Elucidation of the Quinone Methide Tautomer of Riboflavin and Generation of a Flavin Nitroxyl Radical

by

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Department of Chemistry

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Chairman, Departmental Graduate Committee

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Professor W. H. Rastetter — Signature redacted — Thesis Supervisor

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Submitted to the Department of Chemistry on June 1, 1981 in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Chemistry

ABSTRACT

A method for the generation of the quinone methide tautomer of riboflavin free in solution is described. Nucleophilic and electrophilic interception of this species by molecules relevant to naturally occurring 8α-peptidyl flavins is examined. Oxygen transfer from 3-methyl-8-demethyltetraisobutyrylriboflavin-5-oxide to phenolates is demonstrated along with concomitant generation of a flavin nitroxyl.

Thesis Supervisor: Professor W. H. Rastetter

Title: Associate Professor of Chemistry

ACKNOWLEDGEMENT

As my investigative career has advanced, I have discovered that the breakthroughs achieved and successes so treasured are as much a function of my environment and the people contributing to it as they are due to any particular skill of my own. Without any doubt, Professor Christopher Walsh has been one of the strongest intellectual influences in my life. His brilliance and dedication to science has proven to be surpassed only by his personal warmth. Such a combination of qualities shall most certainly set the standard by which I will judge my progress as a scientist.

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Finally, I wish to express my gratitude to the Chemistry Department of M.I.T., the National Institutes of Health, and Professor William Rastetter for financial support.

CONTENTS

INTRODUCTION

Beginning with their isolation as "lactochrome", a yellow pigment in cow's milk, flavins have been the target of intensive investigation. Roughly a century of research might long ago have exhausted the last interesting bit of data were it not for the widespread occurrence and diversity of function of flavins. Though flavins may be one of the most studied biological cofactors, they remain among nature's most ubiquitous catalysts.

Two areas which occupy a large portion of the vanguard of contemporary flavin research[1] include:

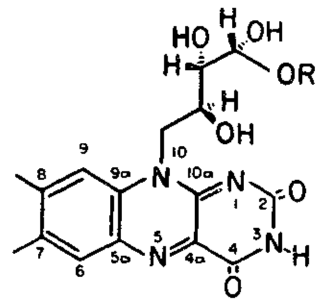
1. flavoenzymes where the nature of cofactor binding is defined by a covalent bond between flavin and apoprotein; and

2. the elusive identity of the flavin/oxygen adduct responsible for flavin monooxygenase activity.

Many of these flavoenzyme functions are restricted in nature to bacteria and at first glance may seem far removed from humans. Closer examination reveals a different picture. Bacteria are responsible for removal of vast quantities of chemical toxins from the environment. In a world being inundated with ever-increasing amounts of industrially spewed chemical wastes, an understanding of the molecular basis for nature's detoxification schemes is critical.

A high degree of sophistication in drug design is pushing the pharmaceutical industry towards greater interest in flavin cofactors. Even though amine-N-oxidase is the only flavin monooxygenase found in humans, its importance is magnified when the number of alkaloid drugs is considered (quinine, codeine, nicotine, lysergic acid). Certainly an understanding of the flavin-mediated first step of degradation is important to predicting the length of action of a drug and its potential physiological side effects. Monoamine oxidase, one of the apoproteins covalently linked to flavin, is of central metabolic importance due to its degradation of monoamines. Selective inhibition of this enzyme in brain tissue is used in treatment of psychic disorders in man.

For nearly thirty years after the structure determination and total chemical synthesis of riboflavin, the only forms of flavin thought to exist in nature were riboflavin (RFl) 1a, flavin mononucleotide (FMN) 1b, and flavin adenine dinucleotide (FAD) 1c. With time a whole range of flavins were recognized to be released from apoprotein only under such forcing conditions as proteolysis and strong acid digestion. These flavins were isolated from sources ranging from mammalian brain tissue to soil bacteria and found to be



Ia R=H

Ib R=PO_3^{-2}

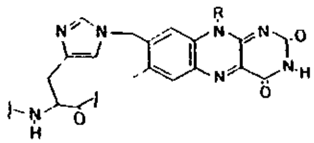
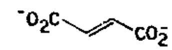
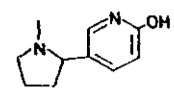
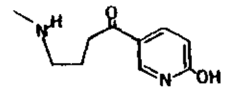
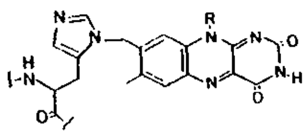
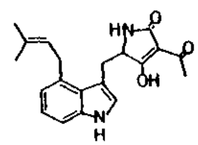
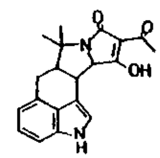
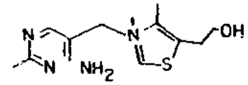
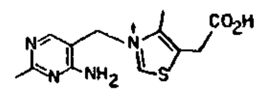
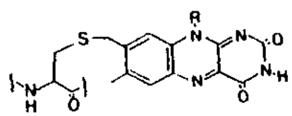
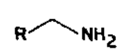
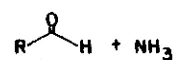
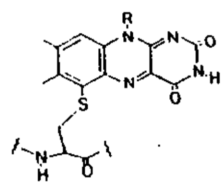
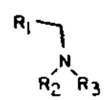
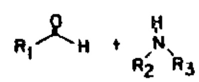
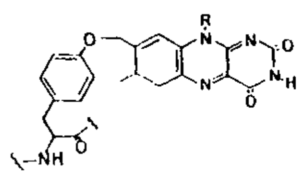
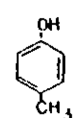
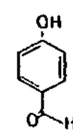
Ic R = $P(=O)(O^-)$-O-$P(=O)(O^-)$-O adenosine

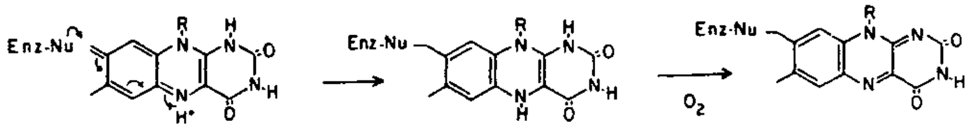
covalently linked to apoprotein. Generally, the linkage is through the 8α methyl group of the isoalloxazine nucleus to an imidazole nitrogen of histidine 2a, 2b,[2] sulfur of cysteine 3,[3] or oxygen of tyrosine 4[4] (see Table I for peptidyl flavin structure and function).

Perturbation of the reduction potential to more positive values is a general phenomenon of electron withdrawing substituents at the 8α position of riboflavin.[5] 8α Peptidyl flavins are in line with this trend.[6] However, their reduction potentials being twenty to thirty millivolts more positive than riboflavin seems of negligible importance *in vivo*.

Recently Walsh hypothesized that peptidyl flavins are generated by interception of a quinone methide tautomer of riboflavin[1a] (Scheme I). This proposal is particularly insightful in relation to the versatile nature of the

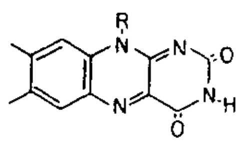
Table I

Cofactor	Enzyme	Substrate	Products
	succinate dehydrogenase		
	D-6-hydoxynicotine oxidase		
	β-cyclopiazonate hydroxylase		
	thiamine dehydrogenase		
	monoamine oxidase		
	trimethylamine dehydrogenase		
	p-cresol hydroxylase		



Scheme I

electronic backbone of the isoalloxazine. Flavins can exist in three oxidation states: the fully oxidized 5, the semiquinone radical 6, or the fully reduced dihydro 7. These differing electronic clouds on the same heterocyclic nucleus enable flavins to shuttle electrons between obligate two electron donors and obligate one electron acceptors and account for flavin reactivity with oxygen.[7] Quinone methide form 8 as proposed by Walsh is a fourth, biologically relevant distortion of the electron cloud.



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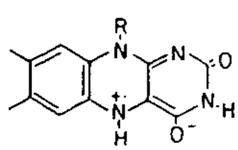

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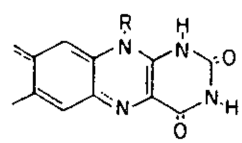

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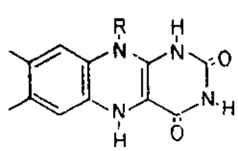


7

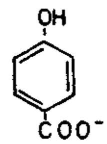
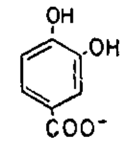
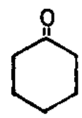
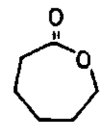
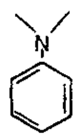
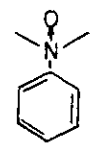
While oxidative states 5, 6 and 7 account for the catalytic activity of the cofactor, the quinone methide 8 dictates the nature of the cofactor-apoprotein binding.

At the time this research was initiated no experimental evidence existed for the proposed attachment mechanism. Hemmerich in the late fifties observed 8α,8α dimerization of riboflavin[8] which he thought involved the quinone methide tautomer. With the exception of a brief communication concerning 8α deuterium exchange when flavin mononucleotide was heated in D_2O,[9] not a single paper over a twenty-year span explored the quinone methide tautomer. Recently, Shinkai[10] has reported a species thought to be the quinone methide tautomer formed under basic conditions on a polymer. The observed species displays no reactivity with thiols.

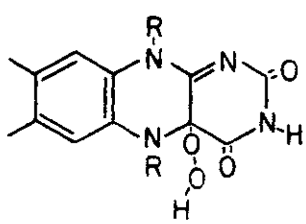
Such a dearth of investigation has not been the case with flavin monooxygenases. Upon two electron reduction, flavin monooxygenases react with oxygen to form what is referred to as an "oxygen gun". This species' reactivity results in one atom of oxygen winding up in water and the other in the oxidized substrates. It is not yet clear whether all flavin monooxygenases use the same oxygen gun. Some examples of oxidations carried out by flavin monooxygenases are shown in Table II.

The flavin hydroperoxide 9a has become a prime candidate for the molecular species responsible for or leading to flavinoid oxygen gun activity. Experiments have

Table II

Enzyme	Substrates		Products	
p-hydroxybenzoate hydroxylase		O_2·Gun	H_2O	
cyclohexanone monooxygenase		O_2·Gun	H_2O	
microsomal amine N-oxidase		O_2·Gun	H_2O	

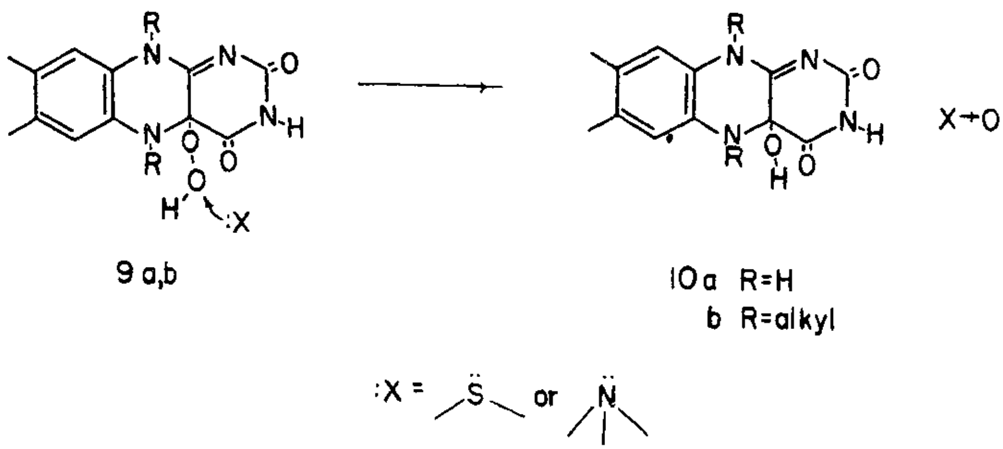
demonstrated the kinetic competence of the 4α-hydroperoxide[11] and independently synthesized flavin 5-ethyl-4α-peroxide 9b[12] has been shown to be spectroscopically extremely close to a species seen in stopped flow techniques.



9a R=H
9b R=alkyl

Several different mechanisms have been proposed for flavin oxygen transfer ability. One proposed in differing forms by Massey and Bruice utilizes the 4α hydroperoxide as the species transferring oxygen. Scheme II shows this proposal in relation to oxidation of amines and sulfides.

Scheme II

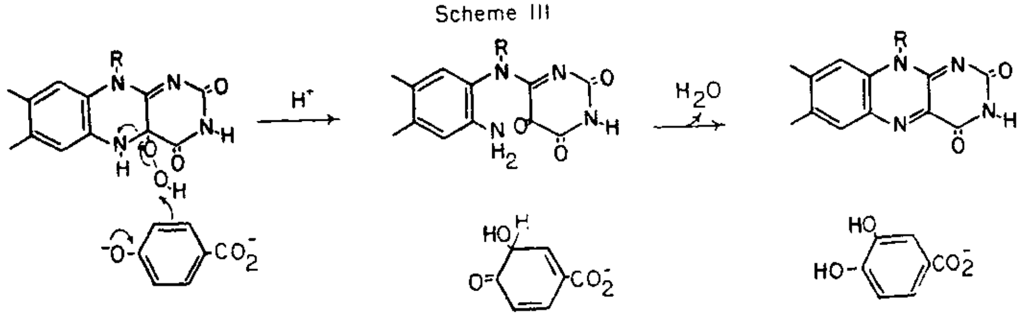


Bruice has found this oxidative chemistry to be incredibly facile.[13] The reaction of dimethyl aniline with 4αFlEtOOH 9b is so much more rapid than with hydrogen peroxide or t-butylhydroperoxide that comparison of rate constants is not possible. Sulfur oxidation of thioxane does facilitate comparison of second order rate constants and shows the ratio of 4αFlEtOOH:H_2O_2:t-BuOOH to be 2 x 10^5:20:1. This vast difference in rate seems to argue against oxidation of sulfides and amines being due to reaction with steady state pools of hydrogen peroxide. Furthermore, S-oxidation of thioxane by hydrogen peroxide and alkyl hydroperoxide is secc order in hydroperoxide reflecting a second molecule of hydroperoxide serving as a proton source. Oxidation of thioxane by 4αFlEtOOH is first order in 4αFlEtOOH, reflecting no apparent requirement for protic assistance.

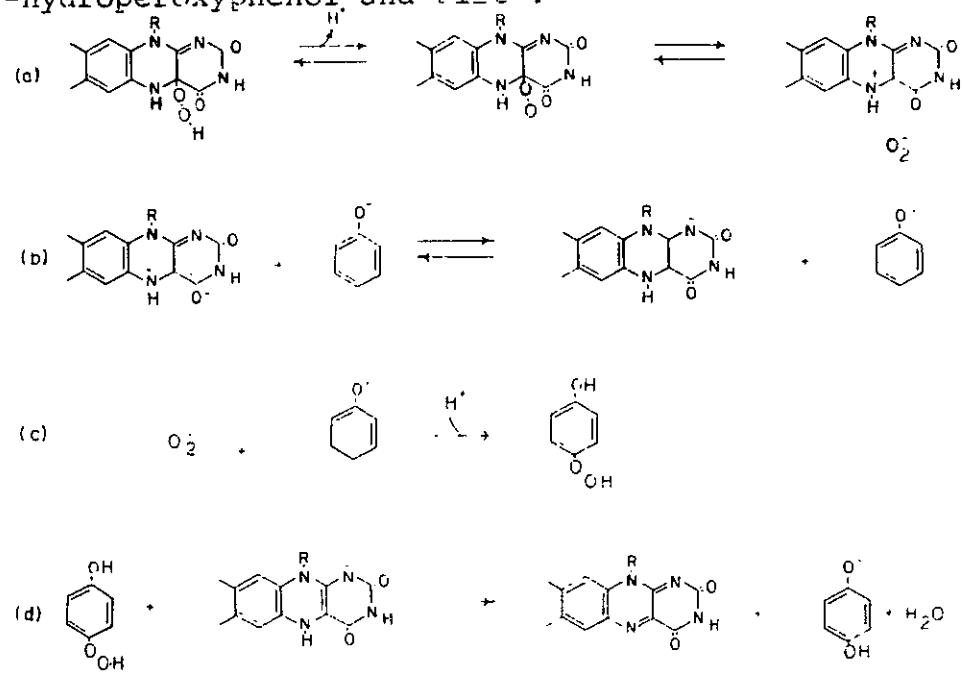
Nucleophilic attack on the terminal oxygen is indicated by spectra of the completed reaction being identical to 4αFlEtOH, 10b. A plot of the logarithm of the second order rate constant versus pK_a of the amine yields separate parallel lines with each line describing one class of amine. Relative reactivity for amines of similar pK_a follows hydroxylamines > tertiary amines > secondary amines. A slope of ∿ .3 corresponds to a $B_{Nu:}$ value consistent with nucleophilic displacement.[13] Radical mechanisms for oxidation are excluded by the absence of a quenching effect on the rate by addition of the free radical inhibitor 2,6-di-tert-butyl-4-methylphenol (although this can not exclude one electron chemistry within the solvent cage).

In relation to flavin hydroxylation of phenols Massey proposes the two electron process in Scheme III whereas Bruice prefers the radical reaction Scheme IV.

Scheme III



Massey's proposals for phenolate oxidation are virtually identical to those for amine and sulfide oxidation. Hence one would expect 4αFlEtOOH to react with phenoxide to give catechol anion and 4αFlEtOH 10b. But reaction of 2,6-di-t-butyl-4-methylphenol yields 2,6-di-t-butyl-4-methyl-4-hydroperoxyphenol and $FlEt^{-}$.[14]



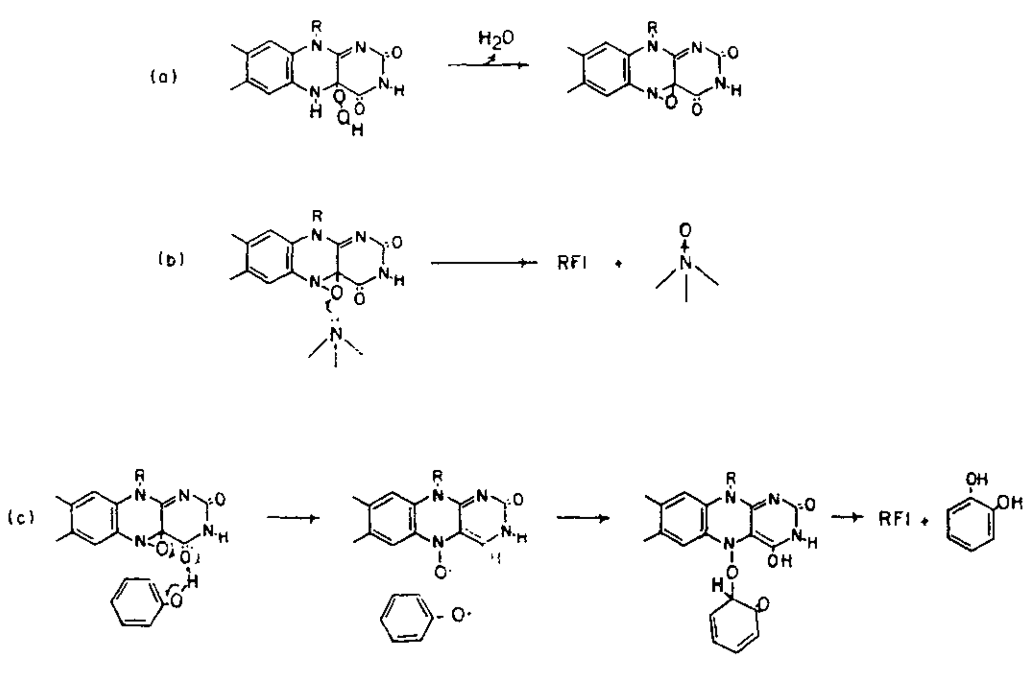
Scheme IV

The lack of formation of products predicted by Scheme III prompted the Bruice proposal of radical intermediates. However, step (c) of Scheme IV constitutes a devastating weakness. As a potassium crown salt, superoxide reduces the radical of 2,6-di-tertbutyl-4-methylphenol producing $^{3}O_2$ and phenolate anion.[15] There appears elsewhere in the literature no documented example of superoxide coupling with a radical species to give a peroxide.[16]

To patch up his hypothesis, Bruice has tried to invoke a dioxetane between 4α and 10α and even singlet oxygen-flavin complexes to account for the oxidative chemistry of the 4α hydroperoxide.[17] None of these proposals seem biologically appealing and all lack precedent for the bond scissions invoked. In fact, Bruice has reported oxygen insertion into only two phenolates.[14,17] An overview of the relevant literature[14,17,18] indicates that while the flavin 4α hydroperoxide adequately explains amine and sulfide oxidation it does not effectively account for phenolate oxidation.

An alternate hypothesis proposed for the identity of the flavin oxygen gun invokes decomposition of the 4α hydroperoxide to an oxaziridine (Scheme V). The idea of the intermediacy of an oxaziridine was initially proposed by Dolphin and Orf[19] and since extensively modified by Rastetter.[20]

Scheme V



Dolphin and Orf proposed their mechanism with only the slightest shred of model system support. Oxaziridines are known to be weak oxidants but as a general class of oxidant their reactivity is unknown.

Precedent for step (c) of Scheme V follows from a report by Rastetter of oxidation of phenols when flavin-5-oxide was photolyzed in organic media with the phenol.[20] The relevance of this result to biological, ground state phenol oxidation is dubious as it is extremely difficult to extrapolate from a model system utilizing 50-70 kcal of photolytic excitation energy. Nonetheless, the proposal for involvement of a nitroxyl radical is particularly intriguing. Nitroxyl radicals are excellent oxidants of phenols and if a biologically relevant ground state generation of such a reactive species were possible, a major impasse in the flavin literature would be broken.

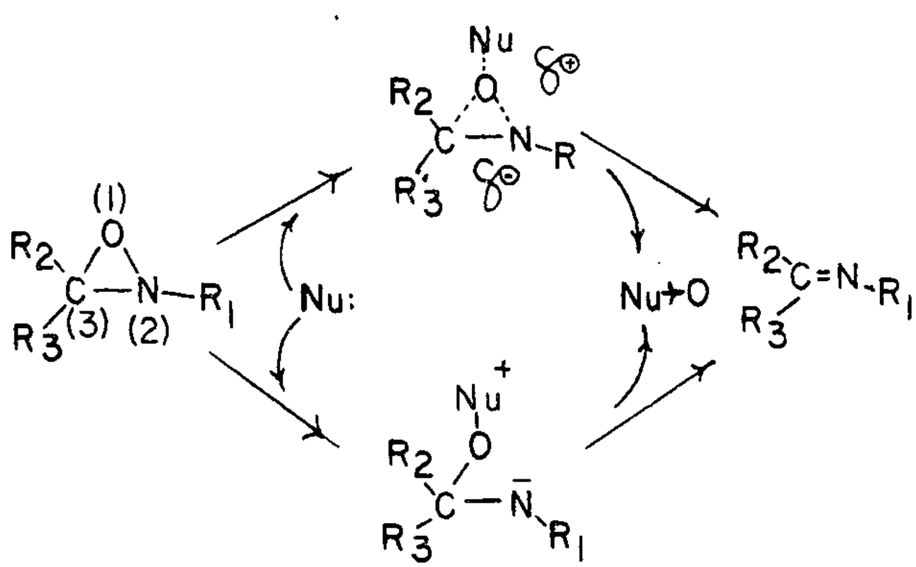
At the time this research was initiated, only one example of oxaziridine oxidation of an amine existed.[21] Research began with an evaluation of the oxidizing power of simple oxaziridines.

CHAPTER I

ASCERTAINING THE OXIDATIVE CAPACITY OF OXAZIRIDINES

Aside from Emmons' pioneering work,[21] oxaziridines have been the target of little comprehensive investigation. Dolphin and Orf proposed their mechanism for flavin monooxygenase activity with virtually no precedent existing in the literature on oxaziridine behavior relative to nucleophiles.

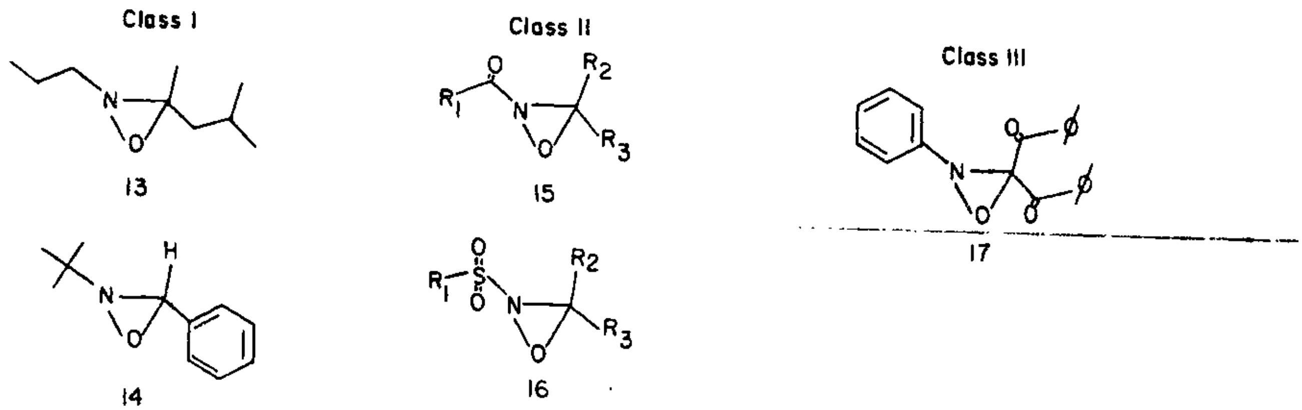
Attack on oxaziridine ring oxygen can be viewed as a concerted or stepwise process (Scheme VI):



Scheme VI

Based on their ability to stabilize the incipient negative charge accompanying nucleophilic attack, a means for categorization of known oxaziridines is possible (Table III).

