Linear Free Energy Substituent Effect on Flavin Redox Chemistry

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Abstract: A systematic study on the effect of various substituents at the 7- and/or 8-position on the redox properties of isoalloxazines (flavins) is reported. The redox properties of these flavin derivatives were studied by cyclic voltammetry in 100 mM, pH 7.4 HEPES and 200 mM, pH 10 borate buffers. The magnitude and direction of the effect was dependent on the nature and location of the substituent. The redox potentials of the substituted flavins were correlated with the Hammett σ value of the substituents.

Flavoenzymes are an ubiquitous and diverse class of biological redox catalysts.[1] The redox active centers of these enzymes are derivatives of riboflavin (vitamin B_2, **1a** (see Figure 1)), which consists of an isoalloxazine ring system substituted with methyl groups at C7 and C8 and a ribityl group at N10; the biologically active forms are derivatized at the 5′-hydroxyl to give FMN (**1b**) or FAD (**1c**). A number of naturally occurring flavin analogues have been isolated from a variety sources, including roseoflavin (**2**) and 8-demethyl-8-hydroxyriboflavin (**3**) in which the C8 methyl group is replaced with a dimethylamino and hydroxyl group.[2] Riboflavin derivatives with substitutents at C6 have also been isolated. Flavoprotein FP_{390} isolated from luminescent bacteria possesses a covalent linkage from C6 of the isoalloxazine ring to C3 of myristic acid.[3] Flavoenzymes in which the cofactor is covalently bound to the protein have also been discovered.[2] The covalent attachment is usually to the C8-methyl group (**4**) via a thioether linkage to cysteine or ether linkage to tyrosine or to either imidazole nitrogens of histidine. Trimethylamine dehydrogenase is covalently bound through a thioether linkage directly to C6 of FMN.[4]

It is well-known that substituents on the benzene subnucleus can change the redox chemistry of flavins with the magnitude and direction of the effect being dependent on the nature and position of the substituent.[1] A systematic study, however, has not been reported. Because of the interest in the preparation of flavin models[5−7] and analogues as structural and mechanistic probes to better understand the redox properties of the holoenzyme,[8] we have studied the effect of substituents on the redox properties of flavins. A series of 7- and/or 8-substituted flavins has been synthesized and their redox chemistry studied by cyclic voltammetry (CV). The reduction potential of flavin analogues is linearly correlated with the Hammett σ value.[9]

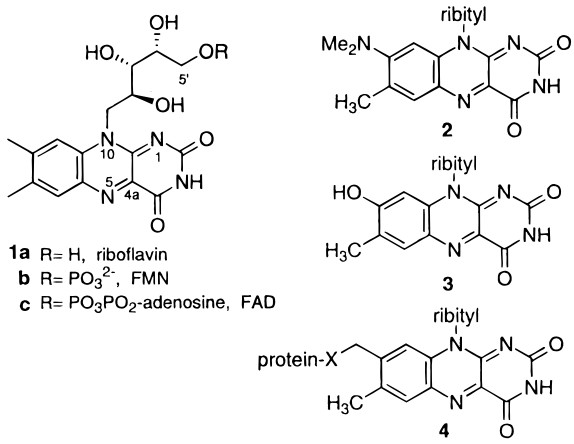

Figure 1.

A series of flavins substituted at C7 and/or C8 was synthesized by the condensation of alloxan with the properly substi-

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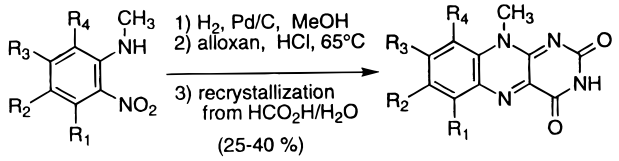
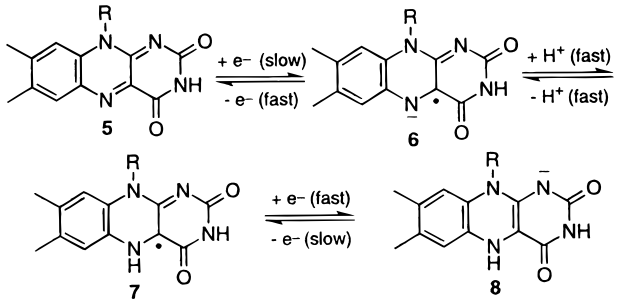
Scheme 1



Scheme 2



tuted *N*-methyl-*o*-phenylenediamines, which in turn were obtained from the reduction of the corresponding *N*-methyl-*o*-nitroaniline.[10] In general, the nitroanilines were reduced by catalytic hydrogenation over palladium on carbon except for those bearing chlorine substitutents. In these cases, hydrogenation resulted in partial reduction of the aryl chloride; thus reduction of the nitro group was accomplished with tin(II) chloride.[11] The *N*-methyl-*o*-phenylenediamines were used without further purification, and condensation with alloxan in 10% aqueous HCl gave the desired flavins in 25−40% overall yield after recrystallization from formic acid/water (Scheme 1).

