collected with Mo Kα radiation on a Siemens P3f diffractometer at −165 °C from a colorless crystal of dimensions 0.40 × 0.40 × 0.60 mm. Unit cell parameters were obtained by least-squares refinement of 24 reflections ($21.00° < 2\theta < 27.00°$). Data were collected in the (*hkl*) range (−11,−15,−16) to (0,15,17) with 3 reflections monitored out of every 100. A total of 5350 independent reflections were collected, with 3722 of $F > 4.00\sigma(F)$. Lorentz and polarization corrections were applied, and the structure was solved under $P\bar{1}$ symmetry by direct methods using SHELXTL PLUS (VMS).[29] Hydrogen atoms were located from difference Fourier syntheses, and full-matrix least-squares refinement was carried out with anisotropic parameters for all non-hydrogen atoms and with isotropic thermal parameters for all hydrogen atoms. Refinement of 524 parameters converged to $R_1(F) = 4.31\%$ and $R_w(F) = 4.25\%$. Results and structural parameters are available as supplementary material.

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Supplementary Material Available: Tables of molecular coordinates and geometries from single-crystal X-ray structure determinations of **1**, **3**, **6**, and **8** (28 pages). Ordering information is given on any current masthead page.

Flavin-6-carboxylic Acids as Novel and Simple Flavoenzyme Models. Nonenzymatic Stabilization of the Flavin Semiquinone Radical and the 4a-Hydroperoxyflavin by Intramolecular Hydrogen Bonding

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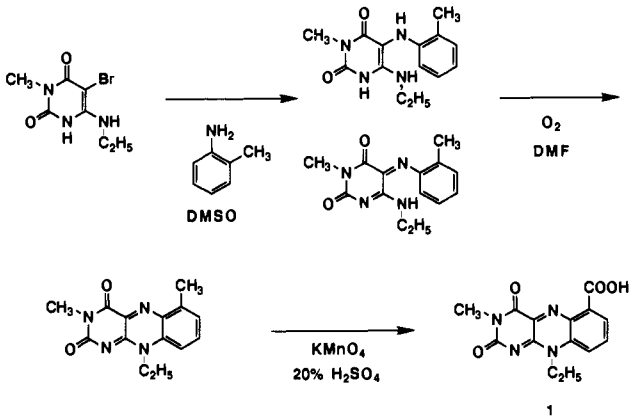
Abstract: Novel flavin derivatives, 10-ethyl-3-methylisoalloxazine-6-carboxylic acid (**1**) and 10-ethyl-3-methylisoalloxazine-6,8-dicarboxylic acid (**2**), which have a carboxyl group at C(6) position, were prepared. Even in the absence of metal cations and under aerobic condition, these flavin derivatives produced the corresponding stable semiquinone radicals by the dithionite reduction in sodium phosphate buffer (pH 6.89). Hyperfine electron spin resonance (ESR) spectra for the flavin semiquinone radicals have been obtained and were stable even after 1 day under ambient circumstances. Further characterization was obtained for these semiquinone radicals by UV-visible spectroscopy. The quantum chemical calculations have shown that the highest spin densities are located at N(5) position, and ESR experiments in D_2O established that the exchangeable protons are attached to N(5). The flavin-6-carboxylic acids **1** and **2** also activated H_2O_2 oxidizing thioanisoles to their sulfoxides. These remarkable reactivities of the flavin derivatives **1** and **2** were ascribed to the intramolecular hydrogen bonding between N(5) and carboxyl group at C(6) position of the flavin nucleus.

Introduction

Flavin coenzymes represented by FAD (flavin adenine dinucleotide) and FMN (flavin mononucleotide) play important roles in versatile redox reactions involving oxygen atom transfer and one-electron transfer as well as hydrogen transfer in many biological systems.[1] In order to catalyze the reactions in such systems, flavin coenzymes can take three readily accessible oxidation states, which include oxidized, semiquinone radical, and reduced forms.

It has been documented that, at the flavoenzymes active sites, there are many hydrogen bondings between heteroatoms of flavin coenzymes and amino acid residues of the apoproteins and that these hydrogen bondings are of importance for promotion of the reactivities of flavoenzymes including the regiospecific activation of flavin skeleton.[2] Massey and Hemmerich[3] have proposed to classify the flavoenzymes into two classes in terms of the position of hydrogen bonding with flavin skeleton; one group has a hydrogen bonding with the N(1) position which activates the C(10a) position, another has a hydrogen bonding with the N(5) position which activates the C(4a) position. Shinkai and co-workers[4] have synthesized a flavin derivative which has an ability of intramolecular hydrogen bonding between N(5) and phenolic hydroxy group. They have shown that thiols oxidation using this flavin involves C(4a) intermediates and was accelerated by activation of the C(4a) position through the above hydrogen bonding.[5]

Scheme I. Synthesis of Flavin Derivative **1**



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[†] Kyoto University.
[‡] Takeda Chemical Industries, Ltd.

(1) (a) Chance, B.; Williams, G. R. *J. Biol. Chem.* **1955**, *217*, 383-393. (b) Pouslen, L. L.; Zeigler, D. M. *J. Biol. Chem.* **1979**, *254*, 6449-6455.
(2) Burnett, R. M.; Darling, G. D.; Kendal, D. S.; LwQuesn, M. E.; Mayhew, S. G.; Smith, W. W.; Ludwig, M. L. *J. Biol. Chem.* **1974**, *249*, 4383-4392.

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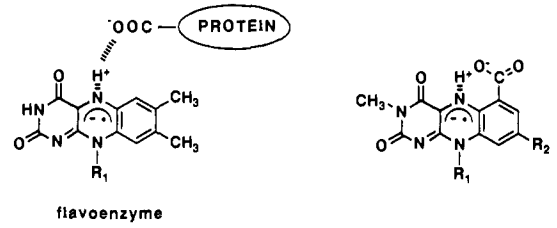
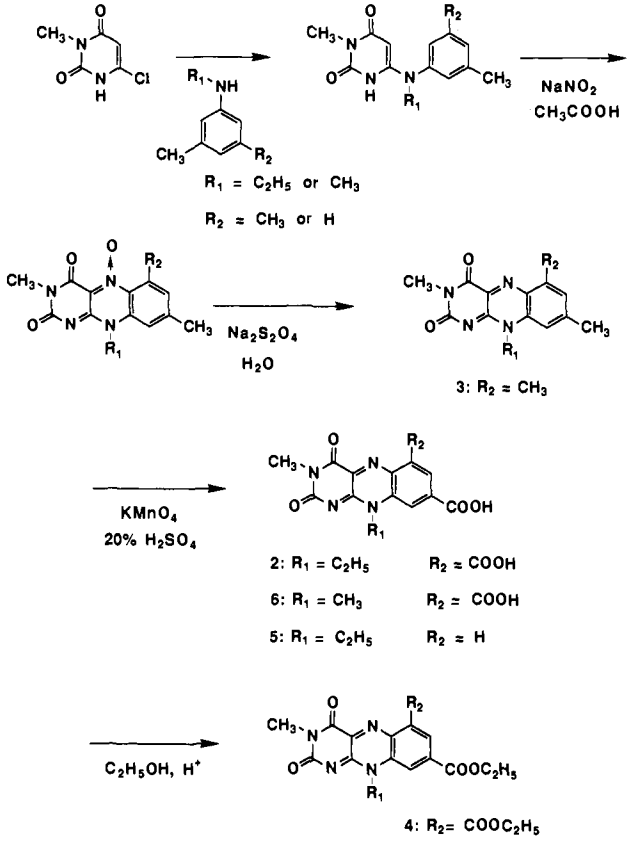