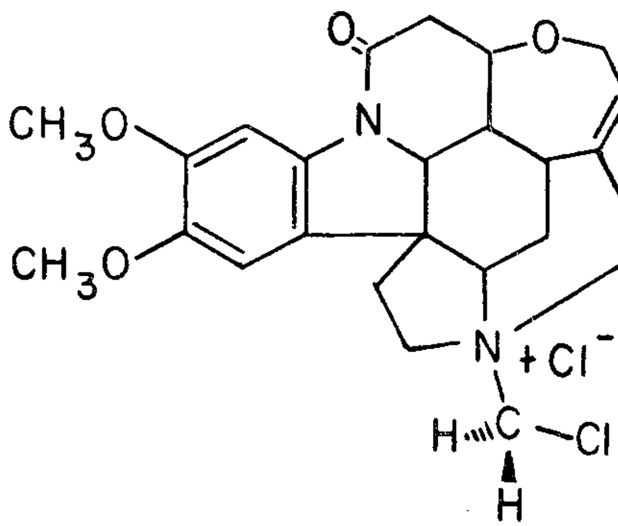
Table III



Class I oxaziridines are quite stable and were the earliest synthesized. Substituents are either alkyl, aryl, or combinations thereof. Class II is defined by substitution at N(2) enabling heteroatom stabilization of developing negative charge. Class III describes heteroatom stabilization at the oxaziridine (3) position.

One of the key points in early oxaziridine (Class I) structure determination was that an oxaziridine, unlike a nitrone, has an asymmetric carbon. Emmons[21] reported a kinetic resolution of oxaziridine 13 with brucine leading to recovery of oxaziridine with α_D^{22} -3.94°. During the course of the resolution a white, crystalline solid precipitated out of solution. Emmons claimed this solid was brucine-N-oxide resulting from attack by the tertiary amine on ring oxygen. For the ensuing twenty years this result was cited as an example of the oxidizing capacity of oxaziridines.

We found that refluxing a methylene chloride solution of 13 and brucine does lead to the precipitation of a white, crystalline solid (MP 194°C, dec).[22] Recovery of remaining 13 indicates preferential destruction of one antipode. After distillation and chromatography, recovered 13 displays α_D^{20} - 4.34°, ℓ=1, neat. Combustion analysis, ^{1}H NMR and field desorption (FD) mass spectroscopy of the white, crystalline precipitate shows the material to be the quaternized amine adduct of brucine and methylene chloride 18.



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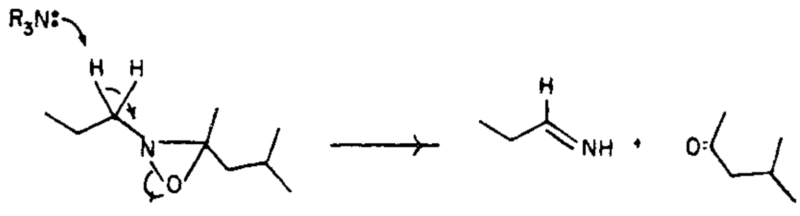
Examination of the kinetic resolution reaction mixture by HPLC shows no formation of brucine-N-oxide.

During chromatographic recovery of 13 from the kinetic resolution we isolated ketone 19 (Scheme VII). Examination of the reaction mixture during kinetic resolution shows the formation of ketone 19 and imine 20, Scheme VII (Table IV).

Scheme VII



The resolution of 13 suggests a brucine catalyzed fragmentation of the oxaziridine (Scheme VII). Aldimines generated in the base catalyzed oxaziridine fragmentation have been isolated by Boyd.[23] Furthermore, this reaction has been used synthetically in an amine to ketone conversion.[24] Oxaziridine 14, lacking α hydrogens, is not resolved by brucine. The alleged formation of brucine-N-oxide from 14 is also incorrect. Again the product formed is the brucine/CH_2Cl_2 adduct.

Simultaneous to our work, Hata and co-workers[25] undertook an exhaustive study of nucleophilic attack on Class I oxaziridines. For oxaziridines with no steric hindrance of ring nitrogen, attack by amines occurs at ring nitrogen. Sulfides and triphenylphosphine reacted at both nitrogen and oxygen with a preference towards ring nitrogen. When the steric bulk on nitrogen increased to a t-butyl substituent, reaction shifted towards oxygen abstraction for sulfur and

Table IV. Kinetic Resolution of Oxaziridine 13; Formation of Imine 5 and Ketone 4

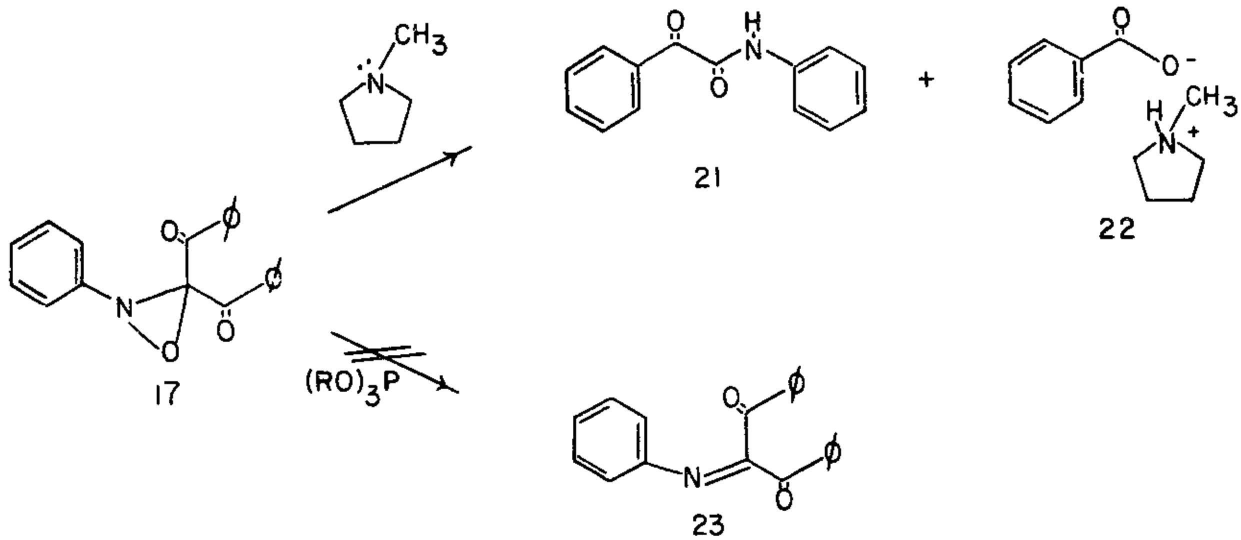
Time Elapsed (hrs)	Percentage 13 Destroyed[b]	Percentage 20 Formed[c,d]	Percentage 19 Formed[b]
1.0	9.6-12.5	11.1-11.8[e]	10.0-11.8
4.0	33.6-34.8	28.6-30.5	30.1-33.8
10.0	52.6-55.5	23.0-25.9	49.3-50.7
16.0	62.5-68.1	14.7-18.0	59.1-62.5

[a]Range represents results of two kinetic runs, performed according to Emmons, reference 21. [b]Determined by GLC vs. dodecane internal standard. [c]Determined by HPLC (C-18 column, 3:1, $DH_3CN:H_2O$) of the 2,4-dinitrophenylhydrazone derivative (2,4-DNP) vs. the 2,4-DNP of heptanal as internal standard. [d]The 2,4-DNP derivative was indistinguishable from an authentic sample by HPLC coinjection, mixture melting point and mass spectral fragmentation pattern. [e]At short reaction times 13 is converted quantitatively to 20 plus 17. Imine 20 is not stable to prolonged heating in the reaction mixture.

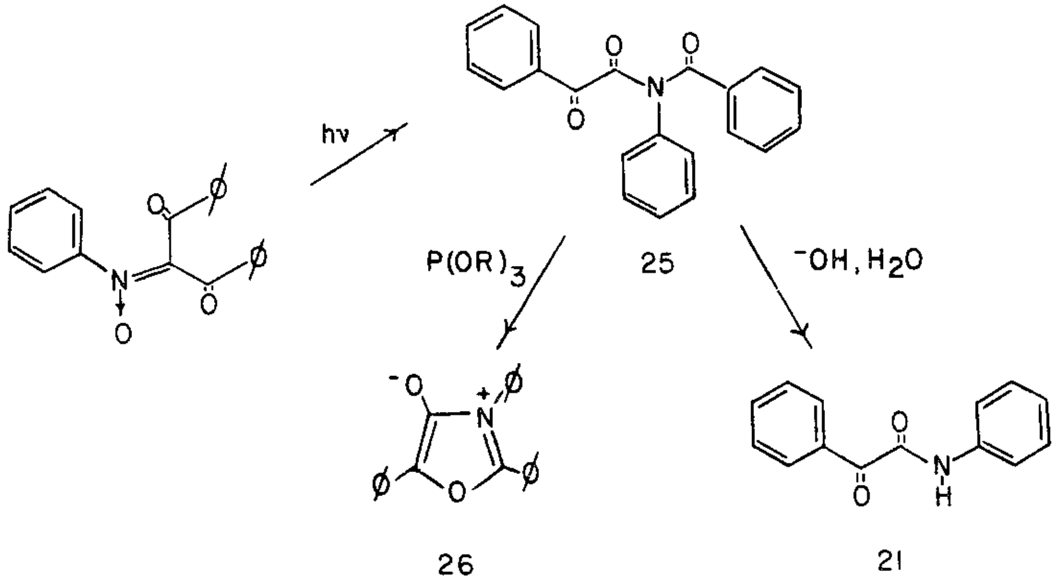
phosphorous nucleophiles while amines no longer reacted. The oxaziridines carbon (C-3) was inert even in the absence of steric hindrance at that center.

A general trend of oxaziridines functioning as aminating agents continues into Class II. Schmitz[26] has found 2-acyl oxaziridines 15 to react vigorously with secondary amines at ring nitrogen. There is an exception to this trend. Davis[27] found 2-sulfonyloxaziridine 16 to be a good oxidant of amines, sulfides, and even olefins.

Oxaziridines of greatest relavance to flavins would be in Class III. An exhaustive search of the literature revealed only one 3-acyloxaziridine 17.[28] Aside from oxaziridine 17 all other attempts to synthesize Class III oxaziridines have resulted in rearrangement of the transiently formed oxaziridine to an amide. We initiated a study of 17 reactivity with the tertiary amine N-methylpyrrolidine. Refluxing 17 in moist tertiary amine leads to the isolation of two molecules, the anilide of phenylglyoxalic acid 21 and the N-methylpyrrolidine salt of benzoic acid 22 (Scheme VIII). As a result of this rather surprising result we reevaluated 17 reactivity towards triethylphosphite. Contrary to the claim of Scheinbaum[28] 17 did not yield any imine 23 upon refluxing neat with triethyl phosphite based on HPLC coinjection. Recently Freeman[29] has communicated results showing that 17 does not have the structure claimed by Scheinbaum (Scheme IX). These results fully explain what we observed. Sadly, this removes the only known example of Class III oxaziridines.



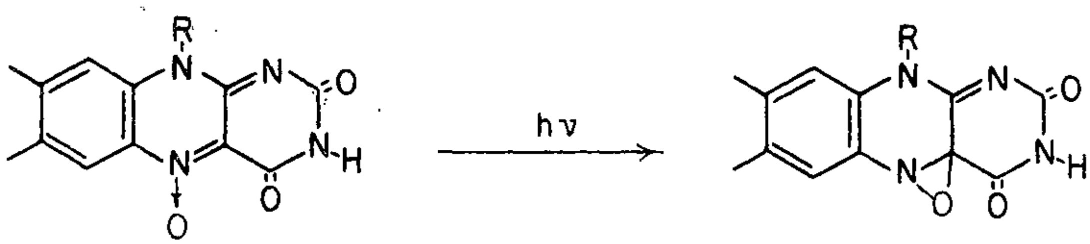
Scheme VIII



Scheme IX

In view of our results and the rapidly expanding oxaziridine literature, it appears that for a flavin oxaziridine to function as a good oxidant of amines and sulfides competitive with the 4αhydroperoxide, it must be an exception to the general trend. Certainly oxaziridine 16 indicates that exceptions are possible.

We tried several ways to make the actual flavin oxaziridine via low temperature photolysis. This approach has been demonstrated to be effective for synthesis of oxaziridines unstable at room temperature.[30] Flavin-5-oxide 27 (Scheme X) was photolized in freeze thaw degassed 2-methyltetrahydrofuran or ethanol glasses with hν = 420-580nm at liquid nitrogen temperatures. Assuming that the flavin oxaziridine has absorbance similar to 4αEtFlOH 9b (λ_{max} 380 nm) one would expect some type of non-uniform bleaching with the 460 nm peak declining faster than the 350 nm peak. We observe a uniform bleaching of both the long wavelength and near ultraviolet flavin absorptions (See Fig. 1).



R=tetraisobutyrylribityl

27

Scheme X

11

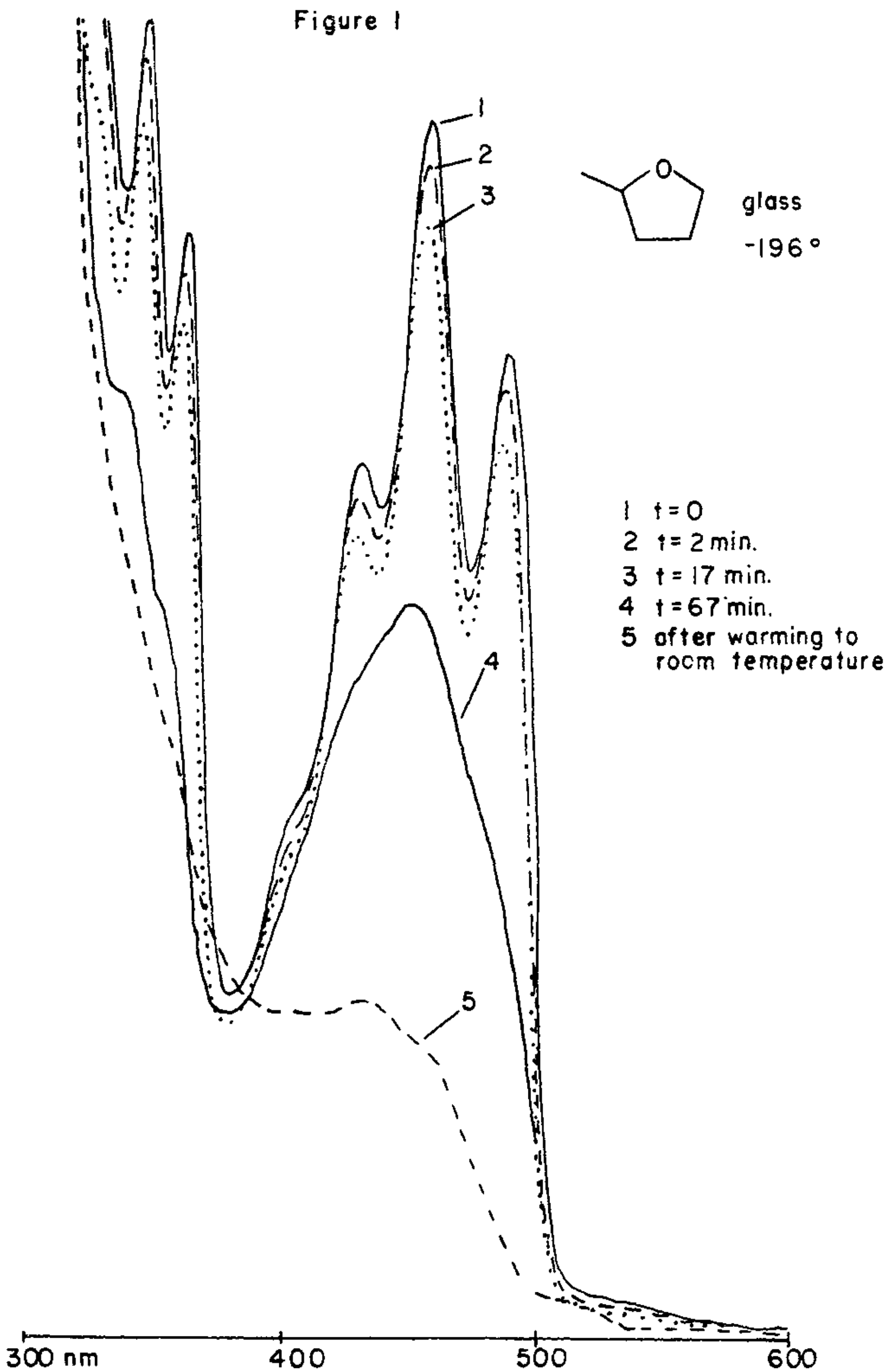
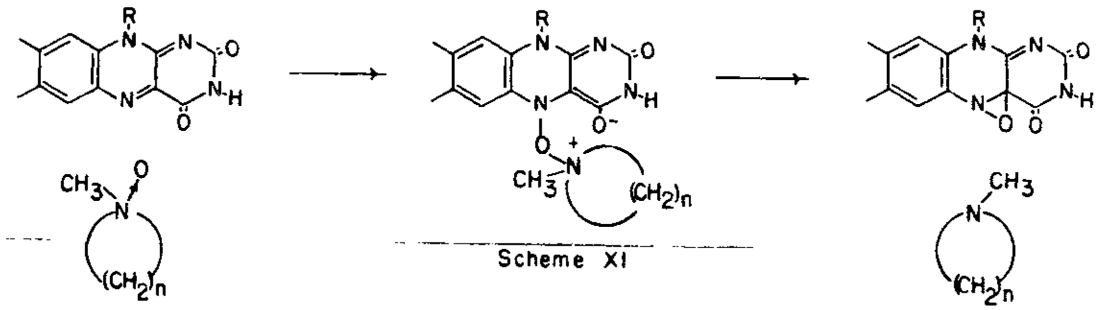


Figure 1
1
2
3
glass
-196°
1 t=0
2 t=2 min.
3 t=17 min.
4 t=67 min.
5 after warming to room temperature
4
5
300 nm
400
500
600

In a second approach to the flavin oxaziridine we tried reacting flavin with cyclic tertiary amine N-oxides (Scheme XI).



Scheme XI

These experiments are based on N-oxide capacity to oxidize benzyl chloride to benzaldehyde[31] (thought to proceed via initial nucleophilic attack by the N-oxide) and the precedented capacity of nucleophiles to attack at N(5)-C(4α) of riboflavin.[32,33] No flavin oxaziridine is formed or any chemistry indicative of it observed. Instead, a reaction occurs which has opened a whole new chapter in flavin chemistry.

CHAPTER II

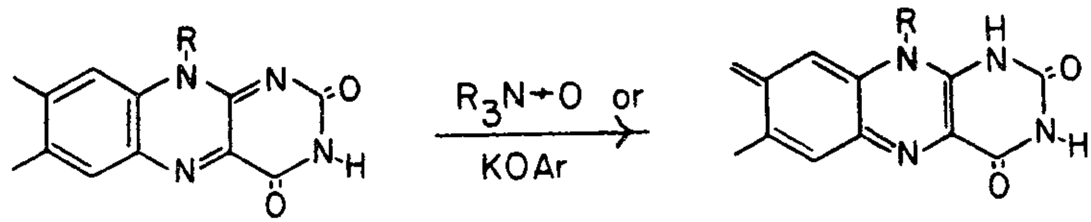
ELUCIDATION OF THE FLAVIN QUINONE METHIDE

Reaction of cyclic tertiary amine-N-oxides 28a-c with tetra-isobutyrylriboflavin 33 leads to formation of brilliant red, crystalline dimer 36a (Scheme XII).[34] N-oxides 28a-c are not alone in their ability to catalyze this transformation (Table V). The conversion is also effected by the tertiary amine base 1,5-diazobicyclo[5.4.0]undec-5-ene (DBU, 29). Potassium phenolates 30, 31 and potassium thiolate 32 convert 33 into a mixture of tautomeric red and orange dimers, 36a and 37, respectively. Dimer 36a upon hydrolysis of the isobutyryl esters forms 36b previously reported by Hemmerich.[8a]

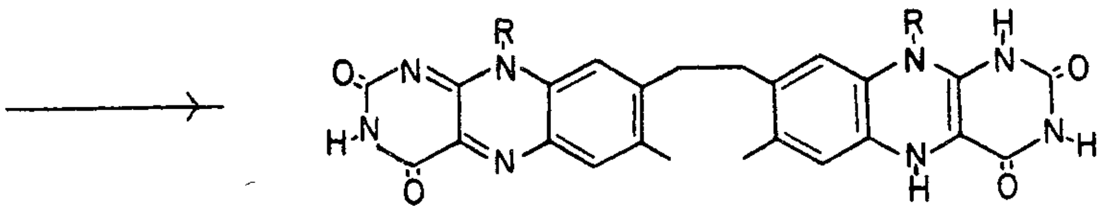
The dimers themselves display interesting secondary chemistry. Dimer 37a is rapidly converted to 36a on addition of N-oxide 28b in anaerobic, organic media. Both dimers 36a and 37 rapidly revert to 33 when treated with thiolate in aerobic organic (Scheme XIII).