The redox chemistry of flavins is shown in Scheme 2.[1] One-electron reduction of flavins to give a delocalized radical anion is the rate-limiting step. In protic media, this radical anion is protonated to give the semiquinone which undergoes a fast, second one-electron reduction to give the reduced flavin anion. The pK_a of the N1 proton of reduced flavin is approximately 6.5 and is largely ionized under the conditions we employed. In protic solvent, the entire electrochemical−chemical−electrochemical (ECE) reaction is faster than the CV time scale and is observed as a single wave, and is electrochemically reversible. For the flavins used in this study, the peak separations were between 40 and 48 mV, indicative of a two-electron process. Brockman and Pearson reported that the polaragraphic half-wave potential ($E_{1/2}$) of a series of substituted benzophenone derivatives could be correlated to the Hammett substituent constant.[12a] Since this initial observation, the $E_{1/2}$ of a number of substituted benzenes carrying reducible groups (carbonyls, thionocarbonyls, and nitroso and azo groups) have been correlated with the Hammett σ and/or $\sigma-$ value of the substitutent.[12b] Of course, the Hammett σ value is dependent upon the location of the substituent relative to the reducible group. For flavins, since the initial radical anion intermediate is coupled to the benzene subnucleus through the N5 position, we reasoned that the position of substituents relative to N5 was an important consideration in determining their influence on redox potential. We thus defined C7 as the meta position and C8 as para. The redox properties of flavins **9**−**25** were studied

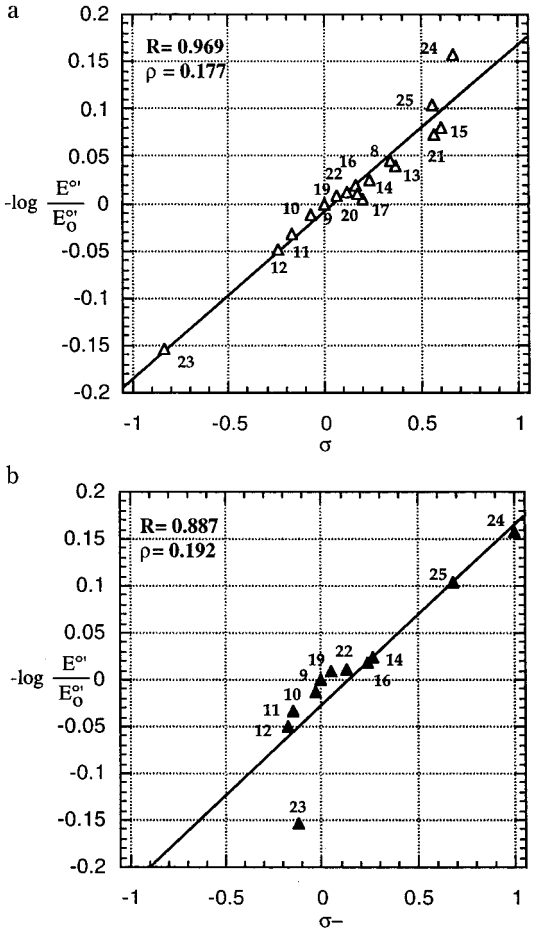


Figure 2. (a) Plot of Hammett σ versus $-\log(E^{\circ\prime}/E_0)$. (b) Plot of Hammett $\sigma-$ versus $-\log(E^{\circ\prime}/E_0^{\circ\prime})$ for 7- and/or 8-substituted flavins. $E_0^{\circ\prime}$ is the reduction potential of **9** (−407 mV vs Ag/AgCl).

by cyclic voltammetry using a standard three-electrode cell with a glassy carbon working electrode, Ag/AgCl reference electrode (+197 mV vs SHE), and platinum wire counter electrode. The reduction potentials were measured in 100 mM, pH 7.4 HEPES and 200 mM, pH 10 borate buffers. The observed reduction potentials of the flavin analogues along with the Hammett σ and $\sigma-$ values are summarized in Table 1. The unsubstituted flavin **9** is taken as the base value (E_0°) and had a reduction potential of −407 and −503 mV at pH 7.4 and 10, respectively. When the log of the normalized reduction potentials were plotted against the Hammett σ value, a linear correlation was observed (Figure 2). For 7,8-disubstituted flavins, the σ values of the substituents were simply added. The influence of the substituent is relatively small as indicated by the ρ value of 0.17. This value is similar to that obtained by Brockman and Pearson for substituted benzophenones ($\rho = 0.25$).[12a]