Figure 1. Concept of flavin-6-carboxylic acid derivatives.

Scheme II. Synthesis of Flavin Derivatives 2–6



In the present paper, we describe the syntheses and reactivities of the flavin derivatives which have a carboxyl group at the C(6) position. The carboxyl group has fairly high acidity and its introduction to the C(6) position will make it possible to cause a hydrogen bonding with N(5) in a stable six-membered ring state. These flavin derivatives are considered as a model compound for the flavoenzymes in which there are strong hydrogen bondings between N(5) position of flavin coenzyme and an Asp or Glu residue of the apoproteins (Figure 1).

Results and Discussion

Syntheses of Flavin-6-carboxylic Acids. The flavin-6-carboxylic acids were synthesized according to the procedures by Maki et al.[6] for **1** and by Yoneda et al.[7] for **2** (Schemes I and II). Condensation of 5-bromo-6-(*N*-ethylamino)-3-methyluracil with *o*-toluidine gave 6-(*N*-ethylamino)-5-(*N*-*o*-toluidino)uracil (concomitant with the corresponding dehydrogenated product), which was oxidatively cyclized by air under heating to give 10-ethyl-3,6-dimethylisoalloxazine. Oxidation of the latter with potassium permanganate in sulfuric acid afforded the desired 10-ethyl-3-methylisoalloxazine-6-carboxylic acid (**1**). 10-Ethyl-3-methylisoalloxazine-6,8-dicarboxylic acid (**2**) was synthesized as follows. Condensation of 6-chloro-3-methyluracil with *N*-methyl-3,5-xylidine gave 6-(*N*-ethyl-3,5-xylidino)-3-methyluracil, which was cyclized by nitrosation to give the corresponding isoalloxazine-5-oxide. Reduction of the 5-oxide by sodium dithionite gave 10-ethyl-3,6,8-trimethylisoalloxazine (**3**). The same oxidation of **3** with potassium permanganate gave the desired **2**.

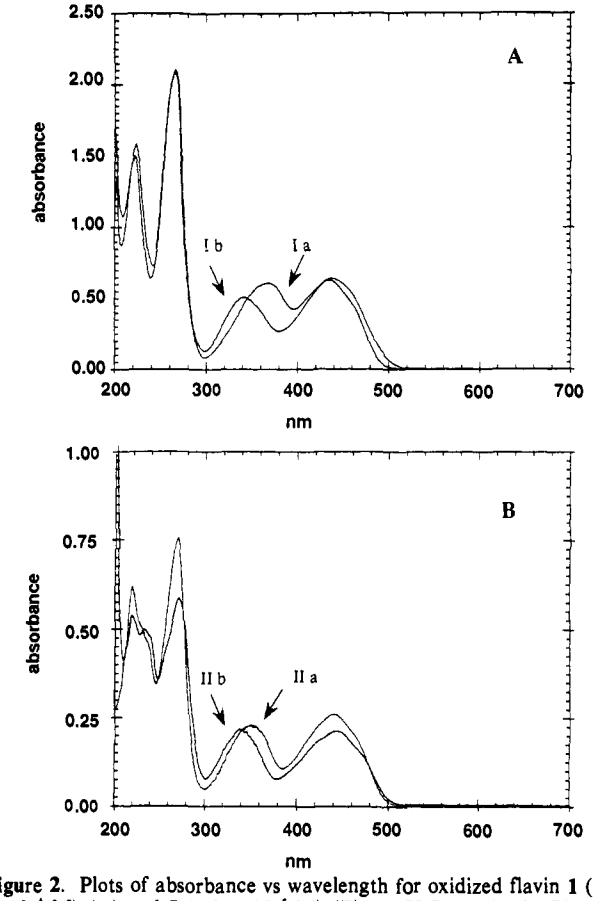


Figure 2. Plots of absorbance vs wavelength for oxidized flavin **1** (1.0×10^{-4} M) (A) and **5** (2.0×10^{-5} M) (B) at pH 7.0 and 1.0. Plots Ia and IIa are at pH 7.0; Ib and IIb are at pH 1.0.

Characterization of Flavincarboxylic Acid Derivatives by UV–Visible Spectroscopy. In order to discuss on the precise electronic structure of flavin-6-carboxylic acid derivatives **1** and **2** and 10-ethyl-3-methylisoalloxazine-8-carboxylic acid (**5**), the pH dependency of the absorption spectra for the oxidized flavincarboxylic acid derivatives was examined. As seen from the UV–visible spectra (Figure 2) of the flavincarboxylic acids at pH 1.0 and 7.0, the secondary peak at around 370 nm was strongly influenced by pH of the solution. In the flavin **1** having a carboxylic acid at C(6) position, the secondary peak was shifted at pH 1.0 toward shorter wavelength (λ_{max}; 340 nm) than that of a typical flavin. At pH 7.0, the secondary peak came out at 370 nm. Considering that the electronic structure of the flavin ring itself may not be strongly influenced by changing pH from 1.0 to 7.0, the above phenomena would suggest that the carboxylic group at C(6) of **1** exists as it is at pH 1.0, while at pH 7.0 it is dissociated to the carboxylate anion. Similar result was observed for flavin-8-carboxylic acid **5** (λ_{max}; 337 nm at pH 1.0 and 349 nm at 7.0) (Figure 2). In this connection, Bruice et al. reported[8] that the secondary peak of a flavin derivative having an electron-withdrawing group at C(8) position was shifted toward shorter wavelength than that of normal flavins.