A likely mechanism for dimer formation (Scheme XII) has as a key intermediate a quinone methide tautomer 8 of tetraisobutyrylriboflavin 33. N-oxide catalyzed dimerization of 33 is quenched in the presence of 2,3,5,6-tetramethyl-phenol. When O-deuterio-2,3,5,6-tetramethylphenol is used

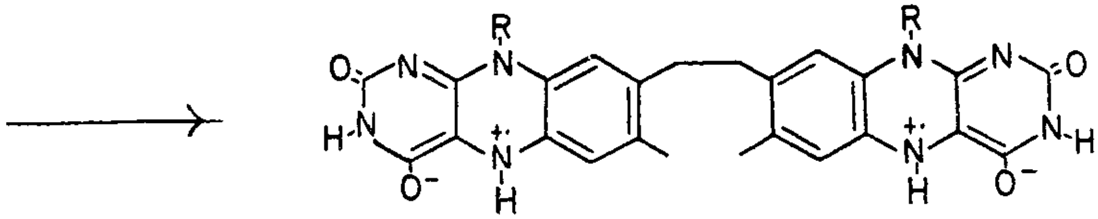
Scheme XII



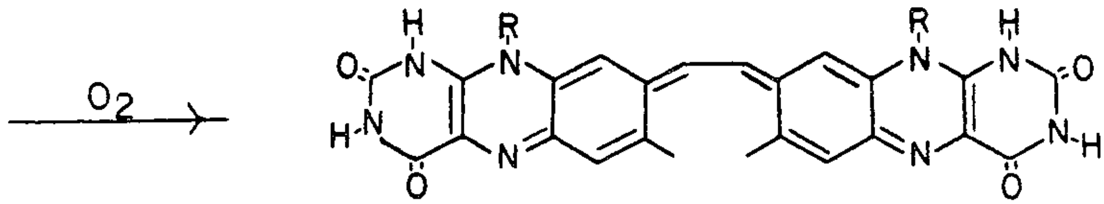
33 8



34

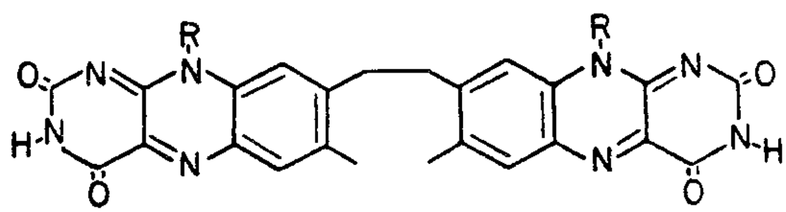


35



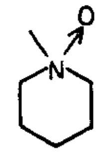
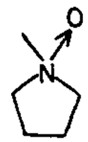
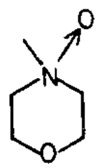
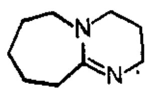
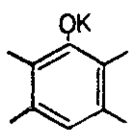
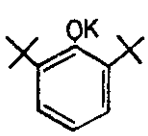
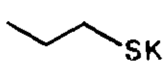
36a R=tetraisobutyrylribityl
b R=ribityl

+

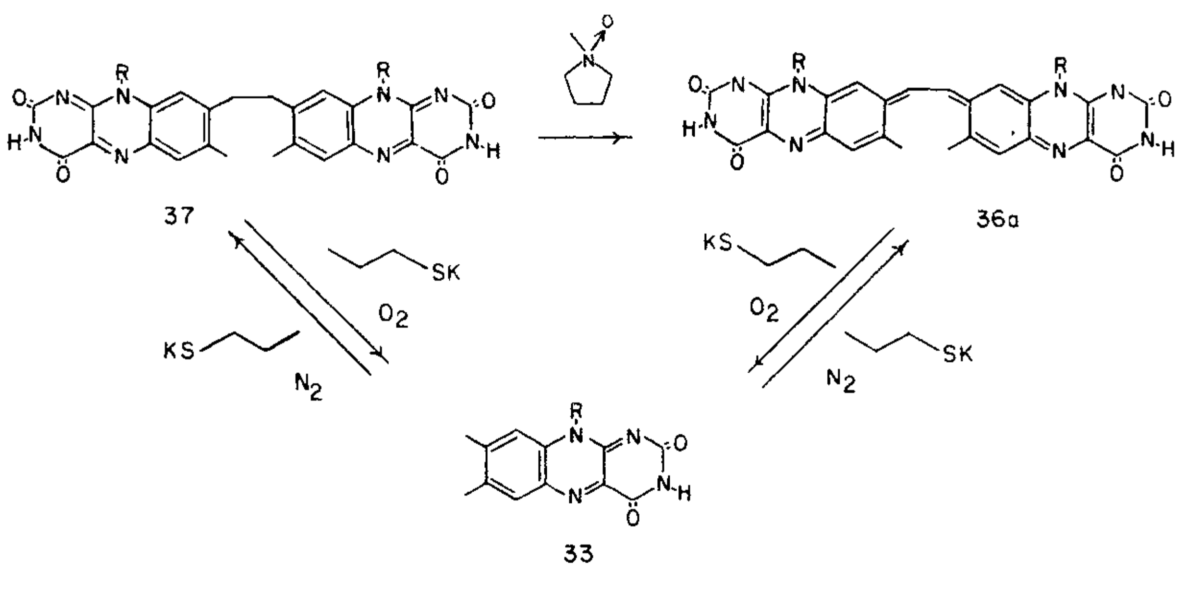


37 R=tetraisobutyrylribityl

Table V

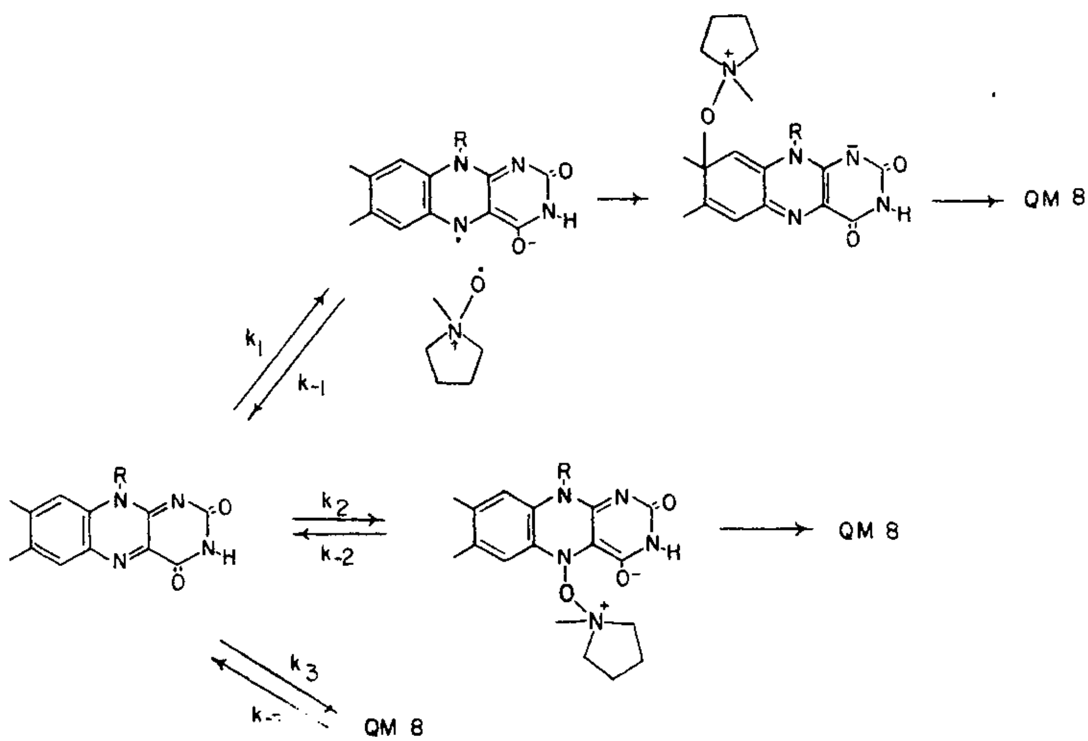
Catalyst	Conditions	HPLC Yield 36a	37	33
 28a	24h	10	—	14
 28b	24h	12	—	4
 28c	24h	trace	—	12
 29	10h	20	—	62
 30	6h	29	19	52
 31	6h	17	38	42
 32	8h	18	trace	29

to inhibit dimerization, recovery of flavin after 24 h reveals (^{1}H NMR) a substantial decrease (∿50%) in the intensity of the 8α methyl absorption relative to the 7α methyl absorption. Prevention of flavin dimer formation by the phenol may reflect the protonation of a quinone methide <u>8</u>. Similar deuterium exchange was observed by Bullock and Jardetzky when flavin mononucleotide was heated at 90-95° in D_2O at pH 6.8-6.9.[9] Dimer <u>37</u> can function as an intramoleculer trap of quinone methide generation. N-oxide catalyzed conversion of dimer <u>37</u> to <u>36a</u> (Scheme XIII) serves as a model for distortion of the normal electron configuration <u>33</u> to that of the quinone methide <u>8</u>.



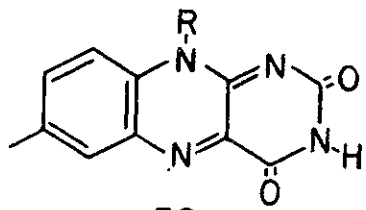
Scheme XIII

In contrast to the generation of 36b with strong base under forcing conditions, the capacity of simple N-oxides to catalyze the same conversion is unexpected. Three mechanisms can be evisioned as responsible for the catalytic activity of the N-oxides (Scheme XIV). Tautomerization via k_1 (Scheme XIV) finds precedent in Bruice's work with the flavin mediated oxidation of methyl-α-hydroxyphenyl acetate anion.[35] Numerous isolations of N(5) covalent adducts in flavin oxidations[33] suggest potential operation of k_2 (Scheme XIV). Finally, k_3 (Scheme XIV) reflects the initial work of Hemmerich.[8]



Scheme XIV

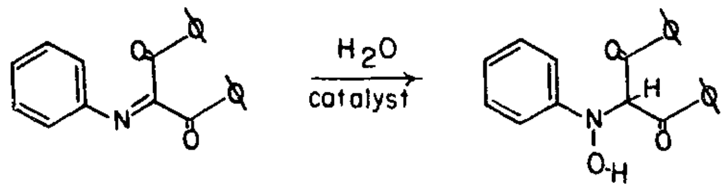
Investigation into the mechanistic basis for the tautomerization made extensive use of 8-demethyltetraisobutyrylriboflavin 38. This molecule, lacking the requisite 8-methyl group for tautomerization to the quinone methide,



38

was prepared via the Merck modification of the Yoneda procedure.[36] Flavin 38 proved to be totally inert to the reaction conditions leading to dimerization of 33. On mixing with N-oxide 28b the reaction mixture did not turn reddish-black like the dimerization reactions. UV monitoring of 38 plus the dimerization catalyst 28b did not reflect any perturbation of the flavin absorptions or formation of anything that could be construed as absorption due to a charge transfer band. The inertness of 38 argues against C(8) or N(5) covalent adducts formed either by recombination of radical species or due to nucleophilic covalent catalysis.

Further evidence relevant to the observed catalysis follows from studies of the hydration of imine 23 to hydroxylamine 39 (Scheme XV).

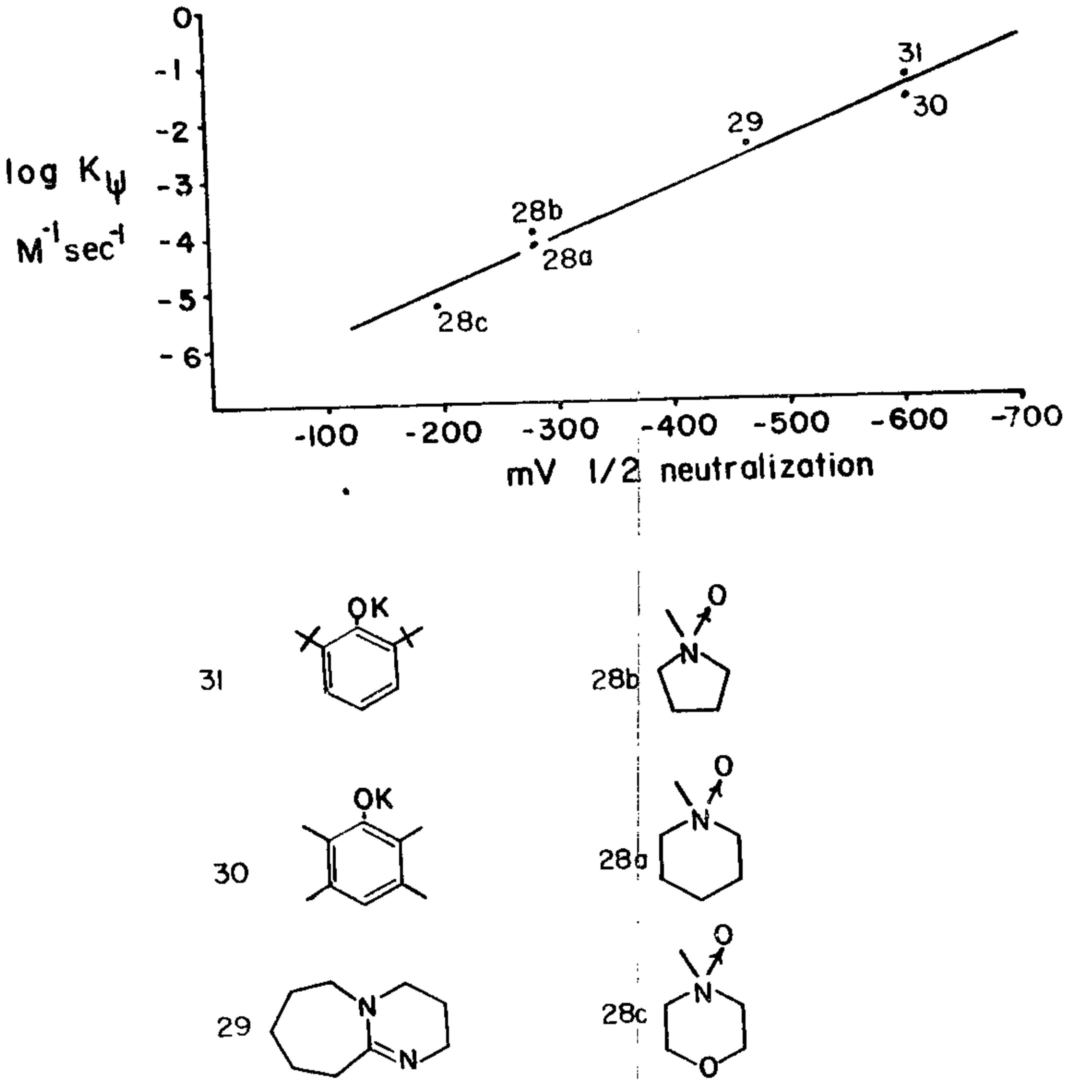


23 39

Scheme XV

As a simple model for the flavin(5)-C(4α) unsaturation, 23 might add bases at nitrogen, at least reversibly, giving a stabilized dibenzoylmethane anion. No adduct formation with the bases of Table V is observed. Instead, hydration of the imine 23 in moist acetonitrile is accelerated by the same catalysts responsible for flavin dimerization. The relative rates of reaction are identical to those in Table V. As with dimerization, inactive catalysts include N,N-dimethylaniline-N-oxide, trimethylamine-N-oxide, and pyridine-N-oxide. A Brønsted plot for the hydration of 23 (fig. 2) is a straight line, which would not be expected if k_1 or k_2 were operative. A striking similarity of both absolute and relative catalyst activity for imine hydration and flavin dimerization suggests that k_3 (general base catalysis) is a sufficient condition for both reactions.

Figure 2

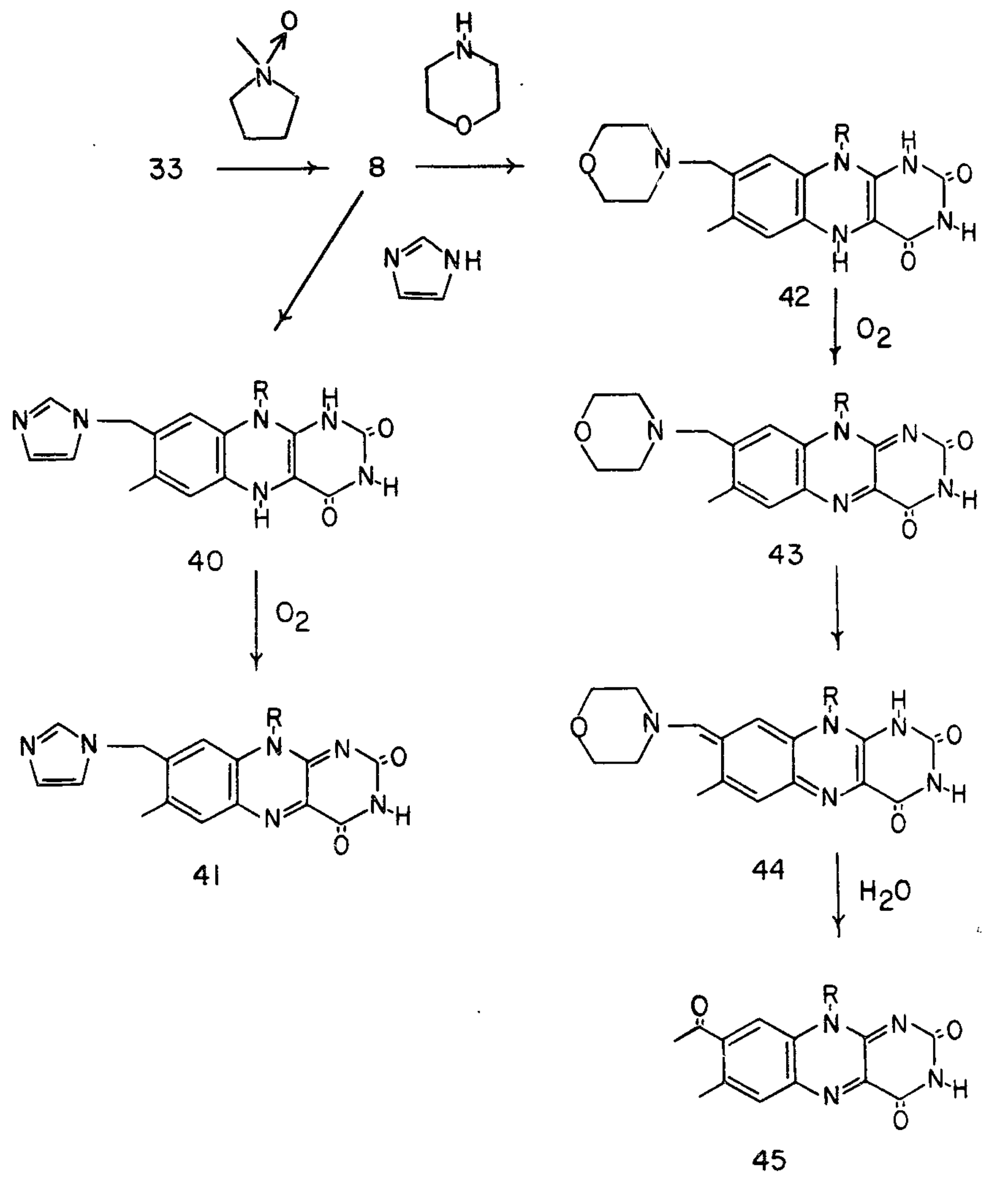


log K_ψ
$M^{-1}sec^{-1}$
0
-1
-2
-3
-4
-5
-6
-100
-200
-300
-400
-500
-600
-700
mV 1/2 neutralization
31
30
29
28b
28a
28c
OK
O
N

The susceptibility of the quinone methide to nucleophilic attack (Scheme XVI) is shown by reaction of tetraisobutyrylriboflavin with a sixfold excess of both imidazole and N-methylpyrrolidine-N-oxide. Reaction at room temperature in dry acetonitrile under anaerobic conditions for 28 h, followed by exposure to the atmosphere, affords yellow-gold, crystalline 8α-imidazoyltetraisobutyrylriboflavin 41 (isolated yield 20%, LC yield 28%, plus 32% unreacted 33 plus 2% 36a).

More complex reactivity is associated with the reaction of tetraisobutyrylriboflavin with morpholine (6 equiv.) and N-methylpyrrolidine-N-oxide (6 equiv.). After reaction under anaerobic conditions (24 h, CH_3CN, ambient temperature) followed by exposure to the atmosphere, these components afford orange, crystalline 8-formyltetraisobutyrylriboflavin 45 in 43% isolated yield (LC yield 82%, plus 4% unreacted 33 plus 1% dimer 36a). The conversion 42→45 (Scheme XVI) may reflect oxidation of the initially formed adduct 42 upon exposure to the atmosphere and further tautomerization (43→44) in the presence of excess morpholine. Upon exposure to water and oxygen, 44 would readily give the observed 8-formylated product 45. Alternatively, N-oxide addition to tautomer 8 may be followed by base mediated 8α proton abstraction with N-O bond cleavage. This seems unlikely as substitution of the tertiary amine, N-methylpyrrolidine for morpholine results in only dimer formation with no 45

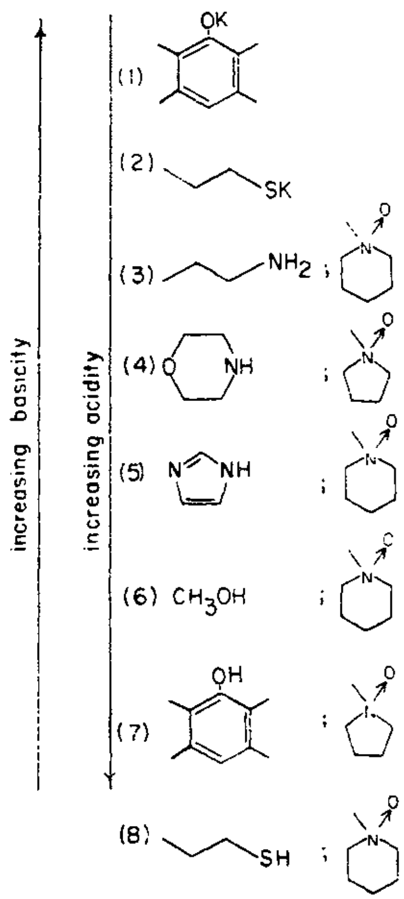
Scheme XVI



observable. The stability of the 8α imidazoyl adduct 41 to similar workup conditions may reflect the lower basicity of imidazole as compared to morpholine.

When the electrophilicity of the quinone methide to a series of nucleophilic models relevant to amino acids is examined (Table VI) a reactivity pattern emerges. Reactions

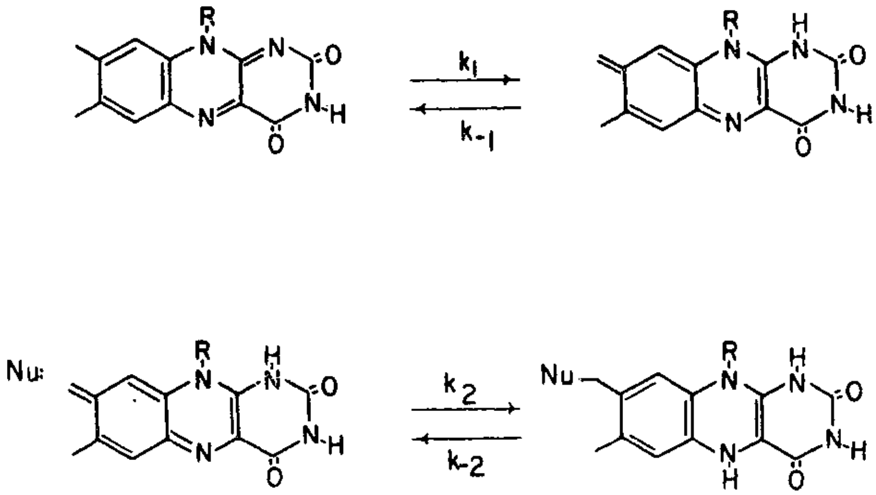
Table VI



HPLC per cent yield

	Dimer 36a	Dimer 37	8-Formyl	8α-Adduct	Quench
(1)	29	19	-	-	-
(2)	18	trace	-	-	-
(3)	10	-	-	-	-
(4)	1	-	82	-	-
(5)	2	-	-	28	-
(6)	-	-	4	-	-
(7)	-	-	-	-	100
(8)	-	-	-	-	100

seem to be following a two-step process (Scheme XVII).



Scheme XVII

When phenolate and thiolate ([1] and [2] Table VI) are functioning as both catalyst for tautomerization (k_1, Scheme XVII) and nucleophile for interception (k_2, Scheme XVIII) only dimeric mixtures of 36a and 37 are seen. Although both thiolate and phenolate may be excellent basic catalysts for the initial base catalyzed tautomerization (k_1) the basicity of the media increases to the point where the acid catalyzed nucleophilic interception (k_2) is strongly disfavored. When a primary amine is used in conjunction with N-oxide (system [3], Table VI) the result is the same. Dimerization in systems (1), (2) and (3), Table VI dominates since intramolecular electron transfer (34 to 35) following dimerization (Scheme XII) removes the requirement for intermolecular protic assistance of the quinone methide interception.

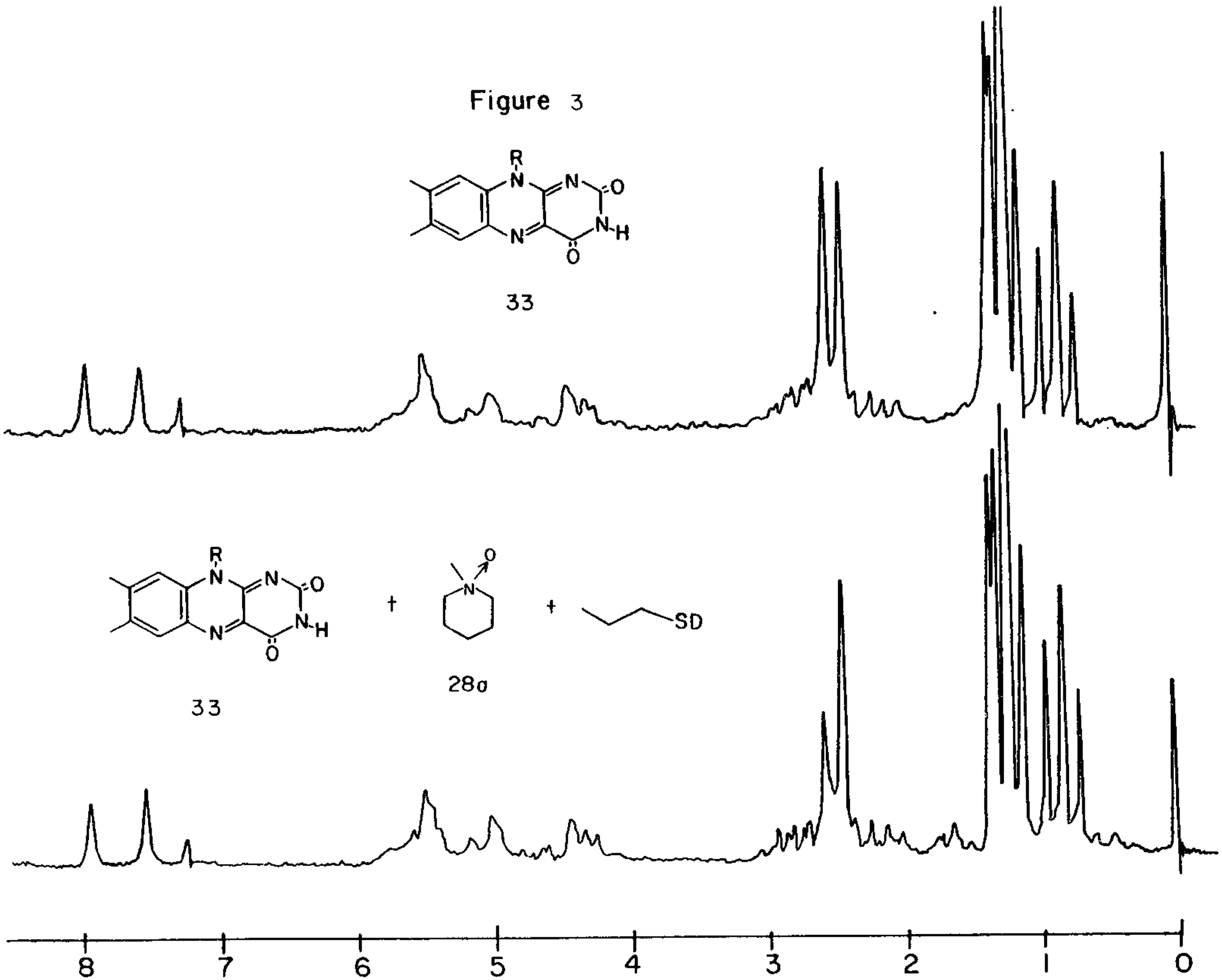
As the acidity of the nucleophile increases as in system (7) using phenol and in system (8) utilizing thiol, not only are no 8α adducts formed, but now dimerization ceases. In both systems run with deuteriophenol or deuteriothiol, recovery of starting material revealed substantial deuterium incorporation at the 8α-methyl position of the flavin (see fig. 3).