While the linear free energy correlation between flavin reduction potential and the Hammett σ is acceptable ($R = 0.97$ and 0.96 at pH 7.4 and 10.0, respectively), given the mechanism shown in Scheme 2, it might be expected that $\sigma-$ would give a better correlation since the C8 substituent could interact directly with the negative charge of the flavin radical anion through the N5 position. When the reduction potentials were plotted against $\sigma-$, the correlation was slightly worse at pH 7.4 ($R = 0.89$); however, it should be noted that for most of our substituents the σ and $\sigma-$ values are nearly the same. There were three substituents in which the σ and $\sigma-$ values are significantly different, the *p*-NMe_2 (**23**), *p*-CN (**24**), and *m*-CN (**25**). For the electron-rich *p*-NMe_2, there was particularly large

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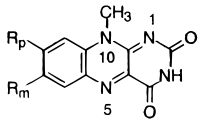
Table 1. Reduction Potentials (mV) of Flavins **9**−**25** in 100 mM, pH 7.4 HEPES and 200 mM, pH 10 Borate Buffers vs Ag/AgCl (+197 mV vs SHE)[a]



			pH 7.4		pH 10.0			
flavin	R_m	R_p	$E^{o\prime}$	$\Delta E^{o\prime}$	$E^{o\prime}$	$\Delta E^{o\prime}$	σ	$\sigma-$
9	H	H	−407	0	−503	0	0.00	0.00
10	CH_3	H	−419	−12	−517	−14	−0.07	−0.03
11	H	CH_3	−439	−32	−535	−32	−0.17	−0.15
12	CH_3	CH_3	−456	−49	−548	−45	−0.24	−0.18
13	Cl	H	−371	+36	−460	+43	0.37	
14	H	Cl	−385	+22	−469	+34	0.23	0.27
15	Cl	Cl	−338	+69	−417	+86	0.60	
16	CH_3	Cl	−390	+17	−485	+18	0.16	0.24
17	Cl	CH_3	−402	+5	−489	+14	0.20	
18	F	H	−366	+41	−454	+49	0.34	
19	H	F	−399	+8	−485	+18	0.06	0.05
20	F	CH_3	−398	+9	−488	+15	0.17	
21	F	Cl	−344	+63	−434	+69	0.57	
22	OMe	H	−396	+11	−483	+20	0.12	0.13
23	H	NMe_2	−580	−173			−0.83	−0.12
24	H	CN	−283	+124	−369	+134	0.66	1.00
25	CN	H	−321	+86	−408	+95	0.56	0.68

[a] The formal standard potential of each substrate was determined by the relationship $E^{o\prime} = (E_p^c + E_p^a)/2$.[13]

deviation when $\sigma-$ was used; however, for the cyano-substituted flavins **25** and **26**,[14] the Hammett $\sigma-$ value appears to give better correlation. Flavin **23** was not sufficently soluble at pH 10 for electrochemical studies. When this substrate was omitted, the $\sigma-$ gave better correlation ($R = 0.99$). It is established that the spin of flavin radical anion and semiquinone is delocalized into the aromatic ring.[15] Our observation that the Hammett σ gave better correlation than $\sigma-$ would suggests that the charge of the radical anion is probably not extensivly delocalized into the aromatic ring except when the substituent is a strong electron-withdrawing group such as cyano.

It has been pointed out that using the log of the redox potential instead of $E^{o\prime}$, is theoretically unjustified.[12b] When we plotted the midpoint potentials of flavins **9**−**25** versus the Hammett σ, a nearly identical correlation was observed, and when $\sigma-$ was used, the correlation was slightly worse. An advantage of using the log of the redox potentials is that both pH conditions could be plotted together to give a linear Hammett correlation so long as the proper $E_0^{o\prime}$ was used (Figure 3). This would suggest that the effect of benzo substitution on redox potential is largely independent of the buffer microenvironment and pH in the pH range used in our study.

Ortho substituents on aromatic rings often show nonlinear Hammett relationships due to steric and dipole effects. In our analysis, the 6-position of flavins is regarded as the ortho position. We have previously examined the redox properties of 9-substituted flavins which show significant steric effects.[6a,b] The conformation of fully reduced flavin is bent along the N5,-N10 axis; in free flavin, the bent conformation places the N10 substituent in a pseudoaxial position above the plane of the isoalloxazine ring system. For 9,10-dimethylflavin (**26**, Figure 4), a better steric arrangement of the methyl groups is achieved upon reduction. As such, we found that the reduction potential of **26** was shifted +50 mV compared to unsubstituted flavin **9**. Thus, Hammett σ values should be regarded as unreliable for predicting the redox potential of 9-substituted flavins. The