The pK_a values of carboxylic group of **1** and **5** were determined by pH dependency of absorption peak at 370 nm for **1** and at 349 nm for **5**, which were found to be 2.8 for **1** and 3.1 for **5**, re-

(3) Massey, V.; Hemmerich, P. *Biochem. Soc. Trans.* **1980**, *8*, 246.
(4) Shinkai, S.; Honda, N.; Ishikawa, Y.; Manabe, O. *Chem. Lett.* **1984**, 327.
(5) Shinkai, S.; Honda, N.; Ishikawa, Y.; Manabe, O. *J. Am. Chem. Soc.* **1976**, *107*, 6268–6269.
(6) Sako, M.; Kojima, Y.; Hirota, K.; Maki, Y. *J. Chem. Soc., Chem. Commun.* **1984**, 1691–1692.
(7) Yoneda, F.; Sakuma, Y.; Ichiba, M.; Shinomura, K. *J. Am. Chem. Soc.* **1976**, *98*, 830–835.
(8) Bruice, T. C.; Chan, T. W.; Taulane, T. P.; Yokoe, I.; Elliot, D. L.; Williams, R. F.; Novak, M. *J. Am. Chem. Soc.* **1977**, *99*, 6713–6720.

spectively. We could not determine the exact pK_a value of **2** having two carboxylic groups at C(6) and C(8) positions, because each carboxylic group indicated an extremely close pK_a value, giving about 3.0. The observed pK_a values are appreciably low, because the electron deficiency of isoalloxazine skeleton makes the acidity of the carboxylic groups higher than that of normal benzoic acids.

Stabilization of Flavin Semiquinone Radicals by the Hydrogen Bonding with N(5). It is well-known that the semiquinone radical form of flavin coenzyme is unstable under aerobic condition in the absence of the apoprotein. In the past, however, there have been many studies concerning the flavin semiquinone radicals[9] because of their importance in biological systems involving flavoenzymes. In order to study the ESR spectra of flavin radicals, 5-alkylated flavin (flavinium) derivatives have usually been employed for the sake of stability of their semiquinones. In cases of nonalkylated flavin derivatives, ESR spectra of their semiquinone radicals can be obtained by dissolving equivalent amounts of reduced flavin and oxidized flavin in anaerobic condition.[10] This manipulation leads to the comproportionation between the reduced flavin and the oxidized flavin through intermolecular association equilibrium to produce the corresponding flavin radical. On the other hand, ESR spectrum of a flavoenzyme can be detected without this complicated manipulation.[11] It is obvious that the difference is ascribed to the presence of apoprotein of the flavoenzyme. The effect of hydrogen bonding in flavin coenzyme was described by Nishimoto and co-workers[12] from a quantum chemical point of view. They have shown that the hydrogen bonds with heteroatoms of flavin ring increase the electron acceptability and change the electronic spectrum of the oxidized flavin. Müller et al.[9a] have suggested that the stabilization of neutral flavin radicals may be explained by the presence of the H^+-donating group in the neighborhood of N(5) position. The facts described above stimulated us to design the novel flavin derivatives **1** and **2** possessing a carboxyl group at C(6) position. The carboxyl substituent at the C(6) position of the flavin skeleton is an excellent H^+-donor and is considered to be capable of strong intramolecular hydrogen bonding with N(5).

In the first place, we have investigated on the effect of intramolecular hydrogen bonding of these flavin derivatives by means of the stabilization of their semiquinone radicals. When flavin-6-carboxylic acid derivatives **1** and **2** having the ability of hydrogen bonding with N(5) were reduced by treatment with 1 equiv of $Na_2S_2O_4$ in sodium phosphate buffer (pH 6.89) under ambient aerobic conditions, the reaction solution exhibited respective clear ESR signals (Figure 3). Under the convenient degassed condition by argon bubbling and sonication, the treatment with 0.5 equiv of $Na_2S_2O_4$ gave the same ESR signal as above. Furthermore, the spectra were observed without change even after 1 day under ambient condition. Full reduction of **1** and **2** with high excess $Na_2S_2O_4$ gave precipitates in a light-brown solution which exhibited no ESR signal. However, the reaction mixture revealed the same ESR signal after air oxidation. The *g* values 2.0033 for **1** and 2.0034 for **2** were in reasonable agreement with the *g* values determined for other flavin radicals. From comparison with ESR signal of equal molar DPPH, it has been evaluated that almost 100% of total flavins were present as the free radicals. The ESR spectra possessed evenly spaced hyperfine lines consisting of an odd number for **1** and an even number for **2**, with a separation between components of 2.3 G for **1** and 2.1 G for **2**.

In the meantime, the same $Na_2S_2O_4$ reduction of flavin-8-carboxylic acid **5**, which has no ability of intramolecular hydrogen bonding with N(5), yielded only the weak ESR spectrum (Figure 4), and this spectrum disappeared completely after 1 h. It has been reported[8] that the presence of an electron-withdrawing group at C(8) position could stabilize a flavin semiquinone form. The observation of the weak ESR spectrum from **5** might be due to the presence of an electron-withdrawing carboxyl group at C(8).

Further information for the structure of the flavin semiquinone radicals was obtained from another flavin-6-carboxylic acid derivative **6** which has a methyl substituent at C(10) position instead of ethyl group. An ESR spectrum of the flavin semiquinone

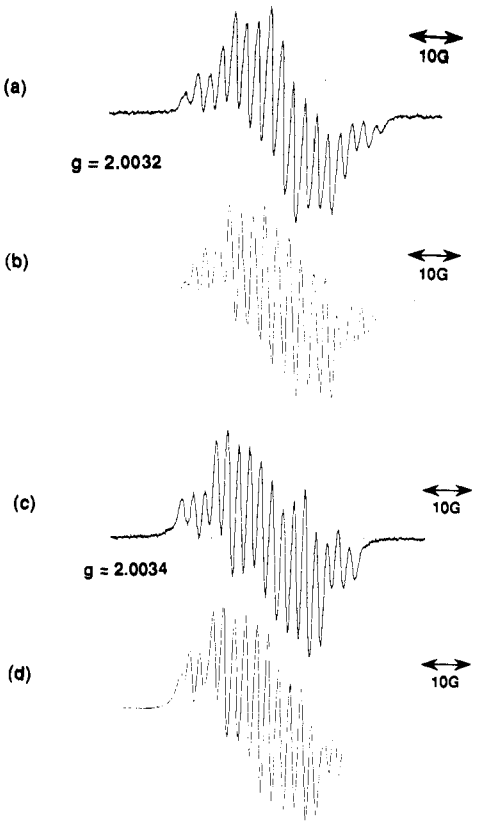


Figure 3. ESR spectra of semiquinone radicals of flavin derivatives **1** and **2** (1×10^{-2} M) in H_2O buffer: (a) observed spectrum of semiquinone radical of **1**; (b) simulated spectrum of semiquinone radical of **1**; (c) observed spectrum of semiquinone radical of **2**; and (d) simulated spectrum of semiquinone radical of **2**.

