_2$), 19.70 (NCH_2), 45.78 ($CH_3\underline{C}H_2$), 52.62 (CH_3), 117.05 (C_9), 126.45 (C_{4a}), 132.54 (C_6), 133.77 (C_{5a}), 134.00 (C_{9a}), 135.52 (C_7), 150.78 (C_{10a}), 155.55 (C_2), 159.35 (C_4), 164.81 ($C_{3\beta}$) and 167.58 ($C_{7\alpha}$); *m/z* (FAB$^+$), (100%, $(M+H)^+$), 273 (60), 232 (20), 137 (14) and 79 (35).



3-N-Ethylaceto-7-methoxycarbonyl-10-N-*n*-propylisoalloxazine. (**76**). - The isoalloxazine (**75**) (1 g, 3.2 mmol) and anydrous potassium carbonate (2.48 g, 17.8 mmol) were suspended in dry DMF (100 ml). To this suspension was added freshly distilled ethyl bromoacetate (1.34 ml, 12 mmol) and the mixture stirred at room temperature. After 5 h the reaction mixture was filtered and the DMF solvent removed by rotary evaporation. Recrystallisation from methanol afforded fine yellow crystals (1.12 g, 88%), m.p. 205 - 207°C (Found: C, 56.9; H, 5.0; N, 13.9. $C_{19}H_{20}N_4O_6$ requires C, 57.0; H, 5.0; N, 14.0%); $\lambda_{max.}$ 432 (ϵ 1150 M^{-1} cm^{-1}) and 285 nm (6700); $\nu_{max.}$ (nujol) 1720m, 1670m, 1600m and 1570 cm^{-1}; δ_H (270 MHz, $CDCl_3$), 1.06 (3H, t, *J* 7.34 Hz, $C\underline{H}_3CH_2CH_2$), 1.21 (3H, t, *J* 7.15 Hz, CH_3), 1.85 (2H, m, *J* 7.9 Hz, $CH_3C\underline{H}_2CH_2$), 3.91 (3H, s, CH_3O), 4.13 (2H, q, *J* 7.15 Hz, $CH_3C\underline{H}_2$), 4.60 (2H, m, *J* 7.5 Hz, $CH_3CH_2C\underline{H}_2$), 4.74 (2H, s, CH_2), 7.66 (1H, d, *J* 9.08 Hz, 9-H), 8.40 (1H, dd, *J* 1.93 & 9.08 Hz, 8-H) and 8.80 (1H, d, *J* 1.74 Hz, 6-H); δ_C (67.9 MHz, $CDCl_3$), 11.17 ($\underline{C}H_3CH_2CH_2$), 14.09 ($\underline{C}H_3CH_2O$), 20.43 ($N\underline{C}H_2CH_2$), 42.85 ($N\underline{C}H_2CO$), 46.66 ($CH_3\underline{C}H_2CH_2$), 52.82 (CH_3O), 61.62 ($CH_3\underline{C}H_2CO$), 115.70 (C_9), 128.08 (C_{4a}), 134.82 (C_6), 135.07 - 135.67 (aromatics), 137.76 (C_7), 149.34 (C_{10a}), 154.69 (C_2), 158.78 (C_4), 164.84 ($C_{7\alpha}$) and 167.58 ($\underline{C}OOC_2H_6$); *m/z* (FAB$^+$), 401 (100%, $(M+H)^+$), 327 (30), 285 (15) and 228 (17).