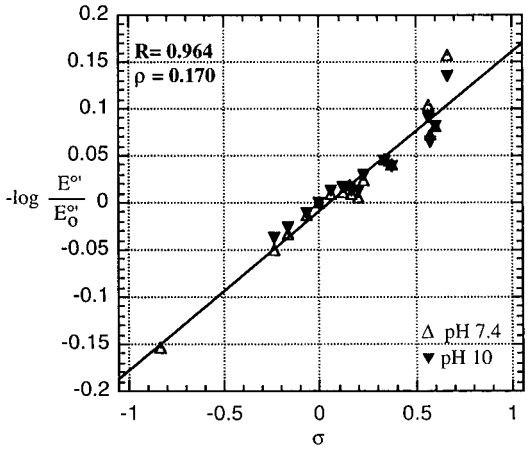


Figure 3. Plot of Hammett σ versus $-\log(E^{o\prime}/E_0)$ in 100 mM, pH 7.4 HEPES (△, $E_0 = 407$ mV vs Ag/AgCl) and 200 mM, pH 10 borate (▼, $E_0 = 503$ mV vs Ag/AgCl) buffers.

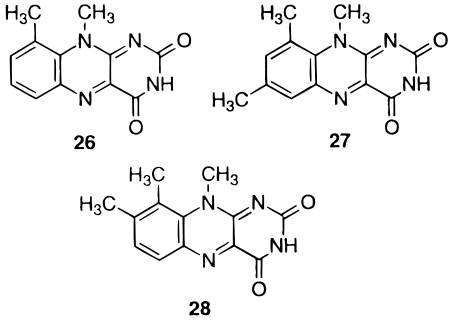


Figure 4.

reduction potential of 7,9,10-trimethylflavin (**27**) was determined to be −370 mV at pH 7.4 ($\Delta E^{o\prime}$= +37 mV) and shows an additive effect for the C7 (**10**, $\Delta E^{o\prime}$= −12 mV) and C9 (**26**, $\Delta E^{o\prime}$= +50 mV) methyl groups. For 8,9,10-trimethylflavin, however, the observed redox potential is −368 mV, which deviates significantly from that predicted by simply adding the individual effects of the 8- and 9-methyl groups (−389 mV at

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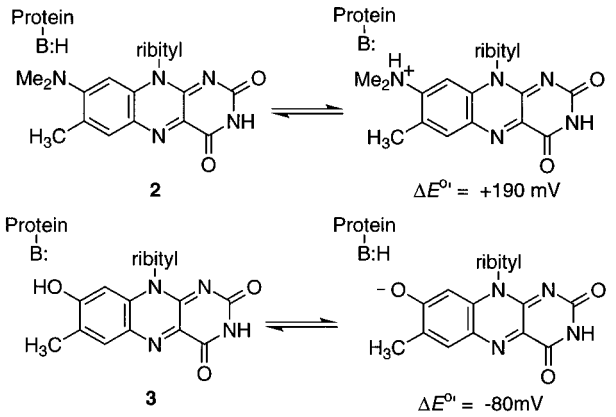
Table 2. Reduction Potentials (mV) of Flavins **26**−**28** in 100 mM, pH 7.4 HEPES and 200 mM, pH 10 Borate Buffers vs Ag/AgCl (+197 mV vs SHE)

	pH 7.4		pH 10.0	
flavin	$E^{\circ\prime}$	$\Delta E^{\circ\prime}$	$E^{\circ\prime}$	$\Delta E^{\circ\prime}$
26	−357	+50	−458	+45
27	−370	+37	−470	+33
28	−368	−39	−463	+40

Scheme 3



pH 7.4) (see Table 2). We attribute this nonlinear behavior to an enhanced steric effect due to steric crowding of the 8,9,10-methyl groups.

There have been a number of studies aimed at determining how the protein modulates the redox and catalytic properties of flavin cofactors.[5−7] A combination of hydrogen bonding, conformational, π-stacking, and desolvation effects contribute to this phenomena. Our results show that benzo substitution shifts the redox potential in a predictable manner and provides insight into how modified flavins such as roseoflavin (**2**) and 8-demethyl-8-hydroxyriboflavin (**3**) could modulate redox potential. For instance, the Hammett analysis predicts that protonation of the C8 dimethylamino group of roseoflavin would significantly alter the redox potential of this cofactor. The Hammett σ value for p-NH_2 is −0.66, while that for p-NH_3^+ is +0.60, representing a shift of nearly 200 mV to more positive values; it should be pointed out that the biological function of roseoflavin is largely unexplored. Similarly, deprotonation of the phenolic group of 8-demethyl-8-hydroxyriboflavin (**3**) is predicted to shift the reduction potential by −80 mV based on the Hammett σ values of p-OH (−0.37) versus p-O^- (−0.81) (see Scheme 3).