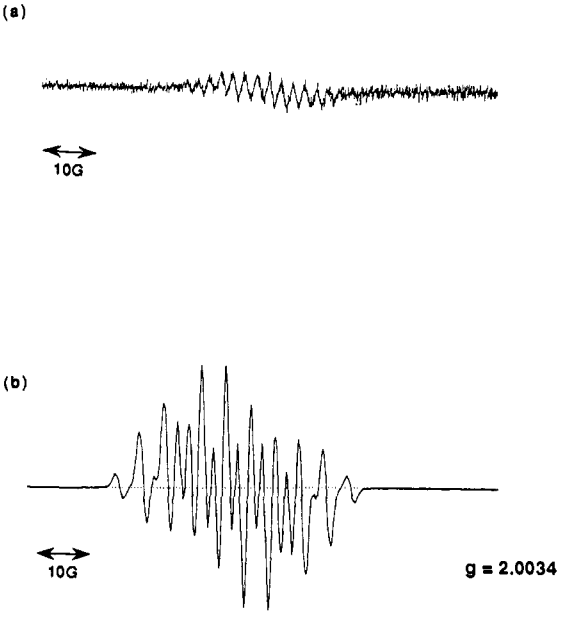


Figure 4. ESR spectra of semiquinone radicals of flavin derivatives **5** (1×10^{-2} M) and **6** (1×10^{-2} M) in H_2O buffer: (a) semiquinone radical of **5** and (b) semiquinone radical of **6**.

(9) (a) Müller, F.; Hemmerich, P.; Ehrenberg, A.; Palmer, G.; Massey, V. *Eur. J. Biochem.* **1970**, *14*, 185–196. (b) Walker, W. H.; Ehrenberg, A. *FEBS Lett.* **1969**, *3*, 315–318.

(10) Ehrenberg, A.; Eriksson, L. E. G. *Arch. Biochem. Biophys.* **1964**, *105*, 453.

(11) Palmer, G.; Müller, F.; Massey, V. In *Third International Symposium on Flavins and Flavoproteins*; Kemin, Ed.; University Park Press: Baltimore, MD, 1971; pp 123–137.

(12) Nishimoto, K.; Fukunaga, H.; Yagi, K. *J. Biochem.* **1986**, *100*, 1647–1653.

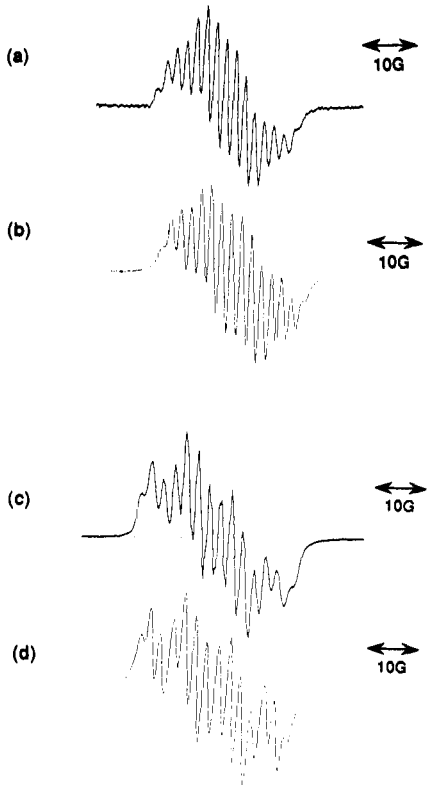


Figure 5. ESR spectra of flavin derivatives **1** and **2** (1×10^{-2} M) in D_2O buffer: (a) observed spectrum of semiquinone radical of **1**; (b) simulated spectrum of semiquinone radical of **1**; (c) observed spectrum of semiquinone radical of **2**; and (d) simulated spectrum of semiquinone radical of **2**.

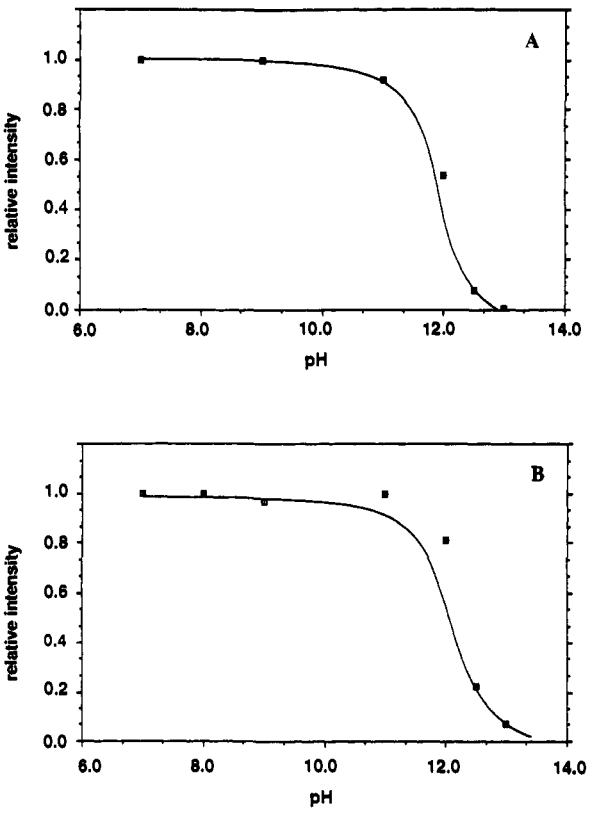


Figure 6. Plots of the relative intensities of ESR signal of semiquinone radicals of **1** (A) and **2** (B) vs pH. The Y axis shows the relative intensities against those obtained at pH 7.0. The flavins **1** and **2** (1×10^{-2} M) were reduced by $Na_2S_2O_4$ (1×10^{-2} M).

radical of **6** was obtained in the same manner with that in **1** and **2**, and its stability was nearly identical. However, the number of hyperfine lines of ESR spectrum of **6** increased complicatedly, which indicated that the spin densities on N(10) of these flavin semiquinone radicals give influence on the hyperfine splits (Figure 4).