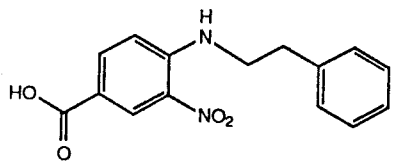


3-N-Carboxymethyl-7-carboxy-10-N-*n*- propylisoalloxazine. (**77**). - The diester (**76**) (567 mg, 1.42 mmol) was dissolved in hydrochloric acid (s.g. 1.18, 4.65 ml) and the resultant mixture stirred at 80 - 90°C for 45 min. The reaction mixture was cooled and ice-water (14 ml) was added to the solution. Yellow crystals that precipitated were collected by suction filtration and washed with water. Yellow needles were obtained on recrystallisation from 2N acetic acid (361 mg, 71%), m.p. 250°C dec. (Found: C, 50.2; H, 4.4; N, 14.2. $C_{16}H_{14}N_4O_6.1½H_2O$ requires C, 49.9; H, 4.5; N, 14.4%); $\nu_{max.}$ (nujol) 3425w, 1750m, 1668m and 1600m cm^{-1}; δ_H (270 MHz, CF_3COOD), 1.37 (3H, t, *J*, 7.0 Hz, CH_3), 2.23 (2H, bm, $CH_3C\underline{H}_2$), 5.04 (2H, bm, CH_2), 5.20 (2H, s, N-CH_2), 8.48 (1H, d, *J* 9.27 Hz, 9-H), 9.08 (1H, dd, *J* 1.7 & 9.3 Hz, 8-H) and 9.35 (1H, d, *J* 1.7 Hz, 6-H); δ_C (67.9 MHz, CF_3COOD), 10.89 (CH_3), 22.90 ($N\underline{C}H_2CH_2$), 44.82 ($N\underline{C}H_2CO$), 53.58 ($CH_3\underline{C}H_2$), 120.00 (C_9), 134.53 (C_{4a}), 136.01 (C_{5a}), 138.40 (C_6), 141.55 (C_8), 142.06 (C_{9a}), 144.59 (C_7), 152.16 (C_{10a}), 160.11 (C_2), 170.36 (C_4) and 173.45 (C_{7a}); *m/z* (FAB$^+$) 359 (100%, $(M+H)^+$), 307 (22), 232 (32), 176 (33), 137 (25) and 79 (26).

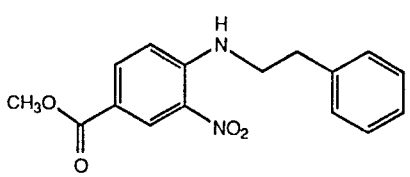


3-(N-Propyl)-N'-acetamido-7-(N-propyl)-formamido-10-N-propylisoalloxazine (**79**). - Triethylamine (85.6 µl, 0.61 mmol) and *iso*butyl chloroformate (40 µl, 0.61 mmol) were added to a degassed solution of the diacid flavin (**77**) (50 mg, 0.28 mmol) in dry DMF (5 ml) at room temperature. After 30 min., propylamine (115 µl, 1.4 mmol) was added and the mixture left stirring for 3h. Excess reactants and DMF were removed *in vacuo* and the residue extracted with dichloromethane (2 x 10 ml), washed with water (3 x 5 ml), dried (Na_2SO_4) and vacuum evaporated. The product was purified by column chromatography (silica gel, CH_2Cl_2/7.5% MeOH), (31 mg, 25%), m.p. 172 - 175°C dec. (Found: M^+, 440.2143. $C_{22}H_{28}N_6O_4$ requires *M*, 440.2172); $\lambda_{max.}$ (DMF) 436 (ϵ 4100 M^{-1} cm^{-1}), 332 (2 950) and 282 nm (16 500); δ_H (270 MHz, DMSO-d_6), 0.85 (3H, t, *J* 7.4 Hz, 3-$CH_2C\underline{H}_3$), 0.94 (3H, t, *J* 7.3 Hz, 7-$CH_2C\underline{H}_3$), 1.07 (3H, t, *J* 7.3 Hz, 10-$CH_2C\underline{H}_3$), 1.43 (2H, sextet, *J* 7.1 Hz, 3-CH_2CH_3), 1.60 (2H, sextet, *J* 7.1 Hz, 7-CH_2CH_3), 1.81 (2H, sextet, *J* 7.1 Hz,

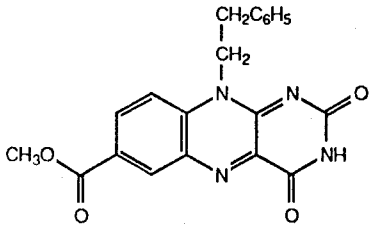
10-CH_2CH_3), 3.05 (2H, q, *J* 5.4 Hz, 3-$NHCH_2$), 3.28 (2H, q, *J* 5.4 Hz, 7-$NHCH_2$), 4.48 (2H, s, NCH_2), 4.65 (2H, q, *J* 5.4 Hz, 10-NCH_2), 8.01 (1H, t, *J* 5.4 Hz, 3-NH), 8.14 (1H, d, *J* 9.1 Hz, 9-H), 8.38 (1H, dd, *J* 1.7 & 9.1 Hz, 8-H), 8.74 (1H, d, *J* 1.9 Hz, 6-H) and 8.82 (1H, t, *J* 5.4 Hz, 7-NH); *m/z* (EI) 440 (37%, M^+), 398 (58), 382 (36), 355 (100), 313 (62), 255 (92) and 104 (23).