The linear relationship between the rate of flavin-catalyzed reactions with the polaragaphic half-wave or redox potential has been well appreciated.[6b,8,16] We have demonstrated that the redox potential for 7- and/or 8-substituted flavins is linearly correlated with the Hammett σ value. This relationship allows for the accurate prediction of the redox potential for substituted flavins and should aid in the design of flavin analogues as mechanistic probes and potential therapeutic agents as well as mechanistic interpretation of flavin-mediated reactions.

Experimental Section

Proton and carbon-13 NMR data were recorded at 300 and 75 MHz, respectively, in CF_3CO_2D. Chemical shifts are reported in parts per million downfield from TMS ($\delta = 0$); coupling constants are given in hertz. NMR samples were prepared with 99.5 atom % D CF_3CO_2D after first evaporating the sample from exchange grade CF_3CO_2D (99 atom % D). TMS ($\delta = 0$) or residual CF_3CO_2H ($\delta = 11.5$ for ^1H and $\delta = 116.6$ and 164.4 for ^{13}C) was used as an internal reference. UV spectra were recorded on a Shimadzu 2101 UV−vis spectrophotometer in 0.1 M NaOH solution. High-resolution mass spectra were obtained from the University of Illinois Mass Spectrometry Center. FAB mass spectra were obtained using "magic bullet" as the matrix. Melting points were collected on a Mel-Temp 3.0 apparatus and are uncorrected. Flavins tend to be high melting compounds and often decompose at their melting temperature. Most of our flavins decomposed without melting, in which case the melting points listed are the temperature at which they decomposed.

The required *N*-methyl-*o*-nitroanilines were prepared as previously described.[10,17] *N*-Methyl-*o*-nitroanilines were purchased from Aldrich and used without further purification. Lumiflavin (**12**) was purchased from Sigma and used as received. Flavin **23** was prepared from **19** under conditions previously described for structurally related flavins.[8] Flavins **24** and **25** were synthesized as described by Bruice et al.[14]

Synthesis of Flavins. The required *N*-methyl-*o*-nitroanilines were reduced to the corresponding *N*-alkyl-*o*-phenylenediamine via catalytic hydrogenation over palladium on carbon in methanol and 1 atm of hydrogen or with $SnCl_2$ in ethyl acetate at reflux.[11] The crude *N*-methyl-*o*-phenylenediamine was dissolved in 10% aqueous HCl, and 1.25 equiv of alloxan monohydrate added. The reaction was heated to 60 °C with stirring for 12 h, during which time a bright yellow precipitate formed. The yellow precipitate was collected by filtration and recrystallized from formic acid/water to give the desired flavin in 25−40% overall yield from the *N*-methyl-*o*-nitroaniline.

10-Methylisoalloxazine (9): decomposed without melting at 337 °C; ^1H NMR (CF_3CO_2D) δ 8.64 (d, $J = 8.3$, 1H), 8.54 (t, $J = 7.4$, 1H), 8.42 (d, $J = 8.3$, 1H), 8.34 (t, $J = 7.4$, 1H), 4.67 (s, 3H); ^{13}C NMR (CF_3CO_2D) δ 161.0, 151.1, 144.4, 143.8, 142.4, 136.1, 135.0, 134.3, 133.9, 119.3, 38.7; UV (0.1 M NaOH) λ 437 ($\epsilon = 7800$), 340 ($\epsilon = 8400$), 264 ($\epsilon = 27\,100$); HRMS (FAB) calcd for $C_{12}H_{11}N_4O_2$ (M + H) 229.0726, found 229.0724.

7,10-Dimethylisoalloxazine (10): decomposed without melting at 336 °C (lit.[18] mp > 280 °C; ^1H NMR (CF_3CO_2D) δ 8.43−8.30 (m, 3H), 4.67 (s, 3H), 2.81 (s, 3H); ^{13}C NMR (CF_3CO_2D) δ 161.1, 151.1, 148.2, 146.2, 143.5, 142.8, 134.6, 133.9, 123.2, 122.3, 118.9, 38.7, 21.8; UV (0.1 M NaOH) λ 448 ($\epsilon = 11\,900$), 341 ($\epsilon = 12\,300$), 269 ($\epsilon = 40\,000$); HRMS (FAB) calcd for $C_{12}H_{11}N_4O_2$ (M + H) 243.0882, found 243.0883.