When the flavin derivatives **1** and **2** were treated with $Na_2S_2O_4$ in D_2O under same conditions, the decrease of two lines in the hyperfine lines number was observed with ESR spectra of the semiquinones of **1** and **2** (Figure 5). These results show the presence of an exchangeable proton near the radical centers of flavin semiquinone radicals. Interestingly, replacing H_2O with D_2O in buffer solution decreased the line width of the ESR signals. It was reported that the line width of the ESR spectrum of the neutral flavin semiquinone was reduced in D_2O, while there was no significant effect on the width of spectrum of the anionic species.[11]

The pH dependency of intensities of ESR signals of semiquinone radical of **1** and **2** were investigated. Thus, the same intensities and line numbers were observed between pH 7.0 and 11.0 as shown in Figure 6. Above pH 12, the intensities of the ESR signal decreased rapidly, because flavin compounds **1** and **2** underwent a change to the other species at high pH condition.

UV-Visible Spectra of Semiquinone Radicals. In order to characterize the semiquinone radicals obtained by dithionite reduction of flavin-6-carboxylic acids **1** and **2**, these UV-visible spectra have been examined. With the exception of concentration of the flavins, we have recorded the spectra under the same condition as that described in the ESR measurements. After degassing by means of argon bubbling and sonication for 1 h, sodium dithionite was added to the flavin solution. As observed in Figure 7, the absorption bands of the oxidized forms for flavins **1** and **2** disappeared completely, and the new absorption bands having λ_{max} at 408 nm for **1** and 416 nm for **2** appeared. Air oxidation of these solutions brought about gradually reappearance of absorption spectra of the oxidized forms through the isosbestic

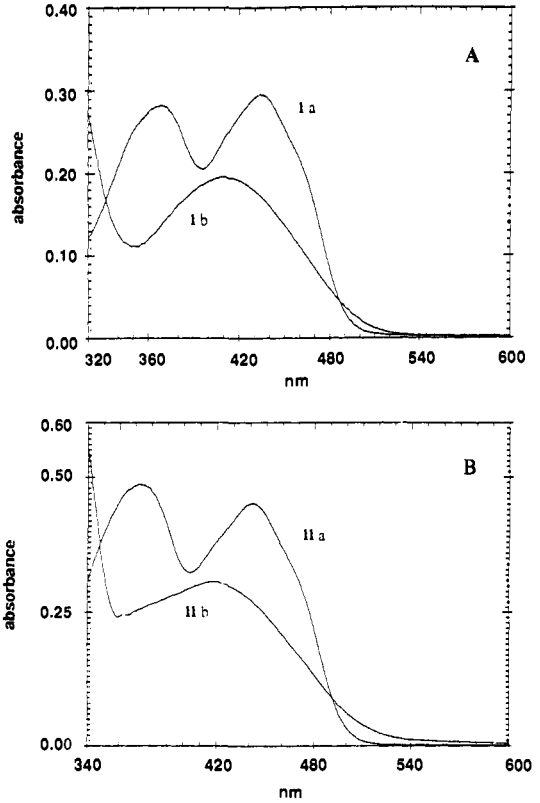
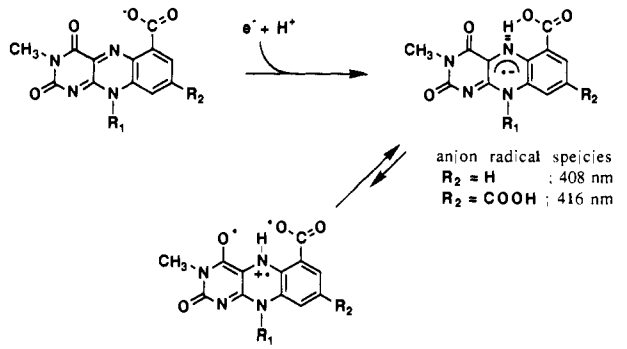


Figure 7. Plots of the absorbance vs wavelength for the oxidized (Ia, IIa) and semiquinone (Ib, IIb) forms of flavin compounds **1** (A) and **2** (B). Flavin-6-carboxylic acids **1** and **2** (5.0×10^{-5} M) were reduced by sodium dithionite (5×10^{-5} M) in 0.1 M sodium phosphate buffer (pH 6.89).

points at 335 and 490 nm for **1** and 350 and 495 nm for **2**, from which we could confirm that the absorption spectra contain only

Scheme III. Proposed Structure of Semiquinone Radical of Flavin-6-carboxylic Acid Derivatives



one species respectively. Furthermore, these absorptions did not change from pH 5.0–11.0. Bruice et al. reported[8] that the anion radical species of the flavin substituted at C(8) position by electron-withdrawing group exhibited the λ_{max} at 406 nm. The flavin derivatives **1** and **2** possessing a carboxylic group at C(6) position exhibited similar patterns to that of the above flavin in the absorption spectra of their semiquinone radicals. In this way, we have presumed that the absorptions having λ_{max} at 408 nm for **1** and at 416 nm for **2** come from the respective anion radical species.

The results obtained from the UV–visible spectra seemed inconsistent with those from the ESR experiments, in which D_2O substitution of buffer solution decreased the hyperfine line numbers and line width (Figure 5). Namely, from the D_2O experiments in ESR spectrometry, these semiquinone radicals would be suggested to be neutral radical species, because anion radical species of normal flavin compounds substituted at N(3) position have no exchangeable proton.

We propose the solution of this discrepancy as depicted in Scheme III. After the flavin-6-carboxylates are reduced to the corresponding anion radicals, they may catch a proton from the buffer solution rapidly. The proton existing between carboxylate anion and semiquinone anion radical must have extremely low acidity for the sake of the considerably strong hydrogen bonding with the two anionic groups. These hydrogen bondings may contribute to stabilization of the flavin semiquinone radicals of **1** and **2**. The proposed mechanism could provide a reasonable interpretation in terms of the same line numbers and intensities of ESR signals between pH 7.0 and 11.0 (Figure 6). We could not determine exact pK_a's of flavin semiquinone radicals of **1** and **2**, because they were unstable above pH 12.0 (Figure 6).

Quantum Chemical Consideration of the Flavin Semiquinone Radicals. In order to study precisely into the structures of the flavin semiquinone radicals of **1** and **2**, the quantum chemical calculations have been carried out in terms of simple HMO method[13] (Figure 8). EHMO and MNDO calculations also showed similar spin densities, however, simple HMO calculations gave more suitable results to explain the ESR spectra. The main spin densities of the flavin radicals of **1** and **2** and their proposed hyperfine coupling constants are tabulated in Table Ia,b. The hyperfine spectrum of the radical of **1** consisting of 17 lines agreed very well with the computer simulation spectrum based on the above proposed coupling constants and the line width of 1.4 G (Figure 3). Quite similarly the ESR spectrum of the radical of **2** consisting of 16 lines agreed with the simulated spectrum using the proposed coupling constants and the line width of 1.7 G. It is interesting to note that the spin densities are considerably located on the benzene ring besides N(5) and N(10). Namely the introduction of carboxylic group on the benzene ring in the flavin nucleus brings about the increase of its spin densities. Usually spin densities of the benzene ring moiety are very low from several MO calculations.[14]

(13) Yonezawa, T. et al. *Introduction to Quantum Chemistry*; Kagaku-Dojin: Kyoto, 1963; Vol. 1, p 55.
(14) Song, P. S. Ann. *N. Y. Acad. Sci.* **1969**, *158*, 410.