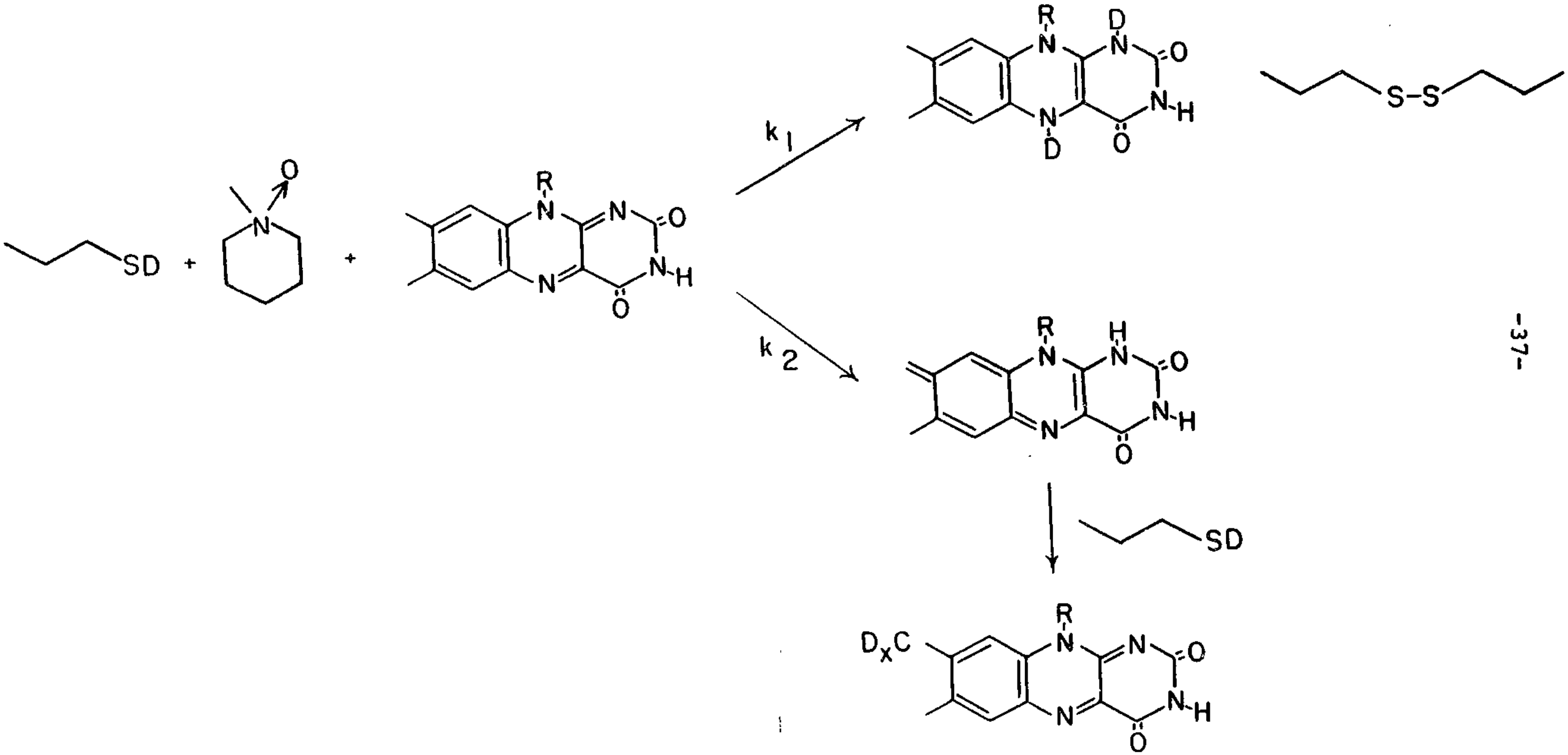
In these instances the system is well set up for quinone methide interception (k_2, Scheme XVII). However, reversal (k_{-1}, Scheme XVII) of the initial tautomerization is now being accelerated. The absence of any kind of adduct formation indicates $k_{-1} >> k_2$ (Scheme XVII) for thiol and phenol nucleophiles.

Only in those cases of intermediate basicity of the tautomerization catalyst and intermediate acidity of the nucleophile are 8α nondimeric adducts formed. Within this pocket of reactivity, the 8α-methoxy and 8α-morpholine adducts do not seem to be stable to reaction conditions. Only 8α-imidazoyl 41 combines the necessary catalyst activity with sufficient product stability for the 8α adduct to be isolated.

Thiol might have quenched 8α adduct formation in two ways (Scheme XVIII). Besides the protic quenching already discussed (k_2, Scheme VIII) tautomerization could be prevented by rapid reduction of the flavin (k_1, Scheme XVIII) thus drastically decreasing the acidity of the 8α protons. Since deuterium incorporation is being observed, the rate constant

Figure 3





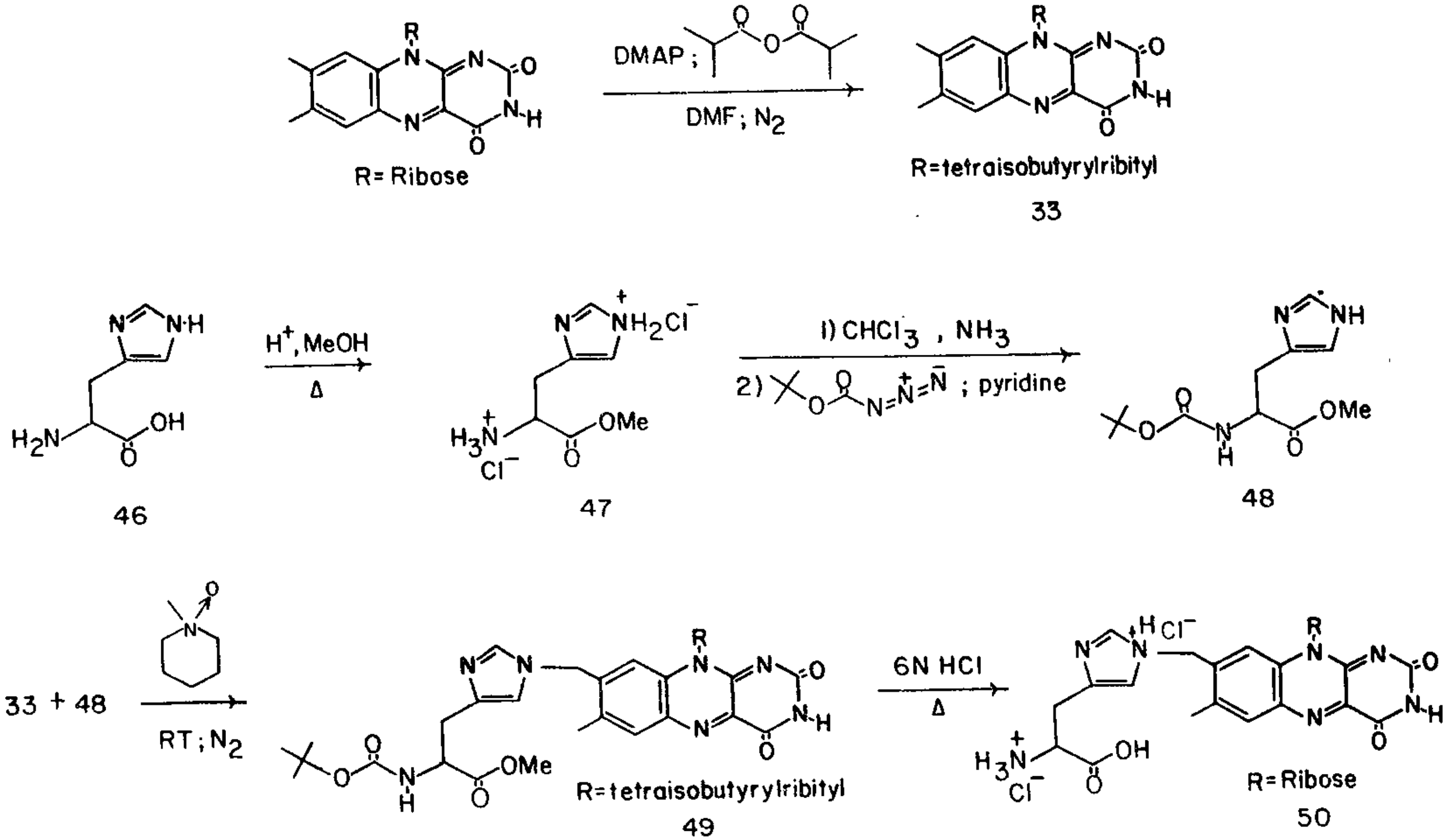
Scheme XVIII

for quinone methide tautomerization must be greater than that for thiol oxidation in polar, aprotic media.

Experience gained with the flavin quinone methide prompted a biomimetic synthesis of succinate dehydrogenase cofactor (Scheme XIX).

Riboflavin is first acylated with isobutyric anhydride and 4-N,N-dimethylaminopyridine (DMAP). Use of phosphorous pentoxide distilled isobutyric anhydride and recrystallized DMAP were found to double the yield of the initial protection step. The key portion of the synthesis is the one-step, biomimetic 8α-functionalization of protected riboflavin. ^{13}C NMR and ^{1}H NMR reveal only one isomer <u>49</u> formed in the coupling step. Hydrolysis of the protecting groups yields compound <u>50</u> identical (fig. 4) to the literature diHCL salt of N_3-histidylriboflavin.[37c] Hence the biomimetic coupling constitutes a 100 percent regioselective process. The best literature synthesis[38] utilizes a two-step coupling consisting of initial 8α bromination followed by histidine nucleophilic displacement of the bromide. This earlier procedure uses coupling conditions which result in 80:20 mixtures of N(3) histidyl to N(1) histidylriboflavin. Two successive electrophoretic separations are required[37a,b,c] to obtain product suitable for characterization. Biomimetic synthesis requires only a simple reprecipitation to obtain clean product in the last step.

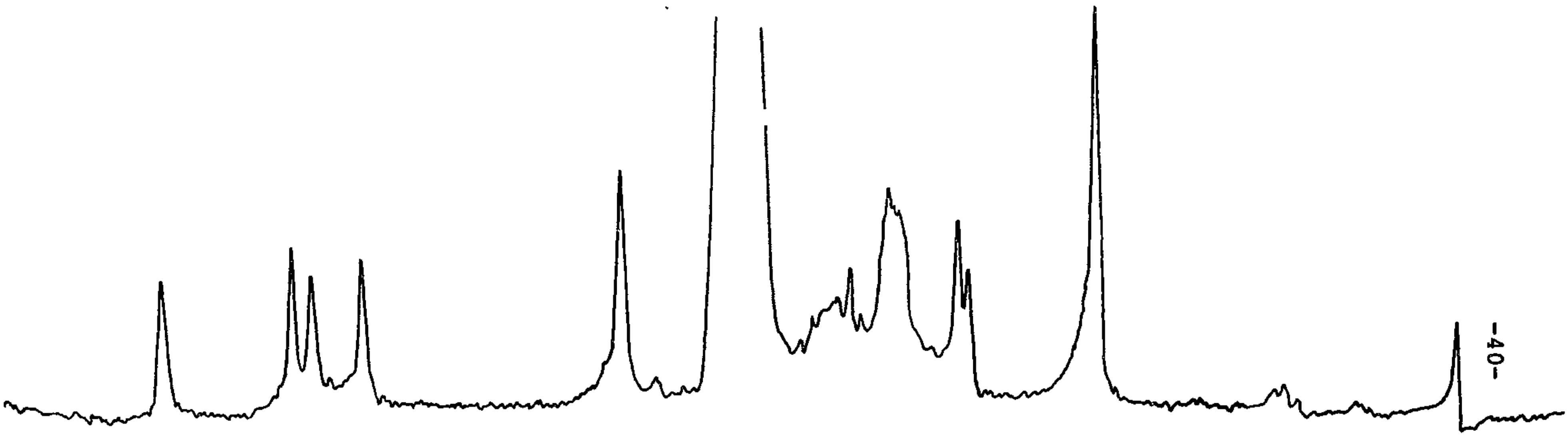
Inspection of the quinone methide structure reveals that <u>8</u> can also be viewed as an enamine. If the quinone methide



DMAP ;
DMF ; N2
R= Ribose
R=tetraisobutyrylribityl
33
H+, MeOH
Δ
1) CHCl3 , NH3
2) ; pyridine
46
47
48
33 + 48
RT ; N2
6N HCl
Δ
R=tetraisobutyrylribityl
49
R=Ribose
50

Scheme XIX

Figure 4

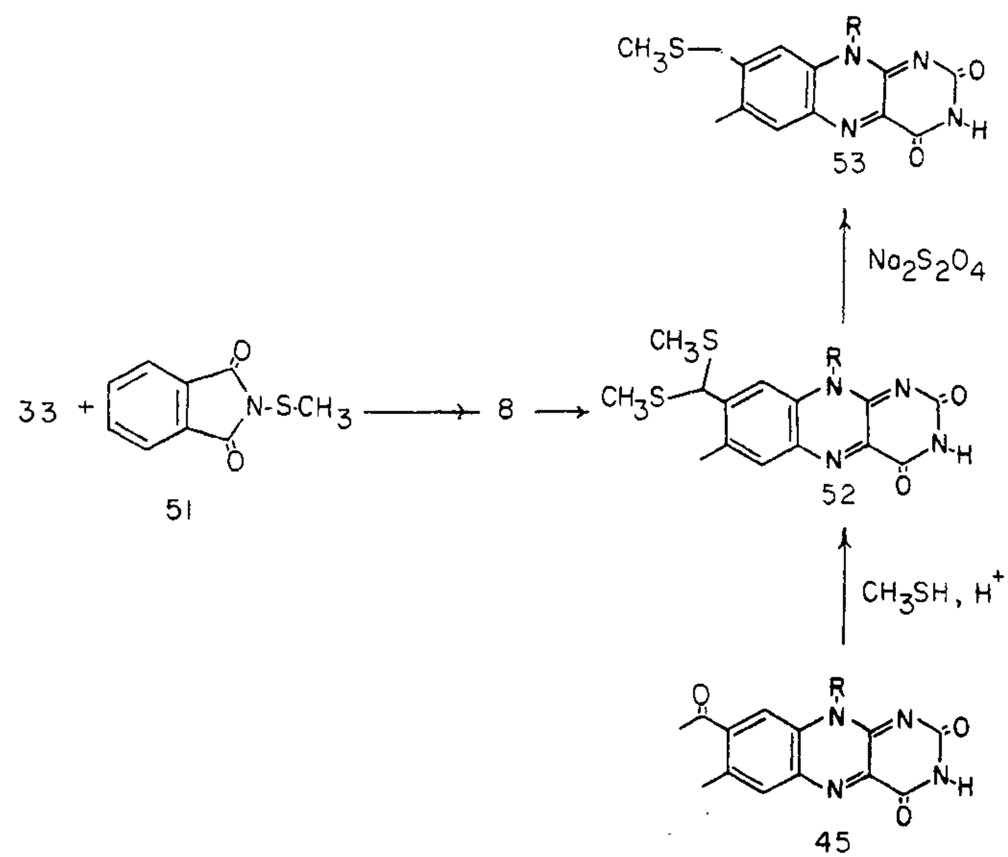


	8α-N(1)-histidylRFl	8α-N(3)-histidylRFl	50
Fl(7)-CH_3	2.58	2.48	2.48
Fl(8)-CH_2-	5.80	5.75	5.74
Fl(9) H	7.39	7.87	7.87
Im(4) H	7.53	7.56	7.51
Fl(6) H	8.10	8.00	8.00
Im(2) H	8.88	8.94	8.89

behaves as an enamine, the resulting nucleophilicity will impart an ambident character to the flavin tautomer.

The nucleophilic capacity of the quinone methide was ascertained with tetraisobutyrylriboflavin reaction with three equivalents of methanesulfenylphthalimide 51 and six equivalents of N-methylpiperidine-N-oxide 28a. Reaction under nitrogen in acetonitrile yields 8α,8α-dimethylsulfenyltetraisobutyrlriboflavin 52 (Scheme XX) in 44% yield. No 8α-methylsulfenyltetraisobutyrylriboflavin 53 was observed.

Scheme XX

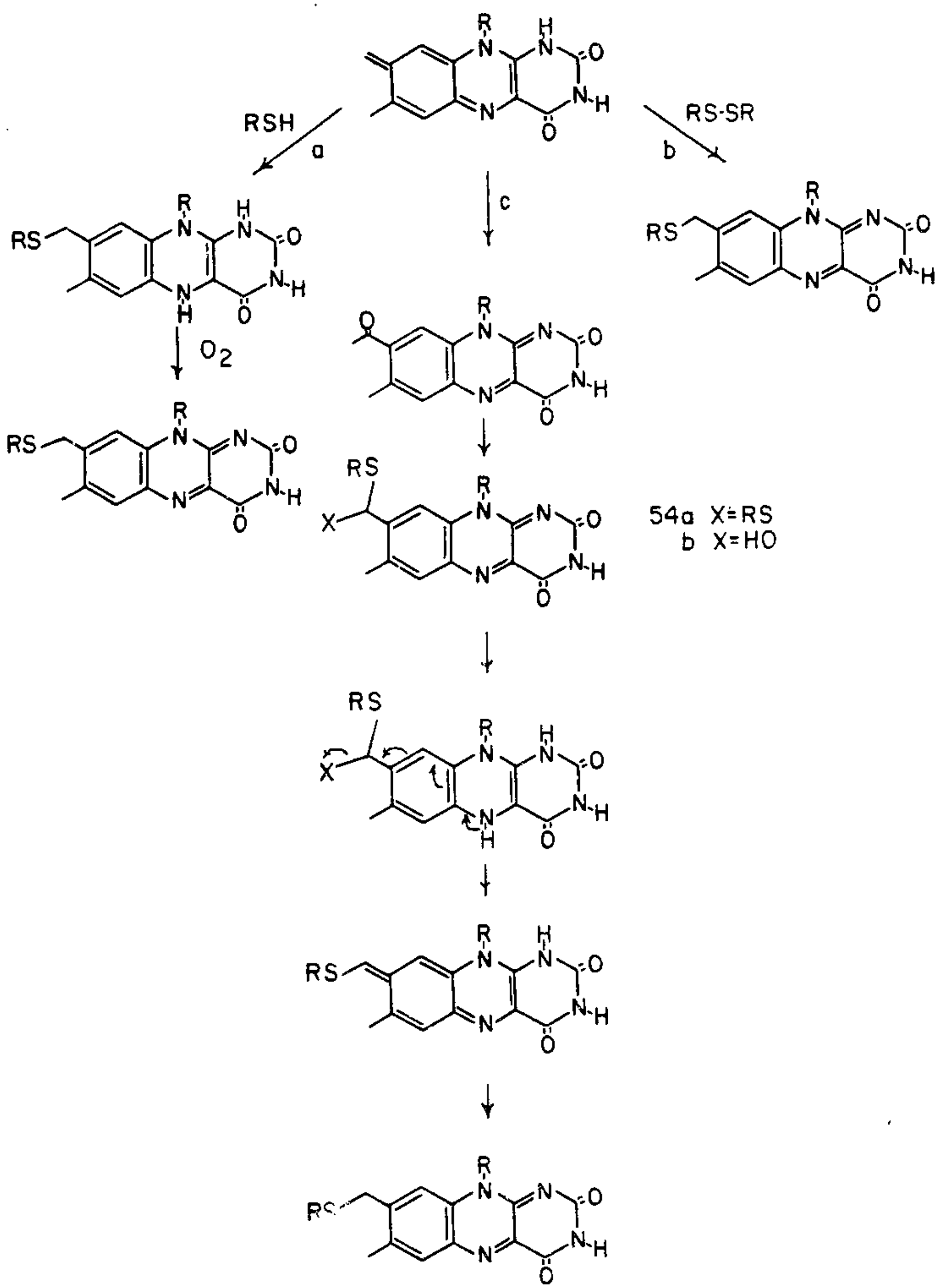


Propyl disulfide did not react under the conditions employed. Subsequent reduction of 8α,8α-disulfenyl 52 with sodium dithionite (Scheme XX) generated 8α-monosulfenyl 53. In related chemistry, the 8-formylflavin 45, formed by action of morpholine and N-oxide in organic media, reacts with excess methanethiol and catalytic trifluoroacetic acid to form dimethanesulfenyl dithiane 52.

Scheme XXI suggests two alternate pathways to the Walsh proposal for biosynthesis of monoamine oxidase. All three utilize the flavin quinone methide but in different manners. Path (a), consistent with the Walsh hypothesis, utilizes the quinone methide as an electrophile. Path (b) depends on the ambident nature of the quinone methide as a nucleophile to open an activated disulfide linkage. Finally, path (c) uses the quinone methide in an indirect fashion to generate 8-formylflavin. Following interception by active site cysteine residues to generate the dimethanesulfenyl dithiane 54a or thiohemiacetal 54b, one reductive cycle produces covalently linked monoamine oxidase cofactor.[39] Paths (b) and (c) find precedent in this work.

As has been seen, neither thiol/thiolate or phenol/phenolate can intercept the base generated quinone methide. Since flavins are known to be linked to active site cysteine and tyrosine residues, an alternate entry to the quinone methide might exist. If the tautomerization of the quinone methide can be accomplished with acid catalysts, interception with thiol or phenol nucleophiles might be possible. Precedent

Scheme XXI



54a X=RS
b X=HO

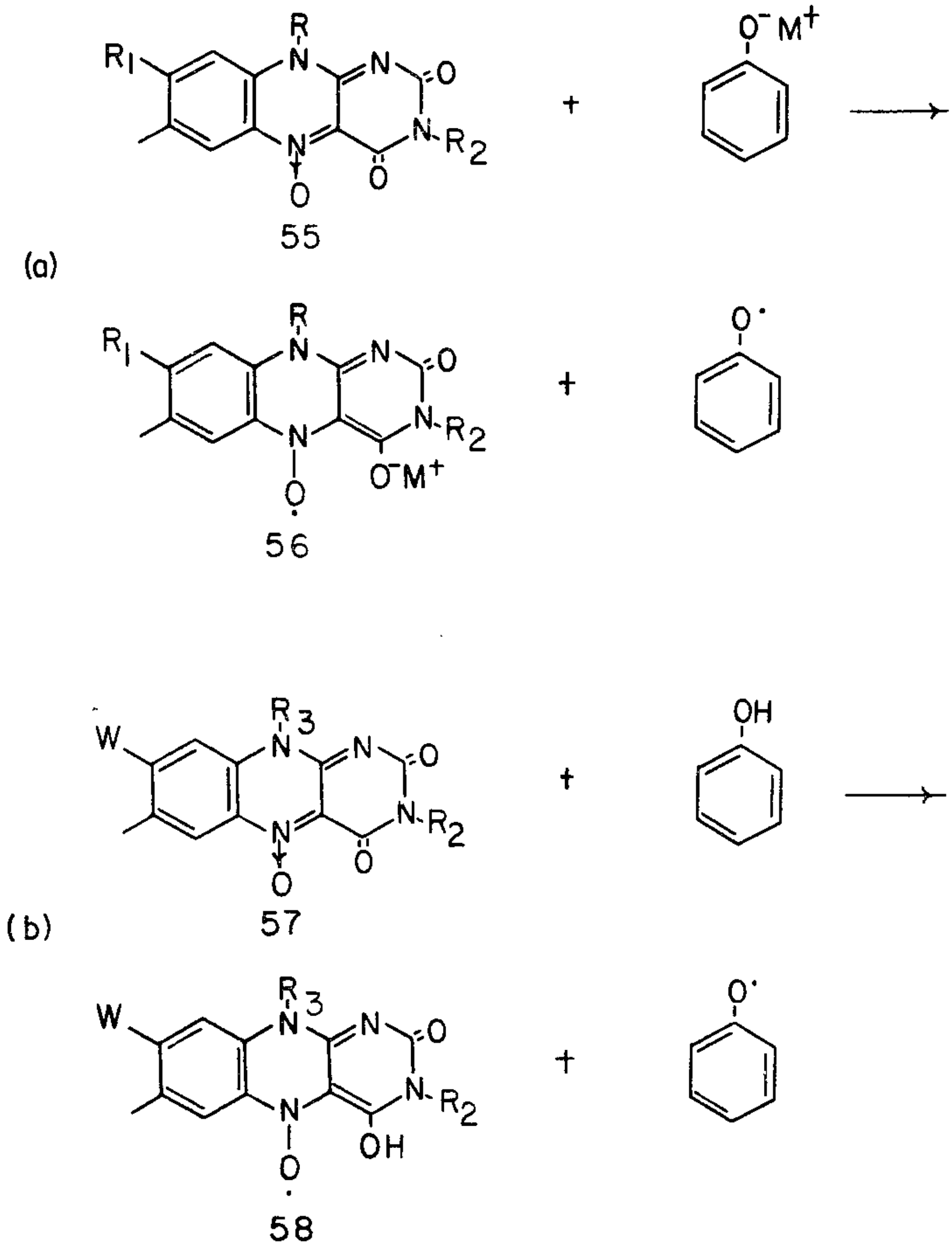
for acid catalyzed quinone methide generation can be found in the literature. Deuterium exchange for 8α-methyl protons occurs under extremely mild acid catalysis.[9] Hemmerich has reported condensation of lumiflavin with p-chlorobenzaldehyde in a H_2SO_4/P_2O_5 mixture.[8a] Finally, conversion of 8α-thioether flavin to the 8-formyl 45 known to occur under acidic conditions may be proceeding via attack by water on an intermediate quinone methide.[40] Certainly these hints justify a future search for an acidic entry into the quinone methide reaction manifold.