8,10-Dimethylisoalloxazine (11): decomposed without melting at 329 °C; ^1H NMR (CF_3CO_2D) δ 8.50 (d, $J = 8.7$ Hz, 1H), 8.22 (s, 1H), 8.13 (d, $J = 8.7$ Hz, 1H), 4.65 (s, 3H), 2.93 (s, 3H); ^{13}C NMR (CF_3CO_2D) δ 161.3, 160.1, 151.1, 144.1, 141.5, 137.3, 135.6, 134.4, 132.5, 118.6, 38.5, 24.5; UV (0.1 M NaOH) λ 437 ($\epsilon = 10\,200$), 352 ($\epsilon = 9900$), 265 ($\epsilon = 28\,100$); HRMS (FAB) calcd for $C_{12}H_{11}N_4O_2$ (M + H) 243.0882, found 243.0883.

7-Chloro-10-methylisoalloxazine (13): decomposed without melting at 330−332 °C; ^1H NMR (CF_3CO_2D) δ 8.53 (s, 1H), 8.37−8.29 (m, 2H), 4.60 (s, 3H); ^{13}C NMR (CF_3CO_2D) δ 160.8, 151.3, 144.6, 143.5, 142.3, 142.2, 135.7, 134.2, 132.5, 120.4, 38.8; UV (0.1 M NaOH) λ 447 ($\epsilon = 10\,900$), 333 ($\epsilon = 10\,000$), 270 ($\epsilon = 38\,400$).

8-Chloro-10-methylisoalloxazine (14): mp 323−324 °C (dec) (lit.[17] mp 315−325 °C); ^1H NMR (CF_3CO_2D) δ 8.70 (d, $J = 8.8$, 1H), 858 (s, 1H), 8.35, (d, $J = 8.8$, 1H), 4.78 (s, 3H); ^{13}C NMR (CF_3CO_2D) δ 160.7, 152.3, 151.3, 145.1, 140.5, 136.6, 135.8, 134.4, 133.9, 119.1, 38.4; UV (0.1 M NaOH); λ 435 ($\epsilon = 11\,500$), 350 ($\epsilon = 10\,800$), 268 ($\epsilon = 31\,900$); HRMS (FAB) calcd for $C_{11}H_8ClN_4O_2$ (M + H) 263.0336, found 263.0337.

7, 8-Dichloro-10-methylisoalloxazine (15): decomposed without melting at 335−336 °C (lit.[19] 346 °C (dec)); ^1H NMR (CF_3CO_2D) δ

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8.59 (s, 1H), 8.47 (s, 2H), 4.53 (s, 3H); ^{13}C NMR (CF_3CO_2D) δ 160.1, 150.2, 145.7, 141.2, 140.5, 139.9, 135.6, 135.4, 132.9, 120.7, 38.6; UV (0.1 M NaOH) λ 445 (ϵ = 8300), 340 (ϵ = 7200), 273 (ϵ = 32 100).

8-Chloro-7,10-dimethylisoalloxazine (16): mp 323−324 °C (dec); ^{1}H NMR (CF_3CO_2D) δ 8.46 (s, 1H), 8.44 (s, 1H), 4.61 (s, 3H), 2.79, (s, 3H); ^{13}C NMR (CF_3CO_2D) δ 161.0, 153.9, 151.2, 146.6, 144.2, 141.1, 135.9, 133.7, 132.8, 119.4, 38.7, 20.9; UV (0.1 M NaOH) λ 445 (ϵ = 8700), 349 (ϵ = 8100), 269 (ϵ = 25 900).

7-Chloro-8,10-dimethylisoalloxazine (17): decomposed without melting at 341−3 °C; ^{1}H NMR (CF_3CO_2D) δ 8.53 (s, 1H), 8.27 (s, 1H), 4.58 (s, 3H), 2.86, (s, 3H); ^{13}C NMR (CF_3CO_2D) δ 161.0, 156.9, 151.2, 144.2, 143.6, 141.4, 134.2, 133.9, 132.7, 120.1, 38.7, 23.4; UV (0.1 M NaOH) λ 446 (ϵ = 7400), 344 (ϵ = 6700), 270 (ϵ = 26 000).

7-Fluoro-10-methylisoalloxazine (18): decomposed without melting at 303−305 °C; ^{1}H NMR (CF_3CO_2D) δ 8.39 (dd, J = 9.2, 4.2, 1H), 8.16 (d, J = 7.1, 1H), 8.13 (m, 1H), 4.59 (s, 3H); ^{13}C NMR (CF_3CO_2D) δ 166.1 (J_{C-F} = 263 Hz), 160.6, 151.2, 144.3, 143.3 (J_{C-F} = 13 Hz), 135.5, 132.7 (J_{C-F} = 27 Hz), 130.9, 121.7 (J_{C-F} = 10 Hz), 119.7 (J_{C-F} = 23 Hz), 39.0; UV (0.1 M NaOH) λ 446 (ϵ = 10 200), 330 (ϵ = 9900), 266 (ϵ = 26 500); HRMS (FAB) calcd for $C_{11}H_7FN_4O_2$ (M + H) 247.0063, found 247.0063.