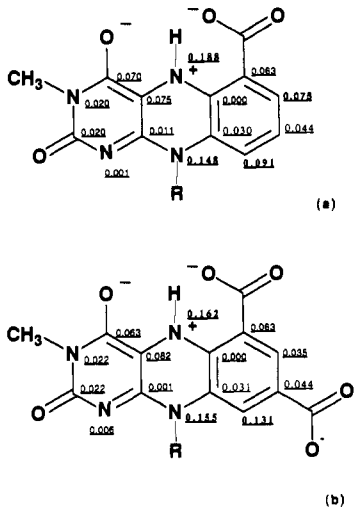


Figure 8. Spin densities of flavin semiquinone radicals calculated with simple HMO method: (a) spin densities of **1** and (b) spin densities of **2**.

Table I. Calculated Spin Densities and Proposed Coupling Constants for Semiquinone of **1** and **2** in H_2O Buffer

position	spin densities	proposed hyperfine coupling constants
	a. **1**	
N(5)	0.188	8.0 G
N(10)	0.148	5.4 G
C(7)–H	0.075	2.3 G
C(8)–H	0.044	not obsd
C(9)–H	0.091	2.3 G
N(5)–H		6.0 G
C(10)–CH_2		2.3 G
	b. **2**	
N(5)	0.162	8.0 G
N(10)	0.155	5.2 G
C(7)–H	0.044	not obsd
C(8)–H	0.035	not obsd
C(9)–H	0.131	2.1 G
N(5)–H		5.6 G
C(10)–CH_2-		2.1 G

Furthermore, the calculations could explain reasonably the assumption that the decrease of hyperfine line number by replacing H_2O with D_2O in buffer solution is due to the exchange of a proton with a large coupling constant attached to N(5) for a deuteron. In fact, the treatment of **1** and **2** with $Na_2S_2O_4$ in D_2O produced the stable ESR spectra bearing 15 and 14 lines, which were simulated very well as shown in Figure 5. In this way, the hyperfine coupling constants proposed for these flavin semiquinone radicals (Table Ia,b) could interpret their ESR spectra.

Oxidation of Thioanisoles with Flavin-6-carboxylic Acids and H_2O_2. Flavin-containing monooxygenases oxygenate a wide variety of substrates including amines and sulfides[15] by using the protein-bound 4a-hydroperoxyflavins.[16] One of the important roles of the proteins has been presumed to stabilize the intermediary 4a-hydroperoxyflavins by a hydrogen bond of the N(5) to the proteins as well. Otherwise the 4a-hydroperoxyflavins undergo spontaneously the elimination of H_2O_2 to yield the oxidized flavins.[17] Thus, the intramolecular hydrogen bonding of N(5) to carboxylate group at C(6) in the flavin nucleus has been anticipated to stabilize the 4a-hydroperoxide intermediate by suppression of the elimination of H_2O_2 (Scheme IV). Up to now, the flavinium derivatives,[18] which were alkylated at the N(5)

(15) (a) Ziegler, D. M.; Mitchell, C. H. *Arch. Biochem. Biophys.* **1972**, *150*, 116–125. (b) Entsch, B.; Ballou, D. P.; Massey, V. *J. Biol. Chem.* **1976**, *251*, 2550–2563.
(16) Bruice, T. C. *Acc. Chem. Res.* **1980**, *13*, 256–262.
(17) For example: Gibson, Q. H.; Hastings, J. W. *Biochem. J.* **1962**, *68*, 368.

Scheme IV. Proposed Mechanism of Oxidation of Sulfides with Flavin Derivatives and H_2O_2

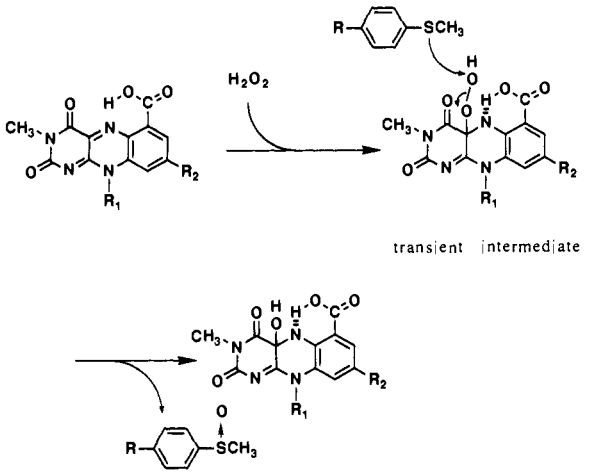
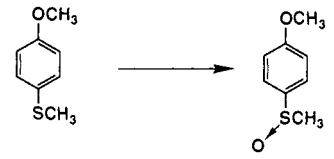


Table II. Oxidation of Thioanisole with Flavin Compounds and H_2O_2 in CH_3CN at 25 °C[a]



flavin derivatives	isolated yields of sulfoxide (%)
1	29.1
2	60.6
5[b]	10.3
3	9.0
4	4.5
none	7.4

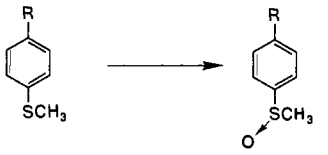
[a] Flavin derivatives = 0.162 mmol, thioanisole = 0.324 mmol, H_2O_2 = 0.324 mmol, CH_3CN = 5 mL. [b] **5** = 0.324 mmol.

position of flavin ring, have been employed as the flavoenzyme models for the purpose of the stabilization of the 4a-hydroperoxyflavins[19] as well as flavin semiquinone radicals.[6a] Also, it was reported that a 5-alkyl-4a-hydroperoxyflavin could oxidize amines and sulfides.[20]

We have now carried out the monooxygenation model reaction in terms of the activation of H_2O_2 by the flavin-6-carboxylic acid derivatives **1** and **2**. Actually, flavin derivatives **1** and **2** oxidized sulfide to its sulfoxides in the presence of H_2O_2 (Table II). In the absence of the flavin derivatives, only small amounts of sulfides were oxygenated, and moreover flavin derivatives **3** and **5** possessing no carboxylic group at C(6) position exhibited no acceleration of oxygenation. The esterification of carboxyl groups of flavin **2** which gives the diester **4** led to the loss of reactivity (Table II). The foregoing findings suggest that intramolecular hydrogen bonding of N(5) to carboxyl group at C(6) is essential for this type of activation of H_2O_2 by flavin derivatives.