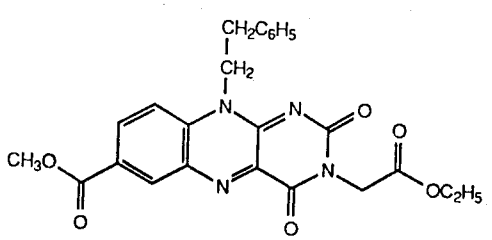
4-N-Phenethylamino-3-nitrobenzoic acid (**80**). - A mixture of 4-fluoro-3-nitrobenzoic acid (**63**) (1 g, 5.4 mmol) and phenethylamine (1.36 ml, 10.8 mmol) were refluxed in ethanol (50 ml) for 2 h. The yellow solution was reduced in volume (to 20 ml) and then acidified with 2N HCl to give a yellow precipitate, which on recrystallisation from methanol gave bright yellow crystals (1.14 g, 74%), m.p. 200 - 202°C (Found: C, 62.8; H, 4.9; N, 9.7. $C_{15}H_{14}N_2O_4$ requires C, 62.9; H, 4.9; N, 9.8%); $\nu_{max.}$ (nujol) 3400m (N-H), 1690 (CO_2H), 1630s (aromatics) and 1550w cm^{-1} (NO_2); δ_H (90 MHz, DMSO-d_6) 2.98 (2H, t, *J* 7 Hz, $C_6H_5C\underline{H}_2$, 3.30 (1H, bs, NH), 3.68 (2H, q, *J* 7 Hz, $NHC\underline{H}_2$), 7.01 (1H, d, *J* 9 Hz, 5-H), 7.31 (5H, m, C_6H_5), 7.95 (1H, dd, *J* 1.7 & 9 Hz, 6-H) and 8.60 (1H, d, *J* 1.7 Hz, 2-H).



4-N-Phenethylamino-3-nitro-methyl benzoate (**81**). - To a suspension of the benzoic acid (**80**) (2 g, 7 mmol) in dry methanol (100 ml), at 0 - -5°C, thionyl chloride (2.6 ml, 35 mmol) was added dropwise over 15 min. After heating to reflux for 1½ h, the excess solvent was removed in vacuo and the product recrystallised from methanol (1.81 g, 86%), m.p. 97 - 99°C (Found: C, 64.1; H, 5.3; N, 9.4. $C_{16}H_{16}N_2O_4$ requires C, 64.0; H, 5.4; N, 9.3%); $\nu_{max.}$ (nujol) 3490w (N-H), 1730m (ester), 1640m (aromatic), 1580w (N-H) and 1530w cm^{-1} (NO_2); δ_H (270 MHz, $CDCl_3$), 3.05 (2H, t, *J* 7.15 Hz, $C_6H_5C\underline{H}_2$), 3.63 (2H, q, *J* 7.15 Hz, $NHC\underline{H}_2$), 3.90 (3H, s, CH_3), 6.87 (1H, d, *J* 9.07 Hz, 5-H), 7.30 (5H, m, $C_6\underline{H}_5$), 8.04 (1H, dd, *J* 1.9 & 9 Hz, 6-H) and 8.88 (1H, d, *J* 1.94 Hz), 2-H); δ_C (67.9 MHz, $CDCl_3$), 35.30 (NCH_2), 44.80 ($C_6H_5\underline{C}H_2$), 52.28 (CH_3O), 113.64 - 147.69 (aromatics) and 165.81 (C=O).