8-Fluoro-10-methylisoalloxazine (19): decomposed without melting at 374−376 °C; ^{1}H NMR (CF_3CO_2D) δ 8.60 (dd, J = 9.3, 5.5, 1H), 8.01 (dd, J = 8.8, 2.1, 1H), 7.92 (dt, J = 7.3, 2.2, 1H), 4.53 (s, 3H); ^{13}C NMR (CF_3CO_2D) δ 172.8 (J_{C-F} = 275 Hz), 161.1, 151.5, 145.5, 139.6 (J_{C-F} = 25 Hz), 139.5, 136.4 (J_{C-F} = 13.6 Hz), 133.4, 125.1 (J_{C-F} = 26 Hz), 106.2 (J_{C-F} = 30 Hz), 38.9; UV (0.1 M NaOH); λ 430 (ϵ = 10 000), 343 (ϵ = 8800), 263 (ϵ = 46 900); HRMS (FAB) calcd for $C_{11}H_7FN_4O_2$ (M + H) 247.0063, found 247.0631.

7-Fluoro-8,10-dimethylisoalloxazine (20): mp 331−332 °C (dec); ^{1}H NMR (CF_3CO_2D) δ 8.23 (d, J = 5.8, 1H), 8.08 (d, J = 7.7, 1H), 4.56 (s, 3H), 2.73 (s, 3H); ^{13}C NMR (CF_3CO_2D) δ 167.2, 160.9, 151.1, 148.7 (J_{C-F} = 22 Hz), 143.7, 142.7 (J_{C-F} = 13 Hz), 133.9, 131.2, 121.3 (J_{C-F} = 6 Hz), 118.7, 39.0, 17.8 (J_{C-F} = 4 Hz); UV (0.1 M NaOH) λ 446 (ϵ = 12 700), 344 (ϵ = 12 000), 266 (ϵ = 30 000); HRMS (FAB) calcd for $C_{12}H_{10}FN_4O_2$ (M + H) 261.0788, found 261.0790.

8-Chloro-7-fluoro-10-dimethylisoalloxazine (21): decomposed without melting at 274−276 °C; ^{1}H NMR (CF_3CO_2D) δ 9.08 (d, J = 6.0 Hz, 1H), 8.31 (d, J = 9.4 Hz, 1H), 4.76 (s, 3H); ^{13}C NMR (CF_3CO_2D) δ 161.3 (d, J_{C-F} = 255 Hz) 160.7, 152.1 (d, J_{C-F} = 21 Hz), 146.0, 143.0 (d, J_{C-F} = 14 Hz), 139.7 (d, J_{C-F} = 18 Hz), 137.7, 131.5, 121.8, 120.0 (J_{C-F} = 24 Hz), 39.0; UV (0.1 M NaOH) λ 448 (ϵ = 4800), 336 (ϵ = 4400), 268 (ϵ = 15 700).

7-Methoxy-10-methylisoalloxazine (22): mp 331−334 °C (dec); ^{1}H NMR (CF_3CO_2D) δ 8.36 (d, J = 9.7 Hz, 1H), 8.18 (dd, J = 9.7, 2.7, 1H), 7.90 (d, J = 2.7 Hz, 1H), 4.67 (s, 3H), 4.18 (s, 3H); UV (0.1 M NaOH) λ 467 (ϵ = 7100), 339 (ϵ = 5700), 277 (ϵ = 19 700); HRMS (FAB) calcd for $C_{12}H_{11}N_4O_3$ (M + H) 259.0831, found 259.0830.

8-Cyano-10-methylisoalloxazine (24): prepared as previously described by Bruice;[14] mp 335−9 °C (lit.[14] mp 341−342 (dec)); ^{1}H NMR (CF_3CO_2D) δ 8.79 (d, J = 0.9 Hz, 1H), 8.70 (d, J = 8.6 Hz, 1H), 8.33 (dd, J = 8.6, 0.9 Hz, 1 H), 4.60 (s, 3H); UV (0.1 M NaOH) λ 445 (ϵ = 8000), 328 (ϵ = 8300), 278 (ϵ = 20 800).

7-Cyano-10-methylisoalloxazine (25): prepared as previously described by Bruice;[14] mp 368−70 °C (lit.[14] mp 360−362 °C (dec)); ^{1}H NMR (CF_3CO_2D) δ 8.91 (d, J = 1.7 Hz, 1H), 8.53 (dd, J = 9.2, 1.7 Hz, 1H), 8.42 (d, J = 9.2 Hz, 1H), 4.55 (s, 3H); ^{13}C NMR (CF_3CO_2D) δ 161.8; 149.8, 141.8, 140.5, 139.6, 138.8, 137.5, 121.0, 116.3, 115.5, 37.3; UV (0.1 M NaOH) λ 433 (ϵ = 8100), 335 (ϵ = 7000), 281 (ϵ = 35 700).