CHAPTER III

GROUND STATE GENERATION OF FLAVIN NITROXYL RADICAL

During the course of the flavin quinone methide investigation, tetraisobutyrylriboflavin-5-oxide was found to react with potassium 2,3,5,6-tetramethylphenolate yielding red dimer 36a and tetraisobutyrylriboflavin. Although no oxidized products were observable, the fact that the flavin-5-oxide could be deoxygenated suggested a route to the flavin nitroxyl radical previously attainable only by photolysis.

Two logical strategies might be envisioned for such an oxidation (Scheme XXII). Ground state oxygen transfer can follow from either enhancing the electron donating capacity of the phenol by making it an anion (Route [a], Scheme XXII), or increasing the electron accepting nature of the flavin 5-oxide via substitution with strong electron withdrawing groups (Route [b], Scheme XXII). The reduction potential of flavin can be perturbed to a value 200 mV more positive by strong electron withdrawing groups at position 8 of the flavin. However, it is not known whether this trend would be true with flavin 5-oxides. In light of this and some major synthetic



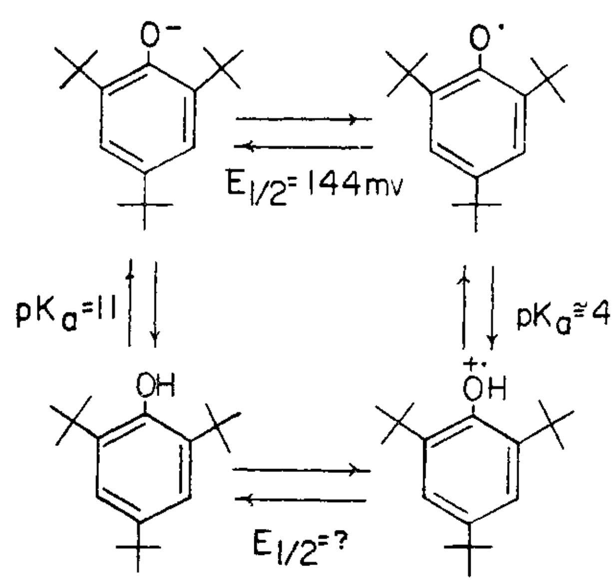
R
R1
N
N
O
N
R2
N
O
O
55
O⁻M⁺
+
(a)
R
R1
N
N
O
N
R2
N
O
O⁻M⁺
56
O
+
R3
W
N
N
O
N
R2
N
O
O
57
OH
+
(b)
R3
W
N
N
O
N
R2
N
O
OH
58
O
+

Scheme XXII

problems associated with synthesis of the model flavin-5-oxide system, Route (b) was not extensively pursued.

Route (a) chances of success can be ascertained via thermodynamic calculations. Differential polarography indicates that the first one-electron reduction potential of flavin 5-oxide occurs at +80 mV (NHE).[41] This enhancement of the flavin electron accepting power by conversion to the 5-oxide is astonishing when compared to the -240 mV (NHE)[42] one-electron reduction potential of FMN.

In discussing the redox chemistry of phenols, attention has to be restricted to sterically encumbered substrates. Steric bulk is required to retard dimerization and polymerization of the phenol radical. As indicated in Scheme XXIII the $\varepsilon_{1/2}$ for 2,4,6-tri-t-butylphenolate 59 is +144 mV (NHE).[43] No clearly defined value for the oxidation of the corresponding 2,4,6-tri-t-butylphenol 61 can be found in the literature. However, a kinetic pKa for 62 derived from reported first-order

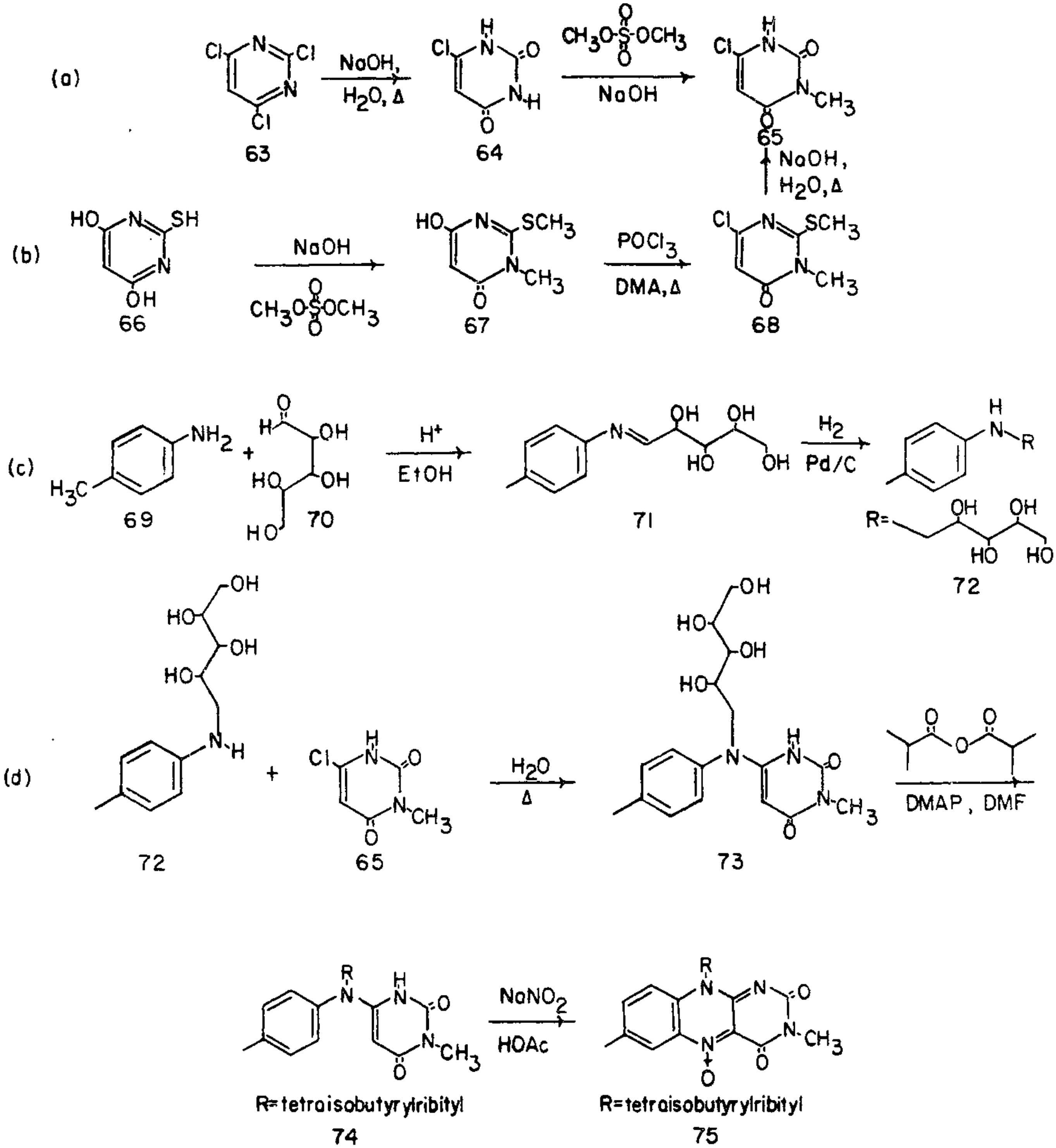


Scheme XXIII

rate constants for deprotonation of protonated phenol radical 62[44] and an estimated thermodynamic pKa for phenol 61[45] allows through Scheme XXIII determination of the one-electron $\varepsilon_{1/2}$ of 61 to be +560 mV. Therefore, reduction of flavin-5-oxide by phenolate is within 64 mV of being an exergonic process compared to the 480 mV barrier for phenol. In terms of equilibria, with phenolate for every 12 molecules of flavin 5-oxide there will be one molecule of flavin nitroxyl radical. With phenol the same ratio will be 1.3×10^8 to one. Clearly, Route (a) stands the best chance for oxygen transfer.

Although the approach to ground state oxygen transfer seems straightforward, electron transfer will not be the only reaction possible in this system. Based on earlier work, rapid tautomerization of the flavin-5-oxide to a quinone methide would be a competing process. Fortunately, earlier studies showing a complete lack of reactivity between phenolate and 8-demethyltetraisobutyrylriboflavin indicate that the use of 8-demethyltetraisobutyrylriboflavin-5-oxide would bypass this problem. Finally, reaction of the flavin-5-oxide could be precluded due to the acidic imido N(3) proton of the iso-alloxazine nucleus. A pKa = 10 for this proton[42] lies easily within the range for deprotonation by phenolate. Alkylation at N(3) would remove this reaction. Based on the foregoing considerations, 8-demethyl-3-methyl-tetraisobutyrylriboflavin-5-oxide 75 is the model system of choice.

Synthesis of the desired model system is shown in Scheme XXIV.


(a)
NaOH, H2O, Δ
CH3OSO2OCH3
NaOH
63
64
65
NaOH, H2O, Δ
(b)
NaOH
CH3OSO2OCH3
POCl3
DMA, Δ
66
67
68
(c)
H+
EtOH
H2
Pd/C
69
70
71
72
(d)
H2O
Δ
DMAP, DMF
72
65
73
NaNO2
HOAc
R=tetraisobutyrylribityl
74
R=tetraisobutyrylribityl
75

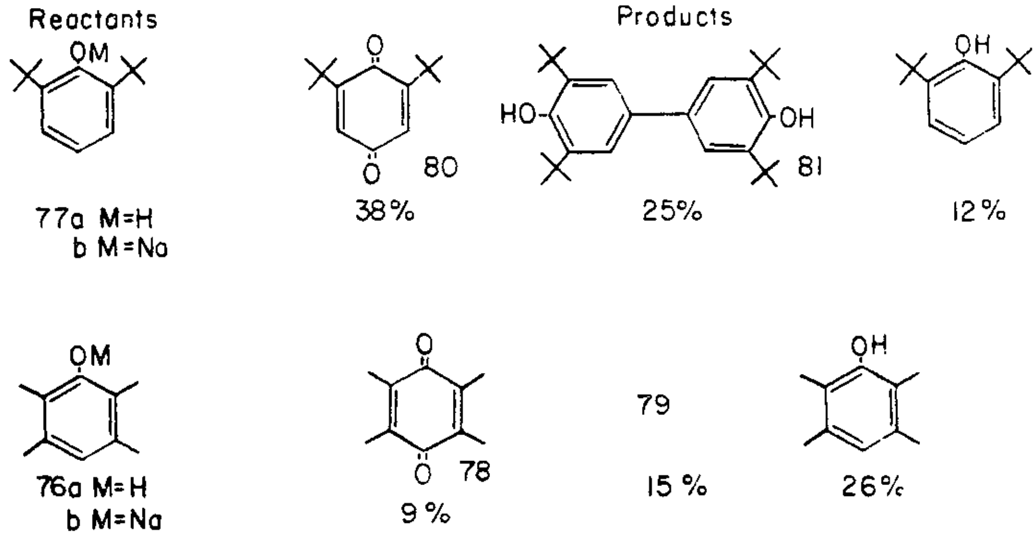
Scheme XXIV

The longer approach (b) to 3-methyl-6-chlorouracil 65 was initially explored since a statistical mixture of dihydro-, mono- and dimethyl adducts was expected from simple base mediated alkylation of the starting 6-chlorouracil 63. However, stirring the chlorouracil in 2N sodium hydroxide with an equivalent of dimethyl sulfate followed by recrystallization afforded only the desired 3-methyl-6-chlorouracil 65. With this shorter route, route (b) to the pyrimidine side of the model system was abandoned. A problem was encountered with the hydrogenation of the ribosyl-p-toluidine 71 with poisoning of the catalyst. Inclusion of approximately one equivalent of glacial acetic acid in the hydrogenation mixture circumvented this obstacle. Crystalline 72 is then fused with uracil 65 in refluxing water. Rather than bothering with intermediate purifications, the hydroxyl groups 73 were immediately acylated with isobutyric anhydride/DMAP and the resulting brittle foam 74 treated with sodium nitrite in glacial acetic acid. Preparative thin-layer chromatography (PTLC) affords the 8-demethyl-3-methyltetraisobutyrylriboflavin-5-oxide 75 as a beautiful orange, brittle foam. Like all the other tetraisobutyryl protected flavins lacking the (3) imido proton, it cannot be crystallized. Model flavin-5-oxide is extremely soluble in organics (even in ether and cyclohexane) and is indefinitely stable on standing at room temperature as long as it is carefully protected from light.

With the model flavin 5-oxide in hand, the earlier thermodynamic calculations can be tested. Addition of a tetrohydrofuran solution of sodium 2,3,5,6-tetramethylphenolate 76b or sodium 2,6-di-t-butylphenolate 77b to a twofold excess of 75 under anaerobic conditions in the absence of light leads on mixing to a change of the orange solution to a reddish-brown coloration. Sodium 2,3,5,6-tetramethylphenolate 76b reaction yields duroquinone 78 and a second oxidation product 79 while sodium 2,6-di-t-butylphenolate 77b produces 2,6-di-t-butylbenzoquinone 80 and dimer 81 (see Table VII).

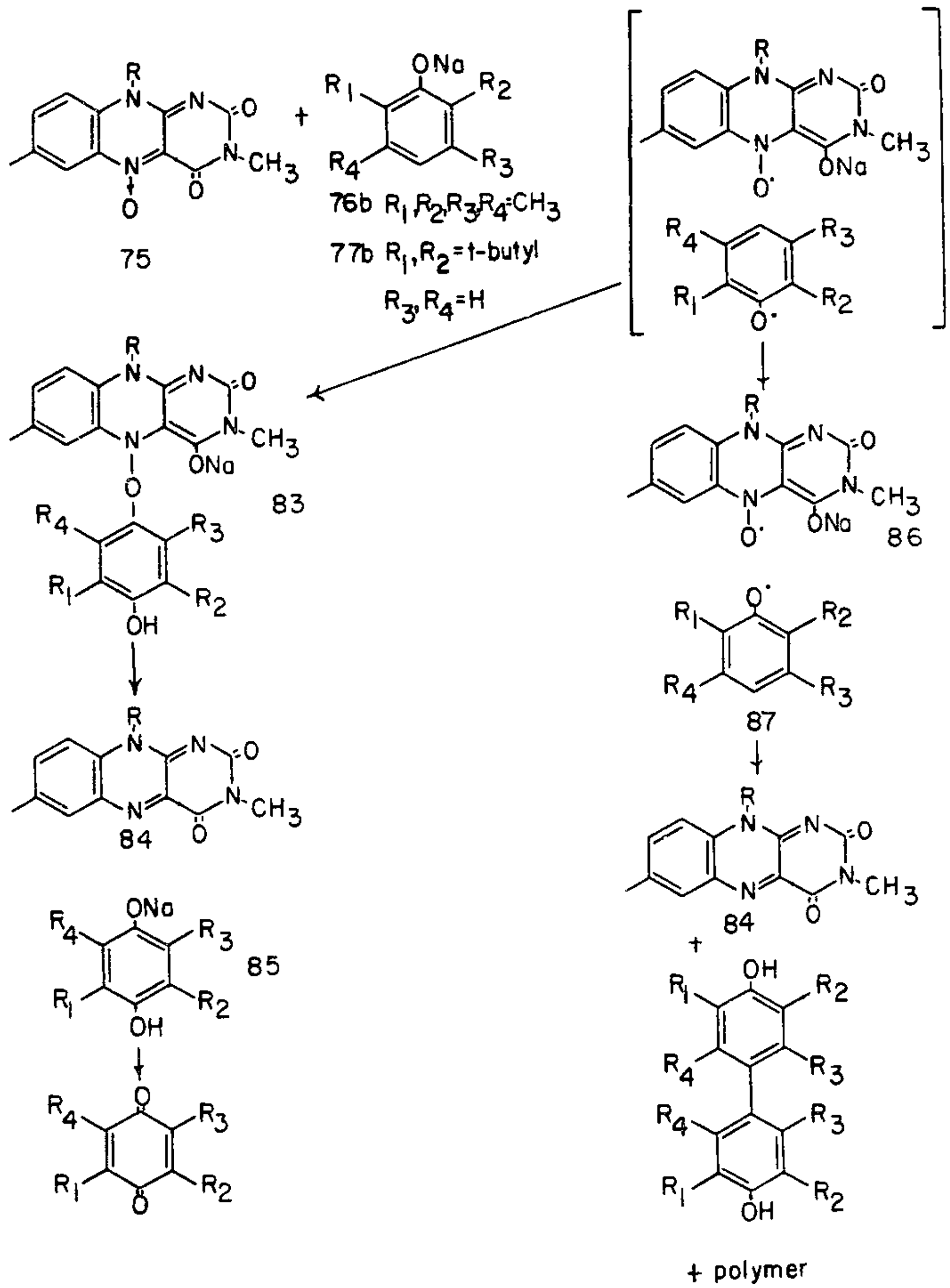
Table VII

Reactivity with 75



Products were verified by GLC coinjection and gas chromatography/mass spectroscopy (GC/MS) correlation to fragmentation patterns of authentic samples. In the oxidation of 76, duroquinone 78 product was isolated from the reaction mixture by PTLC or basic alumina oxide. A likely mechanism for the observed reactivity is shown on Scheme XXV. Observation of

Scheme XXV


75
76b $R_1,R_2,R_3,R_4=CH_3$
77b R_1,R_2=t-butyl
$R_3,R_4=H$
82
83
84
85
86
87
+ polymer

quinones versus hydroquinones as products follows from previous work demonstrating the facile dark chemical oxidation of hydroquinone to quinone by flavin-5-oxide.[20]

The critical transfer of the oxygen from the flavin-5-oxide to phenolate was rigorously established via use of ^{18}O labelled flavin-5-oxide (Table VIII). Variation of the synthesis (Scheme XXVI) by use of nitrosonium tetrafluoroborate and $H_2{}^{18}O$ in the nitrosative ring fusion afforded flavin 5-^{18}O. Percent ^{18}O incorporation was determined by triphenylphosphine deoxygenation of the flavin-5-oxide followed by mass spectral analysis of the resulting triphenylphosphine oxide. ^{18}Oxygen incorporation in the products of the phenol oxidations was assayed by gas chromatography interfaced with alternating voltage scanning mass spectroscopy

per cent ^{18}O incorporation

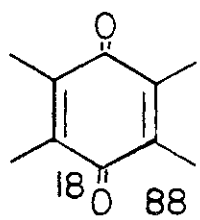
Reactants		Products
77b 75 28.1% I.P.	 88 27.9% I.P.	89 30.5% I.P.
after treatment with pyridine, TMSCl		89-OTMS 90 26.6% I.F.

Table VIII

(GC/AVS MS). The isotopic percent of ^{18}O in 75 is reflected in the two products of 76 oxidation (Table VIII).

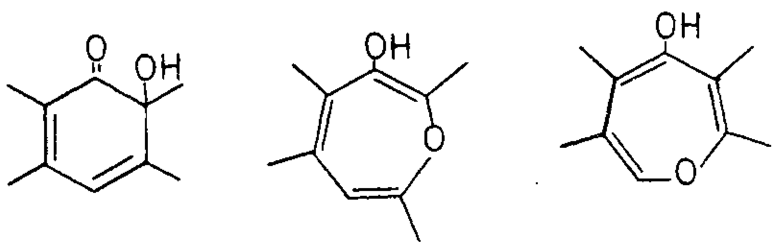
As shown by Table VII, one of the products, 79, of the oxidation of 76 could not be identified. The molecule, 79, is characterized by mass spectral fragmentation in Table IX.

Table IX

78	M^+	164	136 -28	121 -43			
76a	M^+	150	135 -15				
79	M^+	166	151 -15	124 -42	123 -43	109 -57	
92	M^+	166	151 -15	136 -30	121 -45		
93	M^+	222	207 -15	133 -89	73 -149		
91	M^+	238	223 -15	208 -30	195 -43	79 -159	73 -165

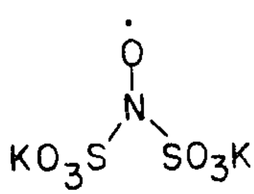
Since molecule 79 has a parent ion equal to starting phenol (M^+ 150) plus oxygen and reflects ^{18}O incorporation, it seems reasonable to label it as material resulting from oxygen transfer. All attempts to isolate 79 have met with little success (analytical HPLC, semi-preparative HPLC, analytical GLC, preparative GLC, alumina PTLC, silica PTLC, diffusion line sublimation). A certain amount of information pertinent to the identity of 79 can be gleaned from mass spectroscopy. Phenol 76 and hydroquinone 92 are characterized by strong peaks due to loss of methyl radicals. Duroquinone 78 lacks this M^+ minus 15 peak and has its major fragmentation at

M^+ minus 43. Potentially, this implies that unknown 79 lacks the aromaticity of a phenol or hydroquinone. Treatment of reaction crude with pyridine and trimethylsilylchloride (TMSCl) leads to formation of the TMS ether 93 of unreacted starting material 76b and a peak indicated by GC/MS to be the TMS ether 91 of unidentified 79 (see Table IX for fragmentation). ^{18}O label is retained on TMSCl treatment in formation of 90 (Table VIII). Structure consistent with disrupted aromaticity and a free hydroxyl group are shown in Scheme XXVI.



Scheme XXVI

Attempts have been made to obtain the unidentified peak by alternate synthetic procedure. Reactions with Fremy's Salt 94



94

failed, however, to produce any product other than duroquinone.

The occurence of dimeric product 81 in the oxidations of 2,6-di-t-butylphenolate 77b suggests that a substantial amount of phenol radical is diffusing out of the solvent cage following electron transfer. If this is indeed the situation, enough flavin nitroxyl radical may survive free in solution for observation by electron spin resonance (ESR) spectroscopy.

When reactants are combined in a flat cell under anaerobic conditions at ambient temperature using concentrations close to those used in Table VII and Table VIII, a strong ESR signal is obtained (fig. 5). With the three-line pattern centered at g = 2.01633 with hyperfine a = 9.94 gauss, the radical observed is signal due to a nitroxyl radical. Examination of the superhyperfine yields even more structural information when used in tandem with the classic structure determinations of the flavin semiquinone radicals.[46] If there were no significant radical density in the pyrimidine ring, one would expect resonance forms 95 through 99 to contribute to the ESR signal. Using an iterative proces, computer simulation yields the spectrum shown in fig. 6. This simulation uses splittings of a = 3.08 gauss for both C(8)H and C(6)H and a = 2.02 gauss due to N-10. Although these are simplistic assumptions, the fact that the derived computer simulation approximates the observed spectra provides further corroboration for a flavin-based nitroxyl radical. Use of $a_1^H = Q\rho^{\pi}$ allows calculation of electron densities in the radical (Table X).