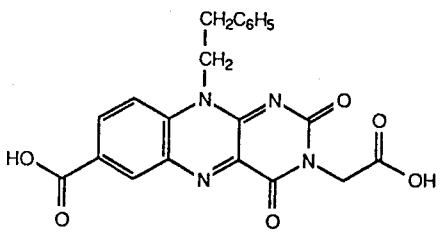


7-Methoxycarbonyl-10-N-phenethylisoalloxazine. (**82**). - The methylester (**81**) (1.75 g, 5.8 mmol) in methanol (150 ml) was hydrogenated over palladium on charcoal (10%, 170 mg) for 24 h. The catalyst was removed by filtration through a celite pad and the solvent evaporated to give a dark residue. This residue was dissolved in absolute ethanol (227 ml) and treated with alloxan monohydrate (1.03 g, 6.38 mmol) in HCl (s.g. 1.18, 11 ml). The mixture was heated to reflux for 15 min, and then left to cool at 4°C overnight. Yellow crystals were filtered, washed with ether and recrystallised from methanol (1.50 g, 64%), m.p. > 300°C (Found: C, 58.8; H, 4.2; N, 13.7; M^+, 376.1145. $C_{20}H_{16}N_4O_4$ requires C, 58.4; H, 4.6; N, 13.6%; M,376.1172); $\nu_{max.}$ (nujol) 1725m, 1600m, and 1574m cm^{-1}; $\lambda_{max.}$ (DMF) 430 (ϵ 2,400 M^{-1} cm^{-1}), and 286 nm (10,500); δ_H, (270 MHz, DMSO-d_6), 3.03 (2H, t, J 7.5 Hz, $C_6H_5C\underline{H}_2$), 3.94 (3H, s, CH_3O), 4.81 (2H, t, J 7.4 Hz, NCH_2), 7.35 (5H, m, C_6H_5), 7.99 (1H, d, J 9.08 Hz, 9-H), 8.26 (1H, dd, J 1.9 & 9.0 Hz, 8-H), and 8.52 (1H, d, J 1.9 Hz, 6-H); δ_C (67.9 MHz, DMSO-d_6), 31.83 (NCH_2), 45.47 ($C_6H_5\underline{C}H_2$), 52.67 (CH_3O), 117.05 (C_9), 126.42 - 137.60 (aromatics), 140.10 (C_7), 150.70 (C_{10a}), 155.59 (C_2), 159.44 (C_4), and 164.84 ($C_{7\alpha}$); m/z 376 (M^+, 2%), 272 (5), 241 (15) and 104 (100).



3-N-Ethylaceto-7-methoxycarbonyl-10-N-phenethylisoalloxazine. (**83**). - A mixture of phenethylisoalloxazine (**82**) (100 mg, 0.27 mmol), potassium carbonate (206 mg, 1.49 mmol) and ethyl bromoacetate (111 µl, 1.01 mmol) were stirred for 4h in DMF (17 ml). After this time the DMF was removed *in vacuo* and the product (**83**) recrystallised from methanol (8.8 mg, 72%), m.p. 195 - 197°C (Found: C, 60.2; H, 4.8; N, 11.5; M^+, 462.1590. $C_{24}H_{22}N_4O_6$ requires C, 60.0 H, 5.0; N, 11.7%; M, 462.1540); $\nu_{max.}$ (nujol) 1750m, 1720m, 1680m, 1640m, 1605m, 1570m, 1540m, and 1430w cm^{-1}; $\lambda_{max.}$ (DMF) 430 (ϵ 1,302 M^{-1} cm^{-1}), and 285 nm (5,100); δ_H, (270 MHz, $CDCl_3$), 1.20 (3H, t, J 7.1 Hz, $CH_2C\underline{H}_3$), 3.11 (2H, t, J 7.5 Hz, $C_6H_5C\underline{H}_2$), 3.91 (3H, s, CH_3O), 4.18 (2H, q,

J 7.1 Hz, $C\underline{H}_2CH_3$), 4.77 (2H, s, NCH_2CO), 4.84 (2H, t, *J* 7.5 Hz, $NC\underline{H}_2CH_2$), 7.19 (5H, m, C_6H_5), 7.46 (1H, d, *J* 9.8 Hz, 9-H), 8.30 (1H, dd, *J* 1.9 & 9.8 Hz, 8-H), and 8.84 (1H, d *J* 1.9 Hz, 6-H); δ_C (67.9 MHz, $CDCl_3$), 14.29 ($CH_2\underline{C}H_3$), 33.08 ($N\underline{C}H_2CH_2$), 43.08 ($N\underline{C}H_2CO$), 46.65 ($NCH_2\underline{C}H_2$), 53.03 (CH_3O), 61.88 ($CH_3\underline{C}H_2O$), 115.51 (C_9), 127.58 - 136.52 (aromatics), 149.39 (C_{10a}), 154.75 (C_2), 158.95 (C_4), 165.03 ($C_{3\beta}$), and 167.74 ($C_{7\alpha}$); *m/z* 462 (M^+, 3%), 358 (5), 285 (10), 228 (10), and 104 (100).