9,10-Dimethylisoalloxazine (26): mp 315 °C; ^{1}H NMR (CF_3CO_2D) δ 8.45 (d, J = 8.2 Hz, 1H); 8.33 (d, J = 7.2 Hz, 1H); 8.14 (t, J = 7.8 Hz, 1H); 4.75 (s, 3H); 3.10 (s, 3H); ^{13}C NMR (CF_3CO_2D) δ 161.0, 151.0, 148.1, 145.7, 144.0, 135.3, 134.6, 134.4, 133.6, 131.9, 45.2, 24.2; UV (0.1 M NaOH) λ 443 (ϵ = 6400), 354 (ϵ = 8600), 267 (ϵ = 22 300); HRMS (FAB) calcd for $C_{11}H_9N_4O_2$ (M + H) 243.0882, found 243.0881.

7,9,10-Trimethylisoalloxazine (27): mp 314−6 °C (dec); ^{1}H NMR (CF_3CO_2D) δ 8.23 (s, 1H), 7.49 3H), 2.55 (s, 3H); ^{13}C NMR (CF_3CO_2D) δ 161.2, 151.0, 150.4, 147.4, 144.8, 144.3, 133.6, 133.2, 133.1, 131.2, 44.9, 24.1, 21.4; UV (0.1 M NaOH) λ 452 (ϵ = 8600), 355 (ϵ = 11 000), 273 (ϵ = 31 500); HRMS (FAB) calcd for $C_{13}H_{13}N_4O_2$ (M + H) 257.1039, found 257.1038.

8,9,10-Trimethylisoalloxazine (28): mp 283−5 °C (dec); ^{1}H NMR (CF_3CO_2D) δ 8.20 (d, J = 8.6 Hz, 1H), 8.10 (d, J = 8.5 Hz, 1H), 4.67 (s, 3H), 2.88 (s, 3H), 2.84 (s, 3H); ^{13}C NMR (CF_3CO_2D) δ 161.6, 160.4, 151.3, 145.9, 143.2, 138.0, 136.5, 133.2, 131.9, 129.8, 46.8, 23.6, 20.1; UV (0.1 M NaOH) λ 443 (ϵ = 14 200), 374 (ϵ = 19 000), 269 (ϵ = 44 800); HRMS (FAB) calcd for $C_{13}H_{13}N_4O_2$ (M + H) 257.1039, found 257.1038.

8-(Dimethylamino)-10-methylisoalloxazine (23). 8-Fluoro-10-methylisoalloxazine (**19**) (45 mg, 0.18 mmol), dimethylamine hydrochloride (58 mg, 0.72 mmol), and sodium acetate (88 mg, 1.08 mmol) in *N*,*N*-dimethylformamide (3.6 mL) were heated at 70 °C for 4 h. During this time, the yellow suspension was converted into a brick red solid. The solid was collected by filtration and washed with water then methanol. Recrystallization from formic acid/water gave **23** (32 mg, 66%) as a brick red powder which decomposed without melting at 389−90 °C (lit.[20] mp > 360 °C): ^{1}H NMR (CF_3CO_2D) δ 8.09 (d, J = 9.9 Hz, 1H), 7.78 (d, J = 9.9 Hz, 1H), 6.90 (s, 1H), 4.25 (s, 3H), 3.63 (s, 6H); ^{13}C NMR (CF_3CO_2D) δ 161.3, 152.1, 144.4, 141.6, 139.3, 137.6, 125.0, 120.0, 96.5, 43.3, 36.9; UV (0.1 M NaOH) λ 487 (ϵ = 14 900), 308 (ϵ = 4600), 254 (ϵ = 18 600); HRMS (FAB) calcd for $C_{13}H_{14}N_5O_2$ (M + H) 272.1148, found 272.1147.

Cyclic Voltammetry. A 0.5 mM solution of the flavin in 100 mM, pH 7.4 HEPES (using HEPES hemisodium salt) or 200 mM, pH 10 borate buffer was prepared. The flavin was dissolved by sonication, and any undissolved flavin was removed by filtration through a kimwipe; the solution was degassed with argon. Electrochemical measurements were performed on an EG&G model 263 potentiostat/galvanostat using a standard three-electrode cell with a glassy carbon working electrode (BAS No. MF-2012), a Ag/AgCl reference electrode (BAS No. MF-2063), and a platium wire counter electrode (BAS No. MW-1032). The sweep rate was 20 mV/s.

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