Figure 5

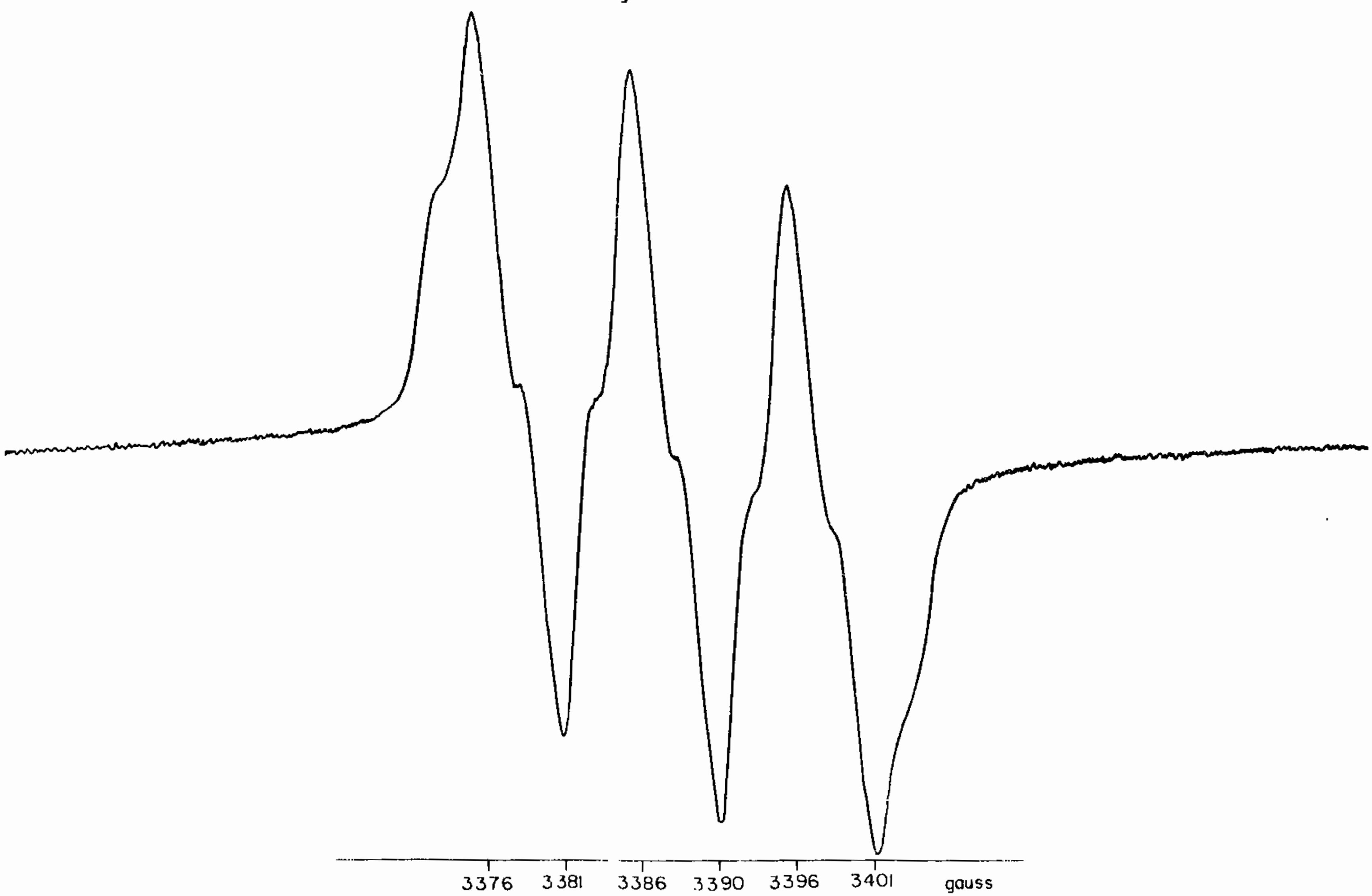
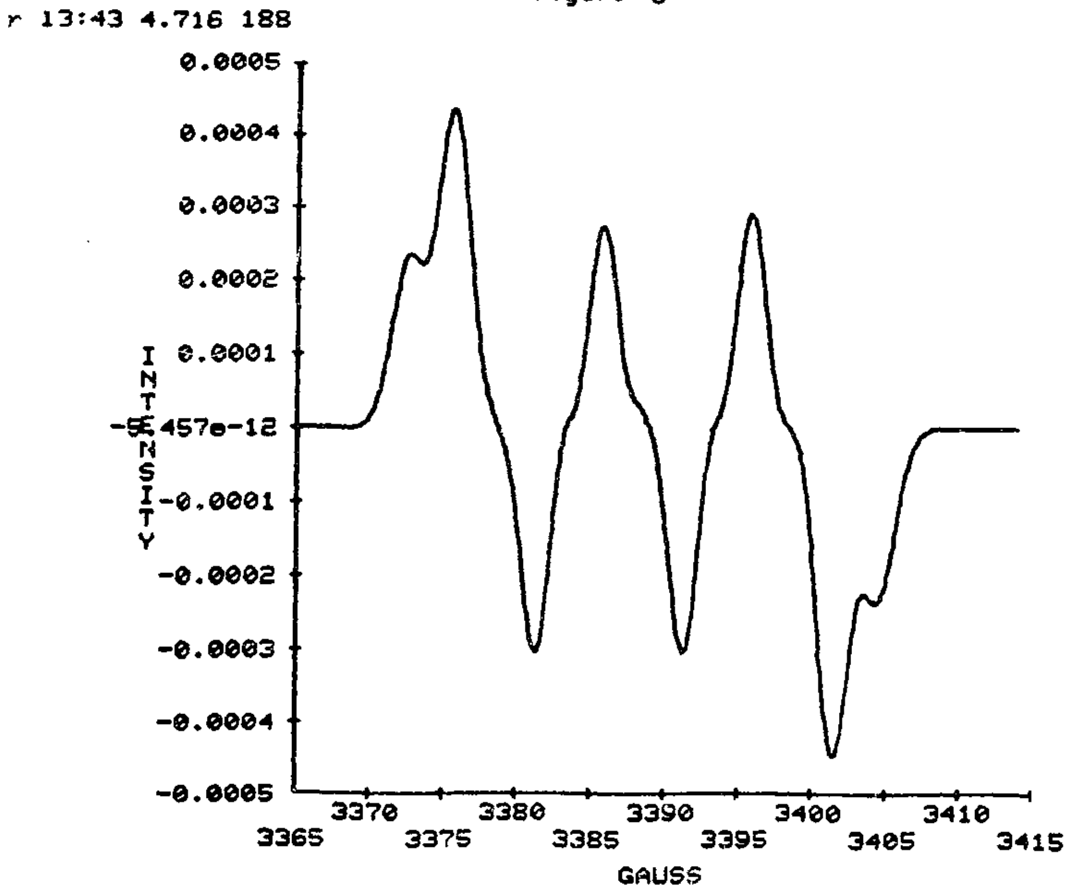
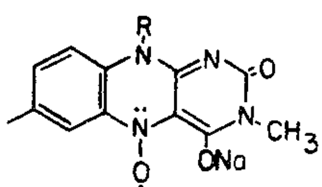


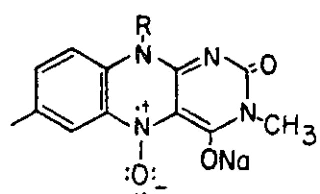
Figure 6



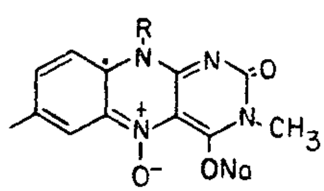
Computer simulation based on ground state contribution from:



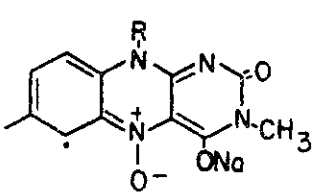
95



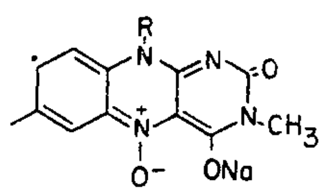
96



97



98



99

Table X

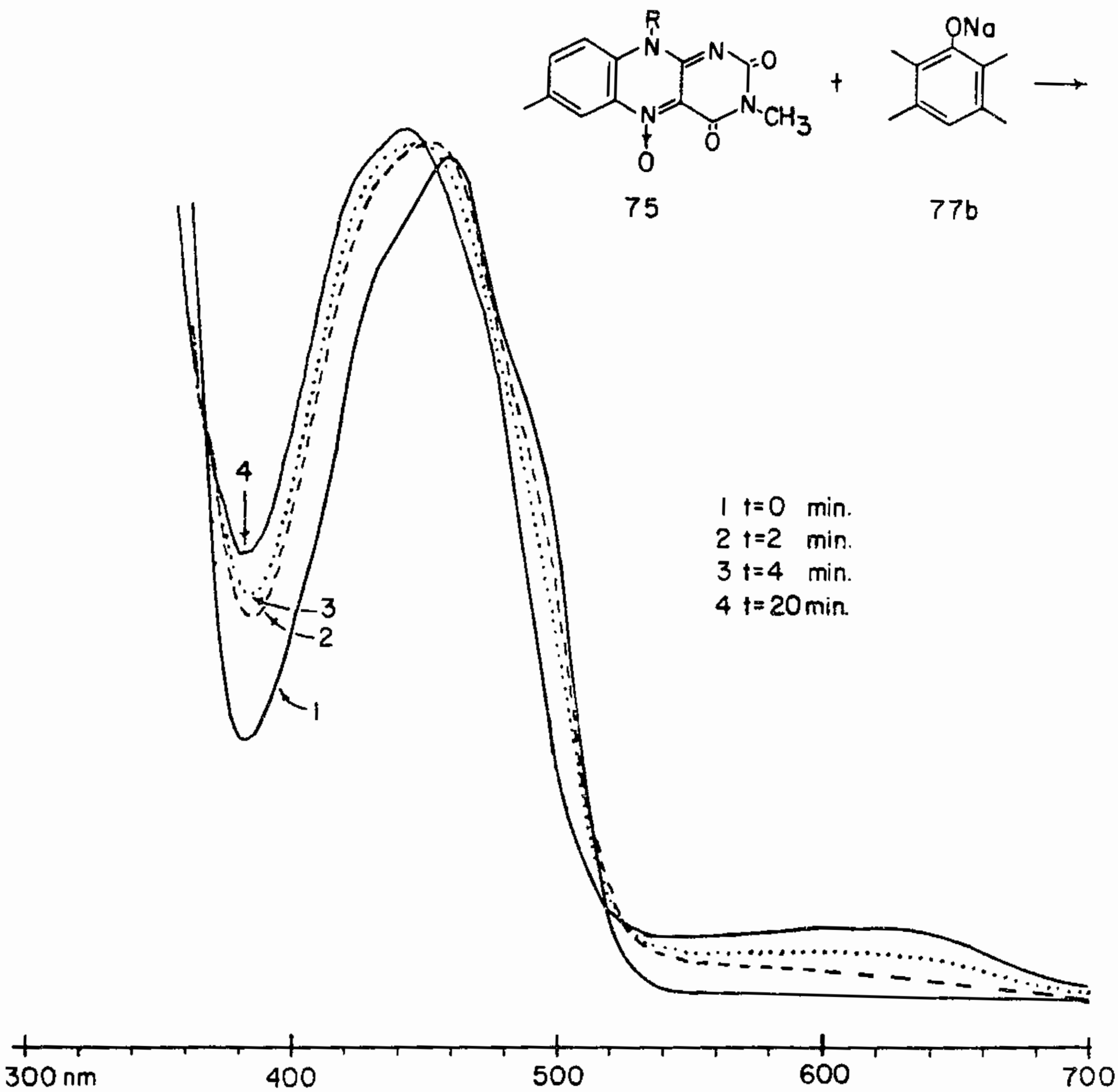
	Semiquinone anion		Nitroxyl radical anion	
position	a	spin density	a	spin density
N (5)	7.3	.256-.394	9.9	.349-.549
C (6)	3.5	.129-.167	3.1	.114-.146
C (8)	4.0	.148-.190	3.1	.114-.146
N (10)	3.2	.112-.173	2.0	.071-.109

When compared to those calculated by Ehrenberg for the flavin semiquinone anion 102, it is apparent that the unpaired electron is more strongly localized on Pz of nitrogen(5). This behavior would be expected for the flavin nitroxyl.

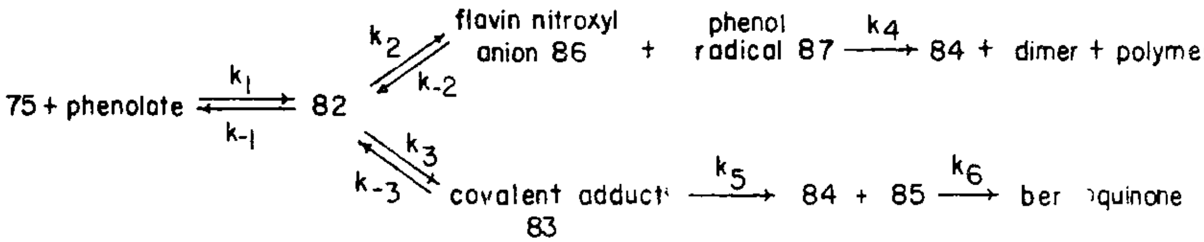
Synthesis of ^{15}N and deuterio flavin-5-oxides in the future should provide actual superhyperfine coupling information for C(6)H, C(8)H, and N(10). Such effort is justified by the uniqueness of this system. The flavin nitroxyl radical anion is quite probably the most complicated isotropic system ever observed for a nitroxyl radical.

Continuous assay of the reaction by UV reveals a reactivity pattern shown in fig. 7. With time, the flavin-5-oxide λ_{max} at 460 nm disappears with a hypsochromic shift to 440 nm occuring with the formation of a charge transfer band at λ_{max} = 620 nm. The overall spectral changes are isosbestic at λ = 372, 448 and 524 n, and the 620 nm absorption forms

Figure 7


R
N
N
O
N
CH3
N
O
O
75
+
ONa
77b
1 t=0 min.
2 t=2 min.
3 t=4 min.
4 t=20 min.
4
3
2
1
300 nm
400
500
600
700

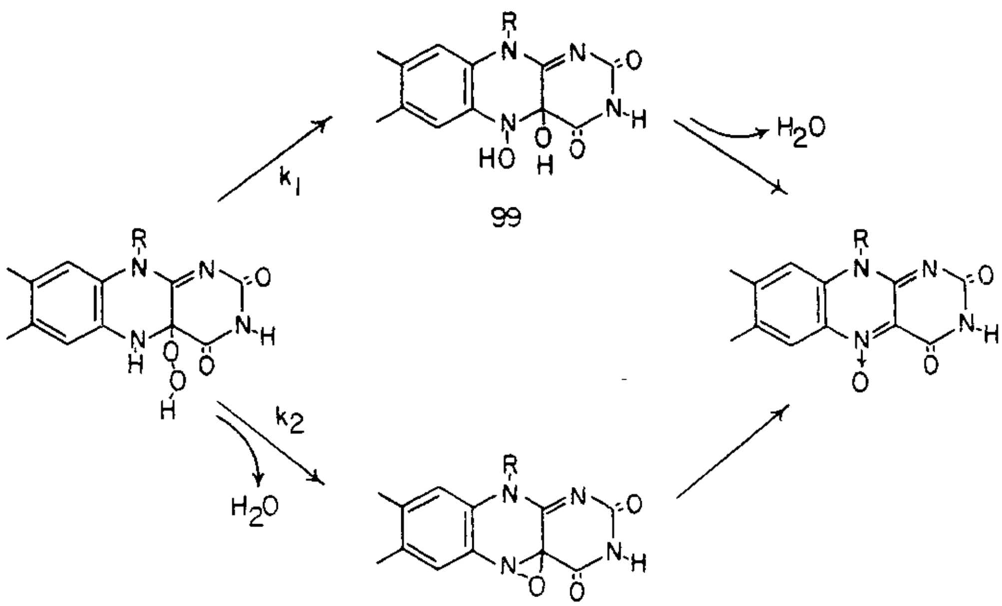
as well on addition of phenolate 76b to flavin 84 (Scheme XXV). Following the change of absorption at 620 nm under pseudo first order concentrations of phenolate 76b shows a first order constant $k = 1.9 \times 10^{-2}$ min^{-1} displaying complex order dependence in phenolate concentration. A kinetic interpretation shown in Scheme XXVII is consistent with the data.



Scheme XXVII

In light of the accumulated data, what role might the flavin-5-oxide play in flavin monooxygenase activity? As shown by Scheme XXVII two paths could lead to formation of the flavin-5-oxide *in vivo.* Starting with the well-characterized 4α-hydroperoxide, either flavin hydroxylamine 99 or flavin oxaziridine could be formed. Both paths would ultimately produce the flavin-5-oxide, a species which this work demonstrates to be a competent oxidant. The biological system could perturb the flavin-5-oxide redox potential to even more positive values on binding to protein[47] or deprotonate the phenol[48] (or both) to initiate oxygen transfer. Furthermore, oxygen transfer from flavin N(5) is consistent with the known geometry of the active site of p-hydroxybenzoate hydroxylase.[49]

Scheme XXVIII



The ultimate oxygen transferred to substrate in the oxaziridine route is the interior oxygen of the 4α-hydroperoxide. Path k_2 (Scheme XXVIII) depends on the electrophilicity of the wrong oxygen of the 4α-hydroperoxide based on the work of Bruice. Furthermore, k_2 must confront an energy barrier of some 30 kcal[50] due to ring strain of the oxaziridine absent in the hydroxylamine path k_1.

The ultimate oxygen transferred in k_1 is the terminal oxygen of the 4α-hydroperoxide. Bond scissions in k_1 are predicted by Bruice's work with the <u>inter</u>molecular oxidation of secondary amines to hydroxylamines by the flavin

4α-hydroperoxide. Essentially, k_1 constitutes a case for intramolecular oxidation of a secondary amine with the enzyme functioning as an effector.

In line with this proposal, the FAD level of riboflavin-5-oxide has been prepared with synthetic riboflavin-5-oxide and ATP being added to FAD-synthetase complex of *Brevibacterium ammoniagenes*[51] in non-nucleophilic morpholinopropane sulfonic acid buffer. Simple Sep Pak filtration removes the enzyme and HPLC separates the FAD-5-oxide **101** from the rest of the reaction mixture. With pure FAD-5-oxide, reconstitution with p-hydroxybenzoate hydroxylase will test the proposed involvement of FAD-5-oxide in flavin monooxygenase activity. The FAD-5-oxide is cleanly cleaved by snake venom phosphodiesterase (*Naja naja*) to FMN-5-oxide.

This work, at the minimum, suggests that models for flavin monooxygenase activity where N(5) of the flavin is alkylated have precluded the migration necessary for flavin mediated oxidation of phenols. Indeed, the mysterious oxygen gun may well be closely related to a species which has existed in the literature ignored by investigators for five years. Highly conjugated nitrones could be utilized in new synthetic oxidative procedures and (or) generally exploited by biological oxidative systems.

If the proposals forwarded by this thesis prove to be true, we stand on the threshhold of a hitherto unexplored general class of oxidants. The attendant biological and synthetic ramifications remain for future investigation.

Kinetic Resolution of 2-propyl-3-methyl-3-isobutyloxaziridine

Brucine dihydrate (33.1 g, 0.077 mol) and oxaziridine (27.5 g, 0.175 mol) were dissolved in 80 mL methylene chloride and refluxed for sixteen hours. The brucine-methylene chloride adduct was filtered off and the cake washed with 2 x 20 mL methylene chloride. Subsequently, the filtrate was passed through a plug of silica gel which was then washed with 2 x 100 mL methylene chloride. Concentration of the filtrate was followed by short path distillation and chromatography (silica, 3:1, hexane:ethyl acetate). GLC and correlation of the IR indicated this material was <u>13</u>.

Optical rotation:

$\alpha_D^{20.1°}$ - 4.343°, ℓ = 1, neat.

Characterization of brucine-methylene chloride adduct <u>18</u>:

Anal. Calcd for $C_{24}H_{28}Cl_2N_2O_4 \cdot H_2O$:C, 57.95; H, 6.08; Cl, 14.26; N, 5.63.

Found: C, 57.66; H, 6.09; Cl, 14.07; N, 5.57.

^1H NMR (270 MHz, D_2O) δ (HOD) 0.73 (2H, ABq, J 9.8 Hz, $^+NCH_2Cl$)

Field-desorption MS $[R_3\overset{+}{N}\text{-}CH_2Cl\ Cl^-]^+$ parent cluster ions, m/e 478, 480, 482; $[R_3\overset{+}{N}\text{-}CH_2Cl]$ m/e 443, 445; $[R_3\overset{+}{N}\text{-}CHCl]$ m/e 442, 444; $[R_3\overset{+}{N}\text{-}CH_2]$ m/e 408; $[R_3N]^+$ m/e 394 (base peak).

Reaction of "17" with N-methylpyrrolidine (Scheme VIII)

2-Phenyl-3,3-dibenzoyloxaziridine (0.314 g, 9.53×10^{-4} mol) dissolved in 6 mL of N-methylpyrrolidine was refluxed under nitrogen for 84 hours. After concentration the oil was taken up in 50 mL chloroform and extracted with 3 x 25 mL water. Drying and concentration of the organic layer was followed by flash chromatography (silica, 3:1, hexane:ethyl acetate) affording 0.083 g (39%) of yellow, crystalline 21. Concentration of the aqueous layer yielded 0.079 g, (40%) of yellow oil 22.

21

M.P. 55-57°

^{1}H NMR (60 MHz, $CDCl_3$)(Me_4Si) δ 7.0-8.0 (m, 8H), 8.3-8.6 (m,2H), 9.1 (br s, 1H).

IR(KBr) 3340, 2500, 2480, 2420, 1695, 1660, 1600, 1580, 1540, 1500, 1470, 1450, 1420, 1325, 1310, 1280, 1245, 1170, 1080, 1040, 1020, 1010, 990, 960, 945, 910, 880, 860, 830, 790, 760, 750, 725, 690, 680, 625, 610, 490, 400, 300 cm^{-1}.

MS m/e 226.

22

^{1}HNMR (60 MHz, $CDCl_3$)(Me_4Si) δ 1.8-2.4 (m, 4H), 2.8 (s, 3H), 3.0-3.5 (m, 4H), 7.2-7.5 (m, 3H), 7.9-8.1 (m, 2H), 9.9 (br s, 1H)

Both 21 and 22 were identical to authentic samples.

1,3-Diphenyl-2-iminophenyl-1,3-propanedione 23[54]

Dibenzoylmethane (1.67 g, 0.0074 mol) and nitrosobenzene 0.80 g, 0.0074 mol) were dissolved in 10 mL warm ethanol and four drops 30% ethanolic potassium hydroxide added. Stirring was continued at room temperature for 1-1/2 hours (until yellow crystals precipitated). The ethanol was evaporated and the residue taken up in a small portion of benzene. Flash chromatography (3:1, hexane:ethyl acetate) of the crude was followed by concentration of the imine 23 band. Recrystallization from ethanol afforded .530 g (23%) of yellow, crystalline 23.

M.P. 84-86°

^{1}H NMR (60 MHz, $CDCl_3$)(Me_4Si) δ 6.8-7.9 (m, 13H), 8.2-8.4 (m, 2H).

IR (Nujol) 1670, 1650, 1595, 1580, 1330, 1315, 1260, 1210; 1185; 1170, 1075, 1030, 1005, 980, 940, 920, 880, 850, 780, 740, 700, 690 cm^{-1}.

UV (CH_3CN) λ_{max} 404 (ε 5.5 x 10^2), 256 (ε 4.2 x 10^4), 222 nm (ε 9.6 x 10^3).

N-Dibenzoylmethyl-N-phenylhydroxylamine 39

1,3-Diphenyl-2-iminophenyl-1,3 propanedione 23 (0.500 g, 1.59 x 10^{-3} mol) and N-methylpyrrolidine-N-oxide (0.97 g, 9.58 x 10^{-3} mol) in 10 mL acetonitrile were freeze thaw degassed three times under nitrogen and reaction allowed to proceed at room temperature under nitrogen in the dark for 24 hours. The reaction was subsequently taken up in 100 mL chloroform and washed with 100 mL pH = 4.0 water followed by 100 mL water. Drying the organic layer followed by concentration and recrystallization from ethanol afforded 0.338 g (53%) of white, crystalline 39.

M.P. 178-179.5°

^1H NMR (60 MHz, $CDCl_3$)(Me_4Si) δ 1.6 (s, 1H, exchangeable), 6.4 (s, 1H), 7.1-7.7 (m, 13H), 8.0-8.3 (m, 2H).

IR ($CHCl_3$) 3440, 1730, 1700, 1600, 1530, 1500, 1450, 1320, 1260, 1245, 1180, 1090, 1070, 1030, 960, 900, 690 cm^{-1}.

General Procedure for Brønsted Plot (fig. 2)

Bases dissolved in acetonitrile were titrated with 0.1M HCL in dioxane and the potential at one-half neutralization determined. Typically, at this point the solution was acetonitrile 10% in dioxane.

Rates for N-oxide, (28a, 28b, 28c) mediated imine 23 hydration were determined by monitoring of loss of imine absorption at 404 nm. As potassium 2,6-di-t-butylphenolate 31 and potassium 2,3,5,6-tetramethylphenolate 30 have substantial absorption at 404 nm, HPLC was used to determine imine loss relative to an internal standard. DBU 29 mediated hydration was followed by UV and HPLC.

With both assay techniques imine was reacted with base and a sixty-fold excess of water in acetonitrile, 10% in dioxane. Initial velocity was plotted against base concentration and this slope divided by imine concentration to give the pseudo second order rate constant. As both methods of following imine loss catalyzed by DBU gave the same rate constant when differing concentrations of imine were utilized, the reaction is apparent first order in imine.

Fig. 2 was generated by plotting log pseudo second order rate constants versus potential at half neutralization.