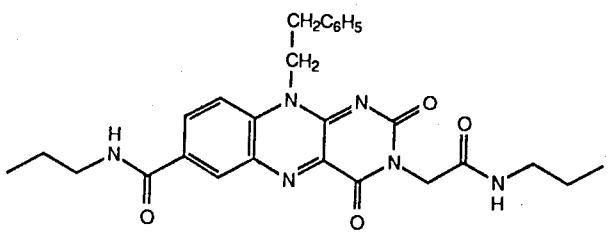


3-N-Carboxymethyl-7-carboxy-10-N-phenethylisoalloxazine. (**84**). - The procedure followed was as for the acid hydrolysis of the 10-N-methyl and 10-N-propyl diacid flavins, (**69**) and (**77**). Yellow crystals (85%) were obtained on recrystallisation from methanol, m.p. 183 - 185°C (Found: C, 58.6; H, 4.2; N, 12.8. $C_{21}H_{16}N_4O_6.\frac{1}{2}H_2O$ requires C, 58.7; H, 4.0; N, 13.0%); $\nu_{max.}$ (nujol) 3425w, 1730m, 1672m, 1600, and 1560 cm^{-1}; *m/z* 418 (M^+, 21%), 404 (30), 272 (37), 214 (40) and 170 (45).

1-Hydroxy-6-(trifluoromethyl) benzotriazole (**85**). - A mixture of 4-chloro-3-nitro trifluorotoluene (6.6 ml, 44 mmol) and hydrazine hydrate (6.4 ml, 132 mmol) in 99% ethanol, was refluxed for 24 h. After removal of the solvent under reduced pressure, the residue was dissolved in 10% aqueous sodium carbonate solution. This aqueous solution was washed with ether to remove any unreacted starting material and acidified with conc. HCl to precipitate the product, which was washed with ether and dried (Na_2SO_4). White crystals were obtained upon recrystallisation from diethyl ether/petrol (5.78 g, 65%), m.p. 142 - 145°C (lit.,[147] 143 - 147°C) (Found: M^+, 203.0295. $C_7H_4F_3N_3O$ requires 203.0307); $\nu_{max.}$ (nujol) 1630m cm^{-1} (aromatic); δ_H (270 MHz, acetone-d_6), 7.67 (1H, dd, *J* 1 & 1.5 Hz, Ar-H), 8.13 (2H, m, Ar-H) and 10.94 (1H, bs, OH).

1,1-Bis [6-(trifluoromethyl) benzotriazolyl] oxalate (BTBO) (**86**). - A solution of 1-hydroxy-6-(trifluoromethyl) benzotriazole (2 g, 9.84 mmol) in dry ether (30 ml) was vigorously stirred with a mechanical stirrer, whereupon oxalyl chloride (1.69 ml, 19.7 mmol) was slowly added at room temperature. After

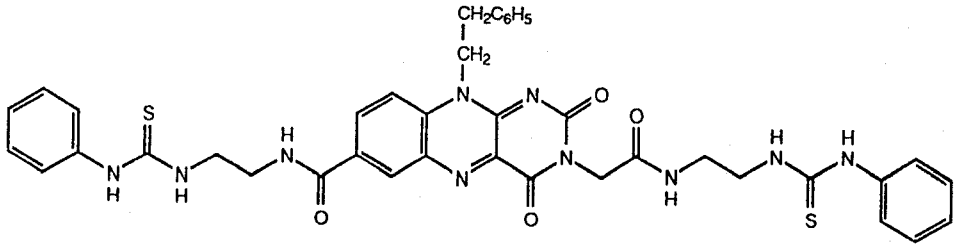
stirring for 3 h, the resulting white precipitate was filtered and washed with dry ether and a trace of dry acetone. Almost pure crystals of BTBO were obtained without recrystallisation, m.p. 151 - 153°C (lit.,[146] 141 - 145°C); $\nu_{max.}$ (nujol) 1750m (C=O), 1730 (C=O) and 1600m cm^{-1} (aromatics); δ_H (270 MHz, acetone-d_6) 7.50 - 8.25 (6H, m, C_6H_6 x 2).