Shinkai et al. have previously reported[5] that the oxidation of thiols to disulfides, which involves nucleophilic attack to C(4a) position, was dramatically stimulated by activation of C(4a) position through the intramolecular hydrogen bonding between N(5) and phenolic hydroxy group. In our case also, it is plausible that the flavins **1** and **2** may activate C(4a) position through the intramolecular hydrogen bonding between the N(5) and carboxyl group at C(6), although no responsible active species have been determined yet. It is also apparent that the electron-withdrawing group attached to flavin ring has little influence for this reactivity, because isolated yields of sulfoxide in the presence of flavin-8-carboxylic acid **5** and flavin-6,8-dicarboxylic acid diethyl ester **4** were nearly the same as those in the absence of the flavin derivatives.

Table III. Oxidation of *para*-Substituted Thioanisoles with **2** and H_2O_2[d]



R	isolated yields of sulfoxide (%)	Hammet value	yields[c] (%)
OCH_3	53.2[a]	−0.28	106.4
CH_3	33.2[b]	−0.14	66.4
H	19.0[b]	0.00	38.0
CN	5.5[b]	0.70	11.0

[a] Deduced blank yields. [b] No sulfoxide was observed in the absence of **2**. [c] On the basis of **2**. [d] *para*-substituted thioanisoles = 0.324 mmol, **2** = 0.162 mmol, H_2O_2 = 0.324 mmol, CH_3CN = 5 mL. In the dark under Ar for 24 h at 25 °C.

Oae and co-workers[21] suggested that oxidation of sulfides by the 4a-hydroperoxyflavin occurs via nucleophilic attack of sulfur on the electrophilic oxygen of the flavin hydroperoxide. In order to obtain further information on the mechanism of this oxidation, the oxidation of a set of substituted thioanisoles by flavin derivative **2** and H_2O_2 has been done. As shown in Table III, the oxidation yield decreased, when Hammet value increased. It is evident that this oxidation involves nucleophilic attack of sulfur to an active species, which is perhaps 4a-hydroperoxy intermediate.

In this way, the intramolecular hydrogen bonding of the N(5) with carboxylic group at C(6) in the flavin nucleus has been proved to activate C(4a) position to receive nucleophilic attack of H_2O_2 producing a stable transient 4a-hydroperoxyflavin intermediate, which oxidizes sulfides to sulfoxides.

Conclusion. The protein bound flavoenzymes yield the stable flavin semiquinone radicals in high yields in oxidation–reduction processes.[22] It has been proposed that this flavin radical stabilization is caused by a strong hydrogen bonding of the flavin N(5) to carboxyl groups in the apoproteins.[9a] This type of hydrogen bond effect has first been realized in the condensed form within the flavin nucleus, in terms of intramolecular hydrogen bonding of the N(5) to the carboxyl group at C(6) position. Thus they produced the corresponding extremely stable semiquinone radicals by the dithionite reduction. Surprisingly, their stable ESR spectra could be observed in sodium phosphate buffer without any manipulation and equipment to maintain anaerobic condition. The intramolecular hydrogen bonding has also been utilized successfully for stabilization of the 4a-hydroperoxyflavin intermediate in the monooxygenation model reaction using the flavin-6-carboxylic acids and H_2O_2. To our best knowledge, these are the first successful examples for nonenzymatic stabilization of the flavin semiquinone radicals and 4a-hydroperoxyflavin intermediates in the *flavoenzyme model reactions by the 5-unsubstituted flavins*. In conclusion, the flavin-6-carboxylic acids derivatives can be regarded as a novel and the simplest and more natural flavoenzyme model, and the intramolecular hydrogen bonding of this type would offer a useful methodology for the execution of other flavin-catalyzed model reactions.

Experimental Section

Proton nuclear magnetic resonance (^1H NMR) spectra were recorded with a JEOL FX200 spectrometer using tetramethylsilane as an internal

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standard. The electron spin resonance (ESR) spectra were obtained on a JEOL JES-FE2XG spectrometer. Mass spectra (MS) were taken on a JEOL JMS01SG-2 instrument at 70 eV. Infrared spectra (IR) were recorded with Shimazu IR-400. UV–visible spectra were recorded with a Shimazu UV-2100 equipped with a thermocontroller Shimzu S-260 SPR-8. All experiments with UV spectroscopy were performed at 20 °C. Melting points were obtained on a Yanagimoto micromelting apparatus and were not corrected. Preparative thin-layer chromatography (PTLC) was run on 20 cm × 20 cm plates coated with a 0.3 mm layer of Merck silica gel PF_{254} and GF_{254}. Syntheses of flavin derivatives shown in Schemes I and II were carried out according to the procedures by Yoneda et al.[7] for **2–6** and by Maki et al.[6] for **1**.

Preparation of ESR Samples of Flavin Semiquinone Radicals. To the solution of flavin derivative (0.01 mmol) in 1 mL of 0.1 M sodium phosphate buffer (pH 6.89) was added $Na_2S_2O_4$ (0.01 mmol). A part of the prepared radical solution was taken in a capillary tube as the sample for electron spin resonance. The ESR spectra were obtained under ambient conditions.

Oxidation of Thioanisoles with Flavin Derivatives and H_2O_2 in CH_3CN. A solution of a flavin derivative (0.162 mmol for **1**, **2**, **4**, and **5** and 0.324 mmol for **3**) and a thioanisole (0.324 mmol) in 5 mL of CH_3CN was treated with 73 μL of 30% H_2O_2 solution (0.324 mmol). The mixture was allowed to stand in the dark for 24 h at 25 °C under argon. The reaction mixture was poured into 100 mL of CH_2Cl_2 and washed with saturated $NaHCO_3$ solution. The organic layer was dried over $Na_2S_2O_4$. Removal of the solvent in vacuo was followed by PTLC (silica gel). Isolated products were identified by the measurement of IR and NMR spectra.