General Procedure for Flavin 8α Functionalization

All cyclic tertiary amine N-oxides of Table V were prepared by dropwise addition of the tertiary amine to 30% hydrogen peroxide at 0°. After reaction at room temperature for twelve hours, the excess hydrogen peroxide was destroyed with manganese dioxide. Following filtration, the water was stripped off and the resulting oil sublimed under vacuum two or three times until the N-oxide was white and crystalline. Typically, N-methylpiperidine N-oxide 28a required fewer sublimations than N-methylpyrrolidine N-oxide 28b. These N-oxides are very hygroscopic and all manipulations were carried out in a glove bag. To guarantee that the requisite amount of catalyst was added to a reaction, a stock solution of the N-oxide was made up before each reaction with acetonitrile (distilled over P_2O_5 and stored under nitrogen) which had been freeeze-thaw degassed under nitrogen three times. Phenolates 30 and 31 were obtained by addition of potassium-t-butoxide (sublimed) to stock solutions of the corresponding phenols which had previously been recrystallized from ethanol/water and dried. Thiolate 32 was obtained in likewise fashion. Reactions were monitored by analytical silica gel TLC (methylene chloride, 5% in ethanol) and HPLC (C-18 column, $CH_3CN:H_2O$ - 60:40 or 50:50, 4cc per min flow rate).

<u>2',3',4',5'-Tetraisobutyrylriboflavin 33</u>

Dimethyl formamide (DMF) was dried over activated 4Å molecular sieves. Isobutyric anhydride was distilled over P_2O_5 under nitrogen. 4-N,N-dimethylaminopyridine (DMAP) was recrystallized from cyclohexane. Riboflavin (15.3 g, .041 mol) and DMAP (.50 g, .0041 mol) were slurried in DMF (67 mL) and isobutyric anhydride (67 mL, .410 mol) added dropwise under nitrogen in the absence of light. The reaction was stirred mechanically for thirty hours at 40° resulting in a virtually homogeneous reaction mixture. After addition of 10 mL water all volatiles were stripped off under high vacuum and the resulting oil azeotroped 6 x 100 mL xylene. After taking up in 100 mL chloroform, the reaction crude was extracted 2 x 50 mL of 1N HCL. The organic layer was dried and concentrated. After drying the oil was dissolved in 25 mL chloroform, 100 mL ether added and recrystallization allowed to proceed at room temperature. On drying these gold crystals were dissolved in 25 mL $CHCL_3$, and 100 mL ether added along with a seed crystal. Following crystallization, filtration and drying afforded 13.51 g (50%) of a brilliant yellow solid.

M.P. 191.2-191.9°

^1H NMR (60 MHz, $CDCl_3$)(Me_4Si) δ 0.7-1.3 (m, 24H), 2.5 (s, 3H), 2.6 (s, 3H), 2.0-2.9 (m, 4H), 4.2-5.8 (bm, 7H), 7.7 (s, 1H), 8.1 (s, 1H), 9.2 (s, 1H).

^{13}C NMR (62.9 MHz)(Me_4Si) δ 175.6-176.6 (multiple lines), 159.5, 154.7, 150.6, 148.0, 136.9, 135.9, 134.4, 132.5, 131.3, 115.9, 69.9, 68.8 (two lines), 61.6, 44.2, 33.9, 33.8, 33.6, 33.5, 21.2, 18.0-19.2 (multiple lines).

IR(KBr) 2980, 2940, 2880, 1740, 1700, 1590, 1550, 1470, 1390, 1350, 1240, 1190, 1150, 1020, 850, 810, 780, 750, 675, 600, 450, 420 cm^{-1}.

UV (HCOOH) λ_{max} 442 (ε9.4 x 10^3), 380 (ε1.2 x 10^4), 272 nm (ε2.9 x 10^4).

8α,8α-2',3',4',5'-Tetraisobutyrylriboflavin dimer 36a

Anaerobic transfer of N-methylpyrrolidine-N-oxide 28b (.93 g, .0092 mol) in 10 mL acetonitrile to tetraisobutylriboflavin (1.0 g, .0015 mol) was followed by an immediate change in color from yellow to red-black. Reaction proceeded twenty-four hours under nitrogen in the absence of light at room temperature. The acetonitrile was subsequently stripped off and the sludge taken up in 100 mL chloroform. Extraction of the organic layer with 3 x 50 mL water was followed by a chloroform back extraction of the combined aqeous layers. Drying and concentration of the organic was followed by chromatography (chloroform, 5% in ethanol). Dimer enriched fractions were concentrated and triturated with ether. After filtration and drying, the residue was recrystallized from acetonitrile affording .1230 g (12%) of a brilliant, red compound.

^{1}H NMR (60MHz, dimethyl-d_6 sulfoxide)(Me_4Si) δ 0.6-1.2 (m, 48H), 1.9-2.9 (m, 8H), partially obscured by solvent), 2.7 (s, 6H), 3.3 (s, exchangeable H plus H_2O), 4.0-5.6 (br m, 14H), 7.9 (s, 2H), 8.1 (s, 2H), 8.3 (s, 2H), 11.6 (s, 2H).

^{13}CNMR (15 MHz, dimethyl-d_6 sulfoxide)(Me_4Si) δ 174.9-175.5 (multiple lines), 170.1, 159.5, 155.0, 150.8, 142.4, 137.7, 135.1, 134.8, 131.4, 112.7 (low intensity, two lines?), 69.4 (multiple lines), 61.6 (multiple lines), 33.2 (multiple lines), 18.4 (multiple lines).

IR(KBr) 2980, 1740, 1575, 1535, 1470, 1400, 1350, 1250, 1185, 1150, 830, 810, 750, 680, 475 cm^{-1}.

UV (HCOOH) λ_{max} 500 (ε6.0 x 10^4), 484 shoulder (ε5.6 x 10^4), 262 (ε6.4 x 10^4), 280 nm shoulder (ε3.6 x 10^4).

Anal. Calcd. for $C_{66}H_{86}N_8O_{20}$: C, 60.43, H, 6.62; N, 8.54.

Found: C, 60.51; H, 6.49; N, 8.33.

Field-desorption MS, M^+ m/e 1311.

8α, 8α-2',3',4',5'-Tetraisobutyrylriboflavin dimer 37

Anaerobic transfer of 2,6-di-t-butylphenol (.346 g, 1.68×10^{-3} mol) and potassium t-butoxide (.171 g, 1.52×10^{-3} mol) to tetraisobutyrylriboflavin (1.00 g, 1.52×10^{-3} mol) led to an instantaneous change in color from yellow to reddish black. Reaction proceeded twelve hours under nitrogen in the absence of light at room temperature. The reaction was concentrated and flashed (silica gel, methylene chloride, 5% in ethanol) followed by recrystallization of the dimer enriched fractions from acetonitrile. Following filtration, the mother liquors were concentrated and pure 37 obtained by semi-preparative HPLC (C-18 column, $CH_3CN:H_2O$, 60:40, 7 cc/min flow rate) with four recycles per separation. Stripping off solvent afforded .0456 g (5%) of the brilliant orange dimeric 37.

^1H NMR (90 MHz, $CDCl_3$)(Me_4Si) δ 0.8-1.2 (m, 48H), 2.5 (s, 6H), 2.2-2.8 (m, 8H), 3.3 (s, 4H), 4.5-5.6 (br m, 14H), 7.7 (s, 2H), 8.1 (s, 2H), 8.9 (s, 2H).

^{13}C NMR (62.9 MHz, $CDCl_3$)(Me_4Si) δ 176.0-176.6 (multiple lines), 159.3, 154.7, 150.7, 150.0 (2 lines), 136.5, 134.5, 133.8, 131.2, 114.8, 70.4, 69.0 (two lines), 61.8, 44.5, 33.7-34.1 (multiple lines), 18.3-19.0 (multiple lines).

IR(KBr) 2980, 1740, 1700, 1580, 1540, 1460, 1380, 1340, 1240, 1180, 1140 cm^{-1}.

UV(HCOOH) λ_{max} 444 (ε6.7 x 10^3), 372 (ε5.5 x 10^3), 252

(ε 2.8 x 10^4), 274 nm shoulder (ε 1.8 x 10^4).
Field-desorption MS, M^+ m/e 1311.

8-Formyl-2',3',4',5'-tetraisobutyrylriboflavin 45

N-Methylpyrrolidine-N-oxide (.926 g, 9.14×10^{-3} mol), tetraisobutyrylriboflavin (1.00 g, 1.52×10^{-3} mol) and morpholine (.796 mL, 9.14×10^{-3} mol) were reacted in 10 mL acetonitrile under nitrogen in the absence of light at room temperature for 23-1/2 hours. The reaction crude was taken up in 100 mL methylene chloride and extracted with 3 x 50 mL water. Drying and concentration was followed by chromatography (silica gel; methylene chloride, 5% in ethanol). Concentration and subsequent recrystallization from cyclohexane gave .4435 g (43%) of orange, crystalline 45.

^{1}H NMR (60 MHz, $CDCl_3$)(Me_4Si) δ 0.6-1.4 (m, 24H), 2.8 (s, 3H), 2.0-2.9 (m, 4H), 4.0-5.9 (br m, 7H), 8.1 (s, 1H), 8.2 (s, 1H), 9.3 (br s, 1H), 10.5 (s, 1H).

^{13}C NMR (22.6 MHz, $CDCl_3$)(Me_4Si) δ 190.5, 175.7-176.6 (Multiple-lines), 158.6, 154.3, 150.8, 139.8, 138.2, 137.6, 137.4, 135.8, 131.4, 118.0, 69.8, 69.0 (2 lines), 61.8, 43.6, 33.9-34.0 (multiple lines), 18.2-18.9 (multiple lines).

IR(KBr) 2980, 1740, 1700, 1615, 1585, 1540, 1460, 1380, 1345, 1235, 1180, 1140, 835, 740, 440 cm^{-1}.

UV($CHCl_3$) λ_{max} 464 ($\varepsilon 1.1 \times 10^4$), 488 shoulder ($\varepsilon 7.5 \times 10^3$), 444 shoulder ($\varepsilon 8.9 \times 10^3$), 340 nm ($\varepsilon 1.2 \times 10^4$).

Anal. Calcd. for $C_{33}H_{42}N_4O_{11}$: C, 59.08; H, 6.32; N, 8.35.

Found: C,58.87; H, 6.35; N, 8.27.

Field-desorption MS, M^+ m/e 670.6.

8α-Imidazolyl-2',3',4',5'-tetraisobutyrylriboflavin 41

Tetraisobutyrylriboflavin (1.00 g, 1.52 x 10^{-3} mol), N-methylpyrrolidine-N-oxide (.926 g, 9.15 x 10^{-3} mol) and imidazole (.623 g, 9.15 x 10^{-3} mol) in 10 mL acetonitrile were reacted anaerobically at room temperature in the absence of light for 28 hours. The reaction crude was subsequently taken up in 100 mL methylene chloride and extracted with 3 x 50 mL water. Following drying and concentration, chromatography (silica gel, methylene chloride 5% in ethanol followed by flushing with straight ethanol) afforded after trituration with ether .215 g (20%) of yellow gold crystalline 41.

^{1}H NMR (60 MHz, $CDCl_3$)(Me_4Si) δ 0.7-1.4 (m, 24H), 2.4 (s, 3H), 2.0-2.8 (m, 4H), 4.0-5.8 (br m, 9H), 6.9 (s, 1H), 7.1 (s, 1H), 7.6 (s, 1H), 7.7 (s, 1H), 8.0 (s, 1H), 10.2 (br s, 1H).

^{13}C NMR (22.6 MHz, $CDCl_3$) (Me_4Si) δ 176.8, 176.2, 175.9, 175.7, 159.1, 154.8, 150.8, 143.2, 137.9, 137.6, 135.6, 135.2, 134.4, 131.4, 130.0, 119.1, 115.9, 70.6, 69.1, 68.7, 61.9, 49.0, 33.7-33.9 (multiple lines), 18.3-18.8 (multiple lines).

IR(KBr) 2975, 1730, 1680, 1580, 1540, 1500, 1450, 1380, 1340, 1230, 1180, 1140, 820, 740, 440 cm^{-1}.

UV($CHCl_3$) λ_{max} 448 (ε1.0 x 10^4), 472 shoulder (ε 7.8 x 10^3), 428 shoulder (ε8.3 x 10^3), 336 nm (ε7.6 x 10^3).

Anal. Calcd. for $C_{36}H_{46}N_6O_{10}$: C, 59.81; H, 6.43; N, 11.63. Found: C, 59.94; H, 6.62; N, 11.42.

Field-desorption MS, M^+ m/e 722.6.

L-Histidine methyl ester dihydrochloride 47[52]

L-Histidine (1.0 g, 6.44 mmol) was slurried in 20 mL methanol and hydrogen chloride gas bubbled through in short bursts until the histidine went into solution. Refluxing for one hour was followed by storing in the freezer for an afternoon. Filtration and drying of the precipitate afforded 1.23 g (79%) of a white crystalline solid.

MP 202°

IR 3800-2100, 2000, 1750, 1620, 1580, 1500, 1450, 1425, 1360, 1350, 1280, 1190, 1135, 1100, 1070, 1055, 990, 950, 920, 895, 860, 820, 795, 710, 650, 635, 610, 530, 405, 345 300 cm^{-1}.

Nα-t-Butyloxycarbonyl-L-histidine methyl ester 48[52]

In 10 mL chloroform was dissolved 47 (5.0 g, .021 mol) and ammonia subsequently bubbled through the solution for five minutes. The precipitated ammonium chloride was filtered off, the filtrate concentrated and subsequently dissolved in 100 mL pyridine. Addition of t-butyloxycarbonyl azidoformate (3.14 mL, .022 mol) was followed by reaction for 96 hrs. under nitrogen. The pyridine was stripped off and the oily residue taken up in ethyl acetate and extracted with .5M citric acid. Basicification of the acid layer to pH=8 by addition of solid bicarbonate was followed by extraction with ethylacetate. Drying and concentration afforded 3.06 g (54%) of white, crystalline 48.

M.P. 122-123°

^{1}H NMR (90 MHz, $CDCl_3$)(Me_4Si) δ 1.4 (s, 9H), 3.09 (d, 2H), 3.7 (s, 3H), 4.5 (br m, 1H), 5.9 (br m, 1H), 6.8 (s, 1H), 7.5 (s, 1H), 9.5 (br s, 1H).

IR(KBr) 3360, 3100, 3000, 2880, 1750, 1680, 1570, 1510, 1450, 1435, 1370, 1295, 1280, 1245, 1225, 1200, 1150, 1085, 1070, 1040, 1020, 995, 975, 960, 860, 845, 830, 780, 760, 680, 655, 620, 580, 450, 430, 340 cm^{-1}.

8α-(N^3-N^α-t-butyloxycarbonylhistidyl)-2',3',4',5'-tetraisobutyrylriboflavin methyl ester 49

N-Methylpiperidine-N-oxide (.64 g, 5.5 x 10^{-3} mol), tetraisobutyrylriboflavin (.610 g, 9.29 x 10^{-4} mol), and 48 (1.50 g, 5.57 x 10^{-3} mol) were reacted in 5 mL acetonitrile under nitrogen at ambient temperature in the absence of light for 27 hours. The reaction crude was taken up in methylene chloride and extracted with water. Drying and concentration was followed by flash chromatography (silica, methylene chloride, 5% in ethanol) and subsequent PTLC (silica, methylene chloride, 5% in ethanol). This afforded .156 g (18%) of yellow, gold 49.

^1H NMR (90 MHz, $CDCl_3$)(Me_4Si) δ 0.7-1.3 (m, 24H), 1.4 (s, 9H), 2.0-2.8 (m, 4H), 2.4 (s, 3H), 3.0 (d, 2H), ~~3.6 (s, 3H),~~ 4.2-5.6 (m, 9H), 6.7 (s, 1H), 7.5 (s, 1H), 7.7 (s, 1H), 8.1 (s, 1H), 9.5 (br s, 1H).

^{13}C NMR 176.7-175.8 (multiple lines), 172.3, 159.0, 155.4, 154.5, 150.6, 142.7, 138.4, 137.8, 137.0, 135.8, 135.1, 134.5, 131.1, 116.5, 79.4, 70.4, 68.8, 61.8, 53.5, 52.0, 48.9, 44.1, 33.8, 30.2, 28.1, 18.7-18.2 (multiple lines).

IR(KBr) 2970, 2930, 2880, 1730, 1580, 1540, 1500, 1450, 1390, 1360, 1340, 1240, 1150, 745, 440 cm^{-1}.

UV($CHCl_3$) λ_{max} 448 (ε1.3 x 10^4), 475 shoulder (1.0 x 10^4), 427 shoulder (ε1.1 x 10^4), 335 (9.9 x 10^3), 270 (ε3.3 x 10^4).

Anal. Calcd. for $C_{45}H_{61}N_7O_{14}$: C, 58.48; H, 6.67; N, 10.61.

Found: C, 58.52; H, 6.95; N, 10.61.

Field-desorption MS, M^+ m/e 924,655.

8α-(N^3-Histidyl)riboflavin dihydrochloride 50

49 was taken up in 2 mL of 6N HCl and refluxed for twenty minutes under nitrogen. Following concentration reprecipitation from ethanol-methanol afforded .0095 g (73%) of yellow, gold crystalline 50.

^{1}H NMR (90 MHz, D_2O)(DSS) δ 2.5 (s, 3H), 2.6-4.8 (m), 5.7 (s, 2H), 7.5 (s, 1H), 7.9 (s, 1H), 8.0 (s, 1H), 8.9 (s, 1H).

IR(KBr) 3650-2500, 1705, 1650, 1580, 1540, 1450, 1400, 1340, 1250, 1180, 1050, 825, 800, 760, 450 cm^{-1}.

UV(H_2O) λ_{max} 445 nm (ε1.5 x 10^4), 355 (2.6 x 10^3), 266 (5.4 x 10^4), 220 nm (ε3.5 x 10^4).

Anal. Calcd. for $C_{23}H_{29}N_7O_8Cl_2 \cdot 2H_2O$: C, 43.26; H, 5.21; N, 15.35.

Found: C, 43.63; H, 4.80; N, 15.23.

Field-desorption MS, M^+ m/e 377.

Methanesulfenylphthalimide 51

Methyldisulfide (22.5 mL, .250 mol) in 50 mL 1,1,2,2-tetrachloroethane was chilled to -5° and sulfuryl chloride (20.2 mL, .250 mol) added under nitrogen. After stirring 1-1/2 hours at -5° and 1/2 hour at room temperature, the reaction crude was distilled two times at room temperature into a flask at -78°. Sulfur dioxide was collected in a liquid nitrogen trap. Subsequently, 4.0 g of the brilliant orange methanesulfenyl chloride was added to a vigorously stirred solution of triethylamine (6.8 mL, 0.048 mol), and phthalimide (3.6 g, .024 mol) in 25 mL dimethylformamide. After 1-1/2 hours 50 mL water was added and the precipitate collected. Recrystallization from ethanol gave 3.55 g (77%) of white, crystalline 51.

^{1}H NMR (60 MHz, dimethyl-d_6-sulfoxide)(Me_4Si) δ 2.6 (s, 3H), 7.9 (s, 4H).

8α, 8α-dimethanesulfenyl-2',3',4',5'-tetraisobutyryl-riboflavin 52

Tetraisobutyrylriboflavin (.500 g, 7.6×10^{-4} mol), methanesulfenylphthalimide (.440 g, 2.28×10^{-3} mol), and N-methylpiperidine-N-oxide (.53 g, 4.57×10^{-3} mol) were dissolved in 5 mL acetonitrile and reaction allowed to proceed 24 hours under nitrogen at room temperature in the absence of light. Product was isolated by semipreparative HPLC (C-18, CH_3CN: H_2O, 50:50, with no recycle and 7 cc/min flow rate) affording .252 g (44%) of yellow, brown solid 52.

^{1}H NMR (90 MHz, $CDCl_3$)(Me_4Si) δ 0.6-1.4 (m, 24H), 2.1 (s, 3H), 2.3 (s, 3H), 2.3-3.1 (m, 4H), 2.6 (s, 3H), 4.1-5.8 (m, 9H), 8.0 (s, 1H), 8.1 (s, 1H).

^{13}C NMR 176.4-175.5 (multiple lines) 159.1, 154.4, 150.7, 147.5, 137.3, 135.1, 134.8, 134.0, 131.7, 114.2, 69.8, 69.1, 68.0, 61.7, 53.3, 44.6, 33.9 (multiple lines), 18.9 (multiple lines), 16.0, 14.8.

IR(KBr) 2970, 2930, 2870, 1730, 1690, 1620, 1580, 1540, 1450, 1380, 1340, 1235, 1180, 1140, 960, 930, 880, 830, 805, 765, 580, 450 cm^{-1}.

UV($CHCl_3$) λ_{max} 450 ($\varepsilon 1.3 \times 10^4$), 475 shoulder ($\varepsilon 1.0 \times 10^4$), 430 shoulder ($\varepsilon 9.9 \times 10^3$), 350 shoulder ($\varepsilon 6.9 \times 10^3$), 273 nm ($\varepsilon 2.7 \times 10^4$).

Anal. Calcd. for $C_{35}H_{48}N_4O_{10}S_2$: C, 56.12; H, 6.47; N, 7.48.
Found: C, 56.44; H, 6.74; N, 7.68.

Field-desorption MS, M^+ m/e 748.

8α-methanesulfenyl-2',3',4',5'-tetraisobutyrylriboflavin 53

8α, 8α-dimethanesulfenyltetraisobutyrylriboflavin (.065 g, 8.69 x 10^{-5} mol) was taken up in methanol and freshly prepared sodium dithionite in water added. After the color changed yellow to green to leuco to green to yellow, the reaction crude was extracted with methylene chloride. Drying and concentration of the organic layer was followed by PTLC (silica, CH_2Cl_2 5% in ethanol) affording .0200 g (33%) of a yellow glass. This material was unstable on standing with substantial conversion to 8-formyltetraisobutyrylriboflavin.

^{1}H NMR (90 MHz, $CDCl_3$)(Me_4Si) δ 0.7-1.3 (m, 24H), 2.2 (s, 3H), 2.3-3.0 (m, 4H), 2.6 (s, 3H), 3.9 (s, 2H), 4.2-5.7 (m, 7H), 7.7 (s, 1H), 8.1 (s, 1H), 8.9 (s, 1H).

IR(KBr) 2970, 2920, 2870, 1735, 1580, 1540, 1455, 1380, 1340, 1235, 1180, 1140, 805, 740, 590, 510, 450 cm^{-1}.

UV($CHCl_3$) λ_{max} 450 (ε1.3 x 10^4), 475 shoulder (ε1.0 x 10^4), 430 shoulder (ε1.0 x 10^4), 345 (ε 8.2 x 10^3), 272 (ε2.9 x 10^4).

Field-desorption MS, M^+ m/e 702.

6-Hydroxy-3-methyl-2-methylthio-4(3H) pyrimidinone 67[53]

Thiobarbituric acid (10.0 g, .069 mol) was dissolved in 100 mL, 2N sodium hydroxide, chilled to 0°C, and dimethyl sulfate (20.0 g, 0.158 mol) added dropwise. The reaction was stirred one hour at 0° and one hour at room temperature. Acidification to pH=1 with concentrated hydrochloric acid led to precipitation of a white, crystalline solid. Filtration and drying afforded 8.27 g (69.7%) of 67.

M.P. ∿ 140° (not sharp)

^{1}H NMR (60 MHz, dimethyl-d_6 sulfoxide) (Me_4Si) δ 2.6 (s, 3H), 3.3 (s, 3H), 3.8 (s, 1H), 5.2 (s, 1H), 5.4 (s, 1H), 11.2 (br s, 1H).

IR(KBr) 2920, 1660, 1620, 1500, 1455, 1410, 1380, 1350, 1310, 1250, 1210, 1170, 1155, 1090, 1020, 970, 945, 880, 800, 755, 730, 710, 660, 640, 620, 600, 570, 460, 430, 410, 390 cm^{-1}.

6-Chloro-3-methyl-2-methylthio-4(3H)pyrimidinone 68[53]

In 35 mL phosphorous oxychloride and 5 mL N,N-dimethylaniline was dissolved 67 (11.5 g, .067 mol). After refluxing for one half hour, the excess phosphorous oxychloride was distilled off at reduced pressure and crushed ice added to the mixture. After cooling for an afternoon the precipitate was filtered and dried. Following trituration with petroleum ether, the solid was filtered and dried affording 6.98 g (67.4%) of off-white, crystalline 68.

M.P. ∿ 109-110.5.

^1H NMR (60 MHz, $CDCl_3$)(Me_4Si) δ 2.6 (s, 3H), 3.4 (s, 3H), 6.2 (s, 1H).

IR(KBr) 3105, 3080, 2920, 1670, 1560, 1485, 1400, 1350, 1310, 1210, 1170, 1085, 1070, 970, 935, 850, 815, 740, 720, 660, 610, 590, 525, 430, 395, 375 cm^{-1}.

6-Chloro-2-hydroxy-3-methyl-4(3H) pyrimidinone 65[53]

Sodium hydroxide (0.105 g), 2.63 x 10^{-3} mol) and (0.250 g, 1.3 x 10^{-3} mol) were dissolved in 2.5 mL water and 2.5 mL ethanol. Refluxing for fifteen minutes was followed by acidification to pH=1. Filtration of the precipitate with thorough washing with water afforded .087 g of 65. Allowing the filtrate to stand resulted after filtration in collection of .034 g (58% combined yield) of white crystalline 65.