3-(N-Propyl)-N'-acetamido-7-(N-propyl)-formamido-10-N-phenylisoalloxazine (**89**). - Suspended 1,1'-*Bis*[6-(trifluoromethyl)benzotriazolyl] oxalate (BTBO) (**86**) (120 mg, 0.26 mmol) in acetonitrile (5 ml) was added to a solution of the diacid flavin (**80**) (50 mg, 0.12 mmol) and pyridine (21 µl, 0.26 mmol) in acetonitrile (10 ml). After evolution of gas had ceased, the mixture was stirred for 1 h at room temperature until clear. Propylamine (49 µl, 0.60 mmol) was added without isolation of the active ester and the resulting orange reaction mixture stirred for 4 h. The solvent was removed *in vacuo* and the product obtained by crystallisation from methanol/diethyl ether (29.8 mg, 51%), m.p. 172°C dec. (Found: M^+, 502.2126. $C_{27}H_{30}N_6O_4$ requires M, 502.2329); $\lambda_{max.}$ (DMF) 434 (ϵ 3 300 $M^{-1}cm^{-1}$) and 284 nm (17 100); δ_H (270 MHz, MeOH-d_4) 0.95 (3H, t, J 7.5 Hz, CH_3), 0.97 (3H, t, J 7.5 Hz, CH_3), 1.55 (2H, sextet, J 7.3 Hz, CH_2CH_3), 1.67 (2H, sextet, J 7.3 Hz, CH_2CH_3), 3.20 (2H, q, J 6.9 Hz, $NHCH_2CH_2$), 4.39 (4H, m, $NHCH_2CH_2$ & $C_6H_5CH_2$), 4.72 (2H, s, $NHCH_2CO$), 4.95 (2H, t, J 7.5 Hz, $C_6H_5C_2CH_2$), 7.35 (5H, m, C_6H_5), 7.88 (1H, D, J 9.27 Hz, 9-H), 8.27 (1H, dd, J 2.13 & 9.08 Hz, 8-H) and 8.55 (1H, d, J 1.93 Hz, 6-H); δ_C (67.9 MHz, MeOH-d_4) 12.51 (CH_3), 12.61 (CH_3), 24.43 (CH_2CH_3), 24.50 (CH_2CH_3), 34.64 (NCH_2), 43.12 ($CH_2CH_2CH_3$), 43.85 ($CH_2CH_2CH_3$), 45.83 ($C_6H_5CH_2$), 118.68 (C_9), 128.89 (aromatics), 169.64 ($C_{7\alpha}$) and 170.60 ($C_{3\beta}$); *m/z* (EI) 502 (M^+, 8%), 445 (13), 398 (22), 313 (31), 255 (16) and 104 (100).

1-(2-Aminoethyl)-3-phenylthiourea (**90**). - A solution of phenylisothiocyanate (1 ml, 8.36 mmol) in dry diethyl ether (2 ml) was added to a stirred solution of ethylene diamine (1.13 ml, 11.7 mmol) in isopropyl alcohol (13 ml) while maintaining the temperature below 15°C. The mixture was stirred until a white precipitate separated, then diluted to about 50 ml with water and allowed to stand overnight at ambient temperature. The mixture was stirred and acidified to pH 2.6 by the dropwise addition of conc. hydrochloric acid and then heated at 70°C for 30 min with stirring. The remaining precipitate in the mixture was removed by filtration and was washed with warm water. The filtrate was evaporated *in vacuo*, producing a light white solid residue which was then dissolved in water. The aqueous solution was cooled and made basic with saturated sodium hydroxide solution which precipitated the monophenylthiourea derivative. The product was collected by filtration, washed with ice-cold water and recrystallised from water as white needles (1.19 g, 73%), m.p. 133 - 135°C (lit.,[149] 135 - 136°C); $\nu_{max.}$ (nujol) 3348s (N-H), 1590w (aromatic), 1540m (CONH), 1500m (CONH) and 1240m cm^{-1} (C=S); δ_H (270 MHz, MeOH-d_4) 2.88 (2H, t, *J* 6.6 Hz, $NH_2C\underline{H}_2$-), 3.36 (2H, t, *J* 6.6 Hz, $CSCHC\underline{H}_2$-), 4.89 (2H, bs, NH_2), 7.22 (2H, t, *J* 6.9 Hz, 2NH), 7.36 (5H, m, Ar-H); δ_C (67.9 MHz, MeOH-d_4) 41.72 ($NH_2\underline{C}H_2$), 47.99 ($NH_2CH_2\underline{C}H_2$), 125.77 (C-Ar), 126.86 (C-Ar) 132.23, (Ar) 139.52 (NHC-Ar) and 182.57 (CS).

1-(2-Aminoethyl)-3-methylthiourea (**91**). - Methylisothiocyanate (0.94 ml, 13.7 mmol) in ethanol (5 ml) was added dropwise with stirring to ethylene-diamine (3.71 ml, 55 mmol) in ethanol (13 ml) at room temperature. The mixture was warmed briefly and the organic solvent removed *in vacuo*. The residue was kept *in vacuo* over conc. H_2SO_4 overnight to remove excess diamine, whereupon off-white crystals where formed. These were filtered and recrystallised from acetonitrile to afford the product as white prisms (1.33 g, 73%), m.p. 102°C (lit.,[218] 103°C) (Found: C, 36.0; H, 8.5; N, 31.7. $C_4H_{11}N_3S$ requires C, 36.1; H, 8.3; N, 31.5%); δ_H (270 MHz, D_2O) 2.62 (3H, t, CH_3), 2.70 (2H, bs, $NH_2C\underline{H}_2$) and 3.28 (2H, bs, $NHC\underline{H}_2$); *m/z* (EI^+) 133 (M^+, 22%), 91 (100), 74 (47) and 30 (62).



[7-(2-Formamido-ethyl)-3-(2-acetamido-ethyl)]-3,3'-*bis* phenyl thiourea-10-N-phenethylisoalloxazine (**92**). - BTBO (**86**) (120 mg, 0.26 mmol) was added to a suspension of the diacid flavin (**84**) (50 mg, 0.19 mmol) and DMAP (32 mg, 0.26 mmol) in acetonitrile (10 ml). After evolution of a gas, the solution cleared on stirring for 1h. 1-(2-aminoethyl)-3-phenylthiourea (**90**) (117 mg, 0.6 mmol) was added to the resultant red solution at room temperature. Excess solvent was removed *in vacuo* and the product purified by column chromatography (silica gel, CH_2Cl_2/7.5% MeOH) (57 mg, 62%), m.p. 155°C dec.; $\lambda_{max.}$ (DMF) 436 (ϵ 16 100 $M^{-1}cm^{-1}$), 332 (12 900) and 280 nm (106 400); $\nu_{max.}$ 1730w, 1670w, 1605m and 1570w cm^{-1}; δ_H (360 MHz, MeOH-d_4/$CDCl_3$) 3.15 (2H, t, *J* 7.98 Hz, N(10)$CH_2C\underline{H}_2$), 3.45 (2H, bt, CH_2), 3.67 (2H, t, *J* 2.76 Hz, CH_2) 3.77 (2H, t, *J* 4.94 Hz, CH_2), 3.92 (2H, bt, CH_2), 4.76 (2H, s, NCH_2CO), 4.88 (2H, t, *J* 7.90 Hz, N(10)$C\underline{H}_2CH_2$), 7.10 - 7.35 (15H, aromatics), 7.70 (1H, d, *J* 9.22 Hz, 9-H), 8.30 (1H, dd, *J* 2.05 & 9.06 Hz, 8-H) and 8.57 (1H, d, *J* 2.08 Hz, 6-H); δ_C (67.9 MHz, MeOH-d_4/$CDCl_3$) 34.22 ($N\underline{C}H_2CH_2C_6H_5$), 41.74 ($NCH_2\underline{C}H_2C_6H_5$), 44.62 ($N\underline{C}H_2CO$), 45.42 ($\underline{C}H_2NHCS$), 45.77 ($\underline{C}H_2NHCS$), 48.07 ($\underline{C}H_2NHCO$), 117.66 ($C_9$), 126.03 - 139.27 (aromatics), 150.86 (C_{10a}), 157.31 (C_2), 161.34 (C_4), 167.62 ($C_{3\beta}$), 170.23 ($C_{7\alpha}$) and 182.61 (C=S); *m/z* (FAB) 776 (33%, $(M+H)^+$), 500 (25), 139 (27), 77 (100) and 63 (41).

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