MP 275-277°

^{1}H NMR (60 MHz, dimethyl-d_6-sulfoxide) (Me_4 Si) δ 3.1 (s, 3H), 5.9 (s, 1H), 14.1 (br s, 1H).

IR(KBr) 3420, 3090, 2900, 2800, 1730, 1700, 1600, 1500, 1445, 1380, 1330, 1270, 1160, 1110, 985, 950, 840, 750, 700, 650, 595, 535, 425, 375 cm^{-1}.

Alternatively, 6-chlorouracil 64 (5.0 g, .034 mol) is dissolved in 100 mL of 2N sodium hydroxide and dimethyl sulfate (4.31 g, 0.034 mol) and reacted overnight. Subsequent acidification to pH=1 leads to formation of a precipitate. Filtration and drying of this solid affords after recrystallization from ethanol 1.62 g (30%) of white, crystalline 65.

^{1}H NMR and IR(KBr) identical to above.

Ribosyl-p-toluidine 71

p-Toluidine (19.7 g, 0.183 mol) dissolved in 180 mL ethanol was added dropwise to a solution of ribose (25.0 g, 0.167 mol) in 330 mL water with catalytic (1 drop) of concentrated sulfuric acid. The reaction was stirred at room temperature with a magnetic stirrer until the entire solution solidified. After storing in the refrigerator for a couple of hours the precipitate was filtered and washed 2 x 20 mL water, 2 x 10 mL ethanol, and 2 x 30 mL ether. Drying under high vacuum afforded 35.9 g (90.0%) of white, crystalline 71. ^{1}H NMR (60 MHz, dimethyl-d_6 sulfoxide) (Me_4 Si) δ 2.2 (s, 3H), 3.1-3.9(m), 4.4-5.0 (m, 4H), 5.5-5.9(m, 1H), 6.5 (d, 2H), 6.9 (d, 2H).

Ribityl-p-toluidine 72

Ribosyl-p-toluidine 71 (35.9 g, 0.15 mol) and 10% palladium on carbon (1.0 g) suspended in methanol containing acetic acid (8.6 mL, 0.15 mol) was hydrogenated to 100% of theoretical hydrogen uptake. CAUTION: Palladium on carbon must be suspended in the methanol under nitrogen atmosphere prior to addition of 71 to avoid fires. Transferring the reaction mixture to a new flask and bringing to reflux under nitrogen was followed by addition of potassium hydroxide (10.1 g, 0.216 mol). The reaction mixture was filtered through Celite and subsequently stored in a refrigerator overnight. Filtration and drying afforded 20.8 g (52%) of white, crystalline 72.

M.P. 142.5-144°

^{1}H NMR (60 MHz, dimethyl-d_6 sulfoxide) (Me_4Si) δ 2.2 (s, 3H), 2.7-3.9 (m, 7H), 4.6 (br s, 5), 6.5 (d, 2H), 6.9 (d, 2H).

IR(KBr) 3500, 3480, 3420, 3300, 2970, 2900, 2860, 1610, 1580, 1510, 1470, 1450, 1410, 1375, 1350, 1310, 1280, 1250, 1220, 1130, 1105, 1075, 1050, 1010, 985, 945, 930, 910, 850, 800, 780, 750, 710, 650, 600, 545, 510, 490, 420, 365, 345 cm^{-1}.

3-Methyl-6-(N-ribityl-p-toluidino)uracil 73

Ribityl-p-toluidine 72 (0.56 g, 2.3 x 10^{-3} mol) and 6-chloro-3-methyluracil 65 (0.13 g, 8.0 x 10^{-4} mol) were refluxed in water under nitrogen overnight. Subsequently, 2N sodium hydroxide (2.0 mL) was added and the reaction crude cooled to 0° for one hour. Filtration was followed by acidification of the filtrate to pH=2 and concentration. Although product was taken immediately on to the next step, some was chromatographed on PTLC (methylene chloride, 10% in ethanol) to afford partial characterization.

^{1}H NMR (60 MHz, d_3 acetonitrile) (Me_4Si) δ 2.2 (s, 3H), 2.4 (s, 1H), 3.1-3.9 (m), 6.7 (d, 2H), 7.1 (d, 2H), 7.3 (s, 1H).

IR(KBr) 3500, 3480, 3420, 3280, 2980, 2920, 1690, 1610, 1510, 1450, 1410, 1380, 1350, 1310, 1280, 1250, 1220, 1080, 1050, 1010, 930, 850, 800, 780, 755, 710, 650, 600, 540, 505, 490, 420 cm^{-1}.

3-Methyl-6-(-N-tetraisobutyrylribityl-p-toluidino)uracil 74

Isobutyric anhydride (14.9 g, 0.094 mol), 4-N,N-dimethylaminopyridine (0.55 g, 0.0047 mol) and 73 (3.5 g, 0.0094 mol) were dissolved in 20 mL dimethylformamide and stirred under nitrogen for twenty-four hours. After addition of some water, the reaction crude was extracted with 1N HCl several times followed by water. Concentration was followed by azeotroping with xylene. The resulting oil was flashed (one column volume 2:1, hexane: ethyl acetate followed by one column volume CH_2Cl_2:ethanol, 95:5). This afforded 4.02 g (66%) of an off-white foam. A small portion was chromatographed on PTLC (silica, hexane:ethyl acetate, 2:1) for characterization. ^{1}H NMR (60 MHz, $CDCl_3$) (Me_4Si) δ 0.9-1.3 (m, 24H), 2.0-2.8 (m, 4H), 2.4 (s, 3H), 3.2 (s, 3H), 3.7-4.3 (m, 4H), 5.0 (s, 1H), 5.0-5.4 (m, 3H), 7.0 (d, 2H), 7.3 (d, 2H), 7.7 (br s, 1H). ^{13}C NMR (22.6 MHz) (Me_4Si) δ 176.4, 175.9, 175.5, 175.2, 163.8, 151.4, 150.8, 146.3, 139.4, 136.4, 131.4 (two lines?), 127.8 (two lines?), 77.7, 69.4, 68.9, 68.7, 61.6, 51.1, 47.6, 33.9-33.7 (multiple lines), 26.6, 21.0, 18.7-18.4 (multiple lines). IR(KBr) 2970, 2940, 2870, 1735, 1700, 1630, 1510, 1460, 1380, 1350, 1240, 1180, 1130, 1060, 1010, 980, 920, 840, 820, 780, 750, 725, 660, 530, 500, 410, 360 cm^{-1}.

8-Demethyl-3-methyl-2',3',4',5'-tetraisobutyrylriboflavin-5-oxide 75

Uracil 74 (2.01 g, 0.0031 mol) and sodium nitrite (1.07 g, 0.0156 mol) in 30 mL glacial acetic acid were reacted at 40° in the absence of light for one hour. The reaction solution was then taken up in toluene and extracted with water. Concentration of the organic layer was followed by azeotroping several times with toluene. Two successive flashes afforded 1.03 g of an orange, brittle foam. Those partially pure fractions were chromatographed on PTLC (ethyl acetate) affording .3347 g of the orange brittle foam (TOTAL YIELD, 66%).

^{1}H NMR (60 MHz, $CDCl_3$) (Me_4Si) δ 0.7-1.4 (m, 24H), 2.0-3.0 (m, 4H), 2.5 (s, 3H), 3.4 (s, 3H), 4.0-5.8 (m, 7H), 7.6 (s, 2H), 8.2 (s, 1H).

^{13}C NMR (22.6 MHz, $CDCl_3$) (Me_4Si) δ 176.3, 175.8, 175.6, 175.2, 156.0, 153.9, 151.1, 136.7 (two lines), 134.4, 131.9, 121.0, 116.2, 69.9, 68.9, 61.5, 43.9, 33.6 (multiple lines), 27.9, 20.7, 18.6 (multiple lines).

IR(KBr) 2970, 2930, 2870, 1730, 1700, 1650, 1585, 1540, 1450, 1400, 1380, 1350, 1270, 1240, 1180, 1135, 1060, 920, 845, 805, 780, 750, 600, 455, 410 cm^{-1}.

UV($CHCl_3$) λ_{max} 460(ε6.6 x 10^3), 485 shoulder (ε5.3 x 10^3), 440 (ε5.5 x 10^3), 345 (ε9.4 x 10^3), 360 shoulder (ε7.1 x 10^3), 330 shoulder (ε7.9 x 10^3), 270 nm (ε2.9 x 10^4).

Anal. Calcd. for $C_{33}H_{44}N_4O_{11}$: C, 58.91; H, 6.61; N, 833.

Found: C, 58.91; H, 6.66; N, 8.05.

Field desorption MS, M^+ m/e 672.

<u>8-demethyl-3-methyl-2',3',4',5'-tetraisobutyrylriboflavin-5-^{18}O-oxide</u>

p-Toluiduidinouracil <u>74</u> (0.322 g, 5.0×10^{-4} mol) and nitrosonium tetrafluoroborate (0.290 g, 2.5×10^{-3} mol) were dissolved in ether and (0.1 mL, 5.0×10^{-3} mol) $H_2{}^{18}O$ added. Reaction for one-half hour was followed by extraction with water. PTLC (silica, ethyl acetate) of the reaction crude afforded 0.086 g (26%) of an orange brittle foam with ^1H NMR identical to <u>75</u> and field desorption MS, M^+ m/e 672, 674.

General Procedure for Oxidation of Phenols with 75

A stock solution of the phenol was prepared in dry, freeze thaw degassed (two times) tetrahydrofuran. The stock phenol solutions were added to the requisite amount of sodium hydride and reaction allowed to proceed until gas evolution ceased. The phenolate solution was subsequently added to a two-fold excess of 75 under nitrogen in the absence of light.

Product structures were ascertained via coinjection with authentic samples on a Varian 3700 gas chromatograph equipped with a flame ionization detector and on column injection (SE-30 column, 4.1% on Chromsorb G,7' x 1/8"). Additional information followed from GC/MS with a Perkin Elmer 990 gas chromatograph interfaced via a glass jet to a Hitachi RMU-6L mass spectrometer (data acquisition and control via IBM 1800 computer). Product fragmentation patterns were correlated to authentic samples. In the oxidation of 2,3,5,6-tetramethylphenol 76, duroquinone 78 was isolated by PTLC (basic alumina, benzene) and shown to be identical to authentic sample by H'NMR and GLC.

Determination of ^{18}O Isotopic Percent

Flavin-5-^{18}O-oxide 75 (0.017 g, 2.5 x 10^{-5} mol) and triphenylphosphine (0.007 g, 2.5 x 10^{-5} mol) were reacted neat as a melt at 180° for five minutes. Semi-preparative HPLC (C-18, $CH_3CN:H_2O$, 50:50) afforded the triphenylphosphine-$^{16,18}O$-oxide. The percent ^{18}O incorporation was determined by mass spectroscopy on a Varian MAT 212 and shown to be 28.1% (See Table VIII.)

Determination of ^{18}O incorporated in Products of Phenol Oxidation

Procedure identical to that used for general oxidation of phenols with 75, except for the nature of the mass spectroscopy used to determine ^{18}O incorporation. To obtain greater accuracy, alternating voltage mass spectroscopy (AVS/MS) was set for a six mass unit window centered on the mass corresponding to the particular parent ion of the phenol oxidation product in question. The contribution of parent-plus-two ion from oxidations with 75 (^{16}O) was subtracted from the peak ratios obtained from 75 (^{18}O) oxidation to give the values listed in Table VIII.

Observation of the flavin nitroxy radical by ESR

A Varian E-9 ESR spectrometer with room temperature flat cell accessory was used in these experiments at 9.56 GHz with a 100 KHz modulation frequency, 0.5 G modulation amplitude, scan rate of 25 G/min., and 0.1 s. time constant. Anaerobic stock solutions of 75 and sodium 2,3,5,6-tetramethylphenolate 76b in dry, degassed tetrahydrofuran were prepared. The 75 stock solution was connected via cannula to the flat cell fitted with a Teflon adapter. A gas tight syringe was used for positive pressure to push the 75 solution into the flat cell for a background spectra. This starting solution was then pulled back into the reaction vessel where the phenolate was added (2:1, 75:76b stoichiometry, 4.93×10^{-3} M 75 concentration after mixing). The reaction was then immediately pushed into the flat cell with positive pressure from the gas tight syringe. Fig. 5 shows the observed ESR signal. All manipulations were carried out in the absence of light. The simulation computer program was provided by Dr. M. Winkler of Professor Solomon's group.

General Procedure for Kinetic Studies of 75 Reaction with 76b

Stock solutions of 75 and 76b in dry degassed tetrahydrofuran were prepared. Reaction was initiated by addition of a ten-fold excess of 76b to 75 in a stoppered, anerobic UV cell. Reaction was followed by increase in absorbance at 620 nm. The apparent first order rate constant was found to be 1.9×10^{-2} $min.^{-1}$

Enzymatic Synthesis of FAD-5-Oxide

FAD-synthetase complex of Brevibacterium ammoniagese (50λ) was combined with 150λ of 100 mM riboflavin-5-oxide, 100λ of 2 mM ATP, 100λ of 8 mM magnesium sulfate and 600λ of 50 mM morpholinopropane sulfonic acid (pH 7.2). Reaction proceeded for 24 hours at 37°. TLC (silica, 12:3:5, butanol: acetic acid:water) indicated quantitative conversion to FAD-5-oxide with no apparent deoxygenation. Filtration of the concentrated sample through two Sep Paks to remove enzyme was followed by HPLC separation (C18, 10:90, methanol:water to 70:30, methanol:water, straight gradient over twenty minutes, water pH = 6, 5 mM ammonium acetate). The FAD-5-oxide seems particularly sensitive to light so careful exclusion of light during workup is essential. Although HPLC gave pure FAD-5-oxide, concentration led to substantial cleavage of the phosphodiester probably due to the ammonium acetate.

REFERENCES AND BIBLIOGRAPHY

1. Recent general reviews and source material:
   (a) Walsh, C. T. Acc. Chem. Res. 1980, 13, 148:
   (b) Bruice, T. C. Acc. Chem. Res. 1980, 13, 256;
   (c) "Flavins and Flavoproteins, Proceedings of the Sixth International Symposium"; Yagi, K.; Yamano, T. (Eds.) University Park Press: Baltimore, 1980;
   (d) Berezovski, U. M.; Zhelina, T. A. Chem. Nat. Prod. 1979, 219.

2. (a) Mohler, H.; Brühmüller, M.; Decker, K. Eur. J. Biochem. 1972, 26, 279.
   (b) Pinto, J. T.; Frisell, W. R. Arch. Biochem. Biophys. 1975, 169, 483.
   (c) Kinney, W. C.; Edmondson, D. E., Seng, R. L. J. Biol. Chem. 1976, 251, 5386.
   (d) Kenney, W. C.; Edmondson, D. E.; Singer, T. P.; Steenkamp, D. J.; Schabort, J. C. Biochemistry 1976, 15, 4931.
   (e) Edmondson, D. E.; Kenney, W. C.; Singer, T. P. Biochemistry 1976, 15, 2937.

3. (a) Walker, W. H.; Kearney, E. B.; Seng, R. L.; Singer, T. P. Eur. J. Biochem. 1971, 24, 328;
   (b) Walker, W. H.; Kearney, W. C.; Edmondson, D. E., Singer, T. P.; Cronin, J. R.; Hendricks, R. Ibid 1974, 48, 439;
   (c) Kenney, W. C.; Singer, T. P. J. Biol. Chem. 1977, 252, 4767;
   (d) Ghisla, S.; Hemmerich, P. F.E.B.S. Lett. 1971, 16, 229.

4. McIntire, W.; Edmondson, D. E.; Hopper, D. J.; Singer, T. P. Biochemistry 1981, In Press.

5. Bruice, T. C.; Chan, T. W.; Taulane, J. P.; Yokoe, I.; Elliott, D. L.; Williams, R. F.; Novak, M. J. Am. Chem. Soc. 1977, 99, 6713.

6. Edmondson, D. E.; Singer, T. P. J. Biol. Chem. 1973, 248, 8144.

7. "Enzymatic Reaction Mechanisms" Walsh, C. T., W. H. Freeman and Company: San Francisco, 1979.

8. (a) Hemmerich, P.; Prijs, B.; Erlenmeyer, H. Helv. Chim. Acta 1959, 42, 2164;
(b) Hemmerich, P. Ibid. 1960, 43, 1942;
(c) Ehrenberg, A.; Müller, F.; Hemmerich, P. Eur. J. Biochem. 1967, 2, 286.

9. Bullock, F. J.; Jardetzky, O. J. Org. Chem. 1965, 30, 2056.

10. Shinkai, S.; Kusano, Y.; Manabe, O. J. Chem. Soc. Perkin I 1980, 1622.

11. Husain, M.; Massey, V. J. Biol. Chem. 1979, 254, 6657.

12. (a) Kemal, C.; Bruice, T. C. Proc. Natl. Acad. Sci. USA, 1976, 73, 995;
(b) Kemal, C.; Chan, T. W.; Bruice, T. C. Proc. Natl. Acad. Sci. USA 1977, 74, 405;
(c) Kemal, C.; Bruice, T. C. J. Am. Chem. Soc. 1977, 99, 7064.

13. (a) Ball, S.; Pruice, T. C. J. Am. Chem. Soc. 1979, 101, 4017;
(b) Ball, S.; Bruice, T. C. J. Am. Chem. Soc. 1980, 102, 6498.

14. Kemal, C.; Bruice, T. C. J. Am. Chem. Soc. 1979, 101, 1635.

15. Nishinaga, A.; Itahara, T.; Tomita, H.; Nishizawa, K.; Matsumara, T. Photochem. Photobiol. 1978, 28, 687.

16. (a) Wilshire, J.; Sawyer, D. T. Acc. Chem. Res. 1979, 12, 105;
(b) Sawyer, D. T.; Gibian, M. J. Tetrahedron 1979, 35, 1471.

17. Bruice, T. C.; Muto, S. J. Am. Chem. Soc. 1980, 102, 4472.

18. Iwata, M.; Bruice, T. C.; Carrell, H. L.; Glusker, J. P. J. Am. Chem. Soc. 1980, 102, 5036.

19. Orf, H. W.; Dolphin, D. Proc. Natl. Acad. Sci. USA 1974, 71, 2646

20. Rastetter, W.H.; Gadek, T. R.; Tane, J. P.; Frost, J. W. J. Am. Chem. Soc. 1979, 107, 2228.

21. Emmons, W. D. J. Am. Chem. Soc. 1957, 79, 5739.

22. Rastetter, W. H.; Frost, J. W. Tetrahedron Lett. 1979, 3353.

23. Boyd, D. R.; Hamilton, R.; Thompson, N. T.; Stubbs, M. E. Tetrahedron Lett. 1979, 3201.

24. Dinizo, S. E.; Watt, D. S. J. Am. Chem. Soc. 1975, 97, 6900.

25. (a) Hata, Y.; Watanabe, M. J. Am. Chem. Soc. 1979, 101, 6671;
(b) Hata, Y.; Watanabe, M. J. Org. Chem. 1981, 46, 610.

26. Schmitz, E.; Ohme, R.; Schramm, S. Tetrahedron Lett. 1965, 1857.

27. (a) Davis, F. A.; Mancinelli, P. A.; Balasubramanian, K.; Nadir, U. K. J. Am. Chem. Soc. 1979, 101, 1044;
(b) Davis F.A., Jenkins, Jr., R.; Yorklovich, S. G.; Tetrahedron Lett. 1978, 5171;
(c) Davis, F. A.; Abdul-Malik, N. F.; Awad, S. B.; Harakal, M. E. Tetrahedron Lett. 1981, 22, 917.

28. Scheinbaum, M. L. Tetrahedron Lett. 1969, 4221.

29. Freeman, J. Unpublished results.

30. Tokumura, K.; Goto, H.; Kashiwabara, H.; Kaneko, C.; Itoh, M. J. Am. Chem. Soc. 1980, 102, 5643.

31. Franzen, V.; Otto, S. Ber. 1961, 94, 1360.

32. MO calculations:
(a) Sun, M.; Song, P. Biochemistry 1973, 12, 4663;
(b) Dixon, D. A.; Lindner, D. L.; Branchaud, B.; Lipscomb, W. N. Biochemistry 1979, 18, 5770.

33. In vitro precedent:
(a) Schonbrunn, A.; Abeles, R. H.; Walsh, C. T.; Ghisla, S.; Ogata, H.; Massey, V. Biochemistry 1976, 15, 1798;
(b) Porter, D. J. T.; Voet, J. G.; Bright, H. J. J. Biol. Chem. 1973, 248, 4400;
(c) Massey, V.; Müller, F. et al, J. Biol. Chem. 1969, 244, 3999;
(d) Maycock, A. L.; Abeles, R. H.; Salach, J. I.; Singer, T. P. Biochemistry 1976, 15, 14;
(e) Yokoe, I.; Bruice, T. C. J. Am. Chem. Soc. 1975, 97, 450;
(f) Loechler, E. L.; Holocher, T. C. J. Am. Chem. Soc. 1975, 97, 3235.
(g) Loechler, E. L.; Holocher, T. C. J. Am. Chem. Soc. 1980, 102, 7312;
(h) Ibid, 7322;
(i) Ibid, 7328.

34. Frost, J. W.; Rastetter, W. H. J. Am. Chem. Soc. 1980, 102, 7157.

35. Novak, M., Bruice, T. C. J. Chem. Soc., Chem. Comm. 1980, 372.

36. Yoneda, F.; Sakuma, Y.; Ichiba, M.; Shinomura, K. J. Am. Chem. Soc. 1976, 98, 830.

37. (a) Edmondson, D. E.; Kenney, W. C., Singer, T. P. Biochemistry 1976, 15, 2937;
(b) Salach, J.; Walker, W. H.; Singer, T. P.; Ehrenberg, A.; Hemmerich, P.; Ghisla, S.; Hartmann, U. Eur. J. Biochem. 1972, 26, 267;
(c) Walker, W. H.; Singer, T. P.; Ghisla, S.; Hemmerich, P. Eur. J. Biochem. 1972, 26, 279.

38. Ghisla, S.; Hartmann, U.; Hemmerich, P. Angew.Chem., Int. Ed. Engl. 1970, 9, 642.

39. This follows from precedent in references (6) and (40). Indeed, use of the thiochemiacetal 54b is proposed (reference 40) as an alternative to synthesis of MAO cofactor reported in 3(d).

40. Kenney, W. C.; Singer, T. P. J. Biol Chem. 1977, 252, 4767.

41. Robin Spencer, Unpublished results.

42. Bruice, T. C. "Progress in Bioorganic Chemistry" Kaiser, E. J.; Kezdy, F. J. (Ed.); John Wiley and Sons: New York, 1976, pp. 3.

43. Steuber, F. W.; Dimroth, K. Chem. Ber. 1966, 99, 258.

44. "Encyclopedia of Electrochemistry of the Elements": Bard, A. (Ed.); Marcel Dekker, Inc.: New York, 1978; Vol. XI, pp. 264.

45. "Handbook of Biochemistry" Sober, H. (Ed.); the Chemical Rubber Co.: Cleveland, 1970; 2nd Edition, pp. J-195.

46. radical anion:
(a) Ehrenberg, A.; Muller, F.; Hemmerich, P. European J. Biochem. 1967, 2, 286.
neutral radical:
(b) Müller, F.; Hemmerich, P.; Ehrenberg, A.; Palmer, G.; Massey, V. Eur. J. Biochem. 1970, 14, 185.
useful overview:
(c) "Biological Applications of Electron Spin Resonance" Swartz, H. M.; Bolton, J. R. (Ed.); Wiley: New York, 1972, pp. 352.

47. Massey, V.; Hemmerich, P. Enzymes, 3rd Ed. 1976, 12, 191.

48. (a) Shoun, H.; Higashi, N.; Beppu, T.; Arima, K.; Nakamura, S. "Flavins and Flavoproteins, Proceedings of the Fifth International Symposium," Singer, T. P. (Ed.); Elsevier: New York, 1976, pp. 124.
(b) Shoun, H.; Higashi, N.; Beppu, T.; Arima, K.; Nakamura, S. F.E.B.S. Lett. 1974, 49, 13.

49. Wierenga, R. K.; de Jong, R. J.; Kalk, K. H.; Hol, W. G.; Drenth, J. J. Mol. Biol. 1979, 131, 55.

50. "Ethylenediamine and other Aziridines" Dermer, O. C.; Hum, G. E.; Academic Press, Inc.: New York, 1969, pp. 89.

51. Spencer, R.; Fisher, J.; Walsh, C. Biochemistry 1977, 16, 3594.

52. Handford, B. O.; Hylton, T. A.; Wang, R.; Weinstein, B. J. Org. Chem. 1968, 33, 4251.

53. Daves, Jr., G. D.; Robins, R. K.; Cheng, C. C. J. Am. Chem. Soc. 1962, 84, 1724.

54. Mirek, J.; Moskal, J.; Moskal, A. Tetrahedron 1975, 31, 2145.