6-(*N*-Ethyl-3,5-xylidino)-3-methyluracil. 6-Chloro-3-methyluracil (24 g, 0.15 mol) and *N*-ethyl-3,5-xylidine (44.8 g, 0.3 mol) was heated at 150 °C without solvent. After 6-chloro-3-methyluracil melted completely, the reaction mixture was cooled to ambient temperature. The resulting green gum was crystallized by addition of ethanol/ether and collected by filtration. Recrystallization from ethanol gave 7.78 g (43.4%) of 6-(*N*-ethyl-3,5-xylidino)-3-methyluracil as white powder: mp 180–182 °C; ^{1}H NMR DMSO-d_6 δ 1.06 (t, J = 6.90 Hz, 3 H), 2.28 (s, 6 H), 3.04 (s, 3 H), 3.67 (q, J = 6.90 Hz, 2 H), 4.34 (s, 1 H), 6.87 (s, 2 H), 10.40 (s, 1 H); high resolution EI-MS calcd for $C_{15}H_{19}N_3O_2$ 273.1466, found 273.1477.

10-Ethyl-3,6,8-trimethylisoalloxazine-5-oxide. A solution of 6-(*N*-ethyl-3,5-xylidino)-3-methyluracil (17 g, 62.3 mmol) in CH_3COOH (50 mL) was stirred in an ice bath, and $NaNO_2$ (12.9 g, 186.9 mmol) was added gradually. After the reaction mixture was stirred at room temperature for 1 h, the orange precipitate was filtered off and washed with ethanol. Recrystallization from ethanol gave orange needles of 10-ethyl-3,6,8-trimethylisoalloxazine-5-oxide (10.86 g, 58.3%): dec 295 °C; ^{1}H NMR ($CDCl_3$) δ 1.48 (t, J = 7.08 Hz, 3 H), 2.54 (s, 3 H), 2.94 (s, 3 H), 3.43 (s, 3 H), 4.71 (q, J = 7.08, 2 H), 7.10 (s, 1 H), 7.29 (s, 1 H); IR ($CHCl_3$) 3000, 1710, 1645, 1545 cm^{-1}; high resolution EI-MS calcd for $C_{15}H_{16}N_4O_3$ 300.1222, found 300.1225. Anal. Calcd for $C_{15}H_{16}N_4O_3$: C, 59.90; H, 5.37; N, 18.74. Found: C, 60.00; H, 5.27; N, 18.66.

10-Ethyl-3,6,8-trimethylisoalloxazine (3). A suspension of 10-ethyl-3,6,8-trimethylisoalloxazine-5-oxide (10.9 g, 33.6 mmol) and $Na_2S_2O_4$ (11.7 g, 67.2 mmol) in H_2O was stirred at room temperature for 2.5 h. The yellow precipitate in reaction mixture was collected by filtration. The yellow precipitate was dissolved in CH_2Cl_2, and the CH_2Cl_2 solution was washed with H_2O. The CH_2Cl_2 layer was dried with Na_2SO_4 and evaporated in vacuo, and the residue was recrystallized from ethanol/chloroform to give 10-ethyl-3,6,8-trimethylisoalloxazine (**3**) (9.88 g, 95.7%) as yellow needles: dec 295 °C; ^{1}H NMR ($CDCl_3$) δ 1.48 (t, J = 7.2 Hz, 3 H), 2.60 (s, 3 H), 2.83 (s, 3 H), 3.52 (s, 3 H), 4.79 (q, J = 7.2 Hz, 2 H), 7.27 (s, 1 H); IR ($CHCl_3$) ν 3000, 1700, 1650, 1555 cm^{-1}; high resolution EI-MS calcd for $C_{15}H_{16}N_4O_2$ 284.1273, found 284.1273.

10-Ethyl-3-methylisoalloxazine-6,8-dicarboxylic Acid (2). A solution of **3** (9.00 g, 34.8 mmol) and $KMnO_4$ (16.5 g, 115 mmol) in 300 mL of 20% H_2SO_4 was stirred at room temperature for 2 days. Ethanol (10 mL) was added to the reaction mixture and neutralized with $NaHCO_3$. After the reaction mixture was filtered, the filtrate was concentrated in vacuo to 1/5 volume, followed by addition with concentrated HCl to give an orange solid. Recrystallization from H_2O gave 10-ethyl-3-methylisoalloxazine-6,8-dicarboxylic acid (**2**) (3.06 g, 28.1%) as orange needles: dec 295 °C; ^{1}H NMR (DMSO-d_6) δ 1.35 (t, J = 7.1 Hz, 3 H), 3.29 (s, 3 H), 4.71 (q, J = 7.1 Hz, 2 H), 8.17 (s, 1 H), 8.39 (s, 1 H), 4–5 (br, 2 H); MS m/z 344 (M^+). Anal. Calcd for $C_{15}H_{12}N_4O_6 \cdot H_2O$: C, 49.72; H, 3.89; N, 15.46. Found: C, 49.64; H, 3.79; N, 15.50.

10-Ethyl-3-methylisoalloxazine-6,8-dicarboxylic Acid Diethyl Ester (4). A solution of **2** (1.5 g, 4.36 mmol) in 20 mL of ethanol was treated with 2 mL of concentrated H_2SO_4 and refluxed for 3 h. The reaction mixture was poured into cold $NaHCO_3$ saturated water and extracted by CH_2Cl_2. The organic layer was dried with Na_2SO_4 and evaporated in vacuo. Recrystallization of the residue from ethanol gave 10-ethyl-3-methylisoalloxazine-6,8-dicarboxylic acid diethyl ester (**4**) as yellow needles (964 mg, 55.2%) mp 197–199 °C; ^{1}H NMR ($CDCl_3$) δ 1.48 (t, J = 7.1 Hz, 3 H), 1.50 (t, J = 7.1 Hz, 3 H), 1.52 (t, J = 7.3 Hz, 3 H), 3.50 (s, 3 H), 4.51 (q, J = 7.1 Hz, 2 H), 4.56 (q, J = 7.1 Hz, 2 H), 4.83 (q, J = 7.3 Hz, 2 H), 8.36 (d, J = 1.7 Hz, 1 H), 8.40 (d, J = 1.7 Hz, 1 H); IR ($CHCl_3$) ν 2990, 1715, 1660, 1250 cm^{-1}. Anal. Calcd for $C_{19}H_{20}N_4O_6$: C, 57.00; H, 5.03; N, 13.99. Found: C, 56.84; H, 4.88; N, 13.88.

6-(*N*-Methyl-3,5-xylidino)-3-methyluracil. 6-Chloro-3-methyluracil (2.08 g, 13.0 mmol) and *N*-methyl-3,5-xylidine (3.5 g, 26.0 mmol) was heated at 180–190 °C without solvent. After 6-chloro-3-methyluracil melted, the reaction mixture was cooled to room temperature. The resulting brownish gum was crystallized by addition with ethanol/ether and collected by filtration. Recrystallization from ethanol gave 1.11 g (32.9%) of 6-(*N*-methyl-3,5-xylidino)-3-methyluracil as white plates: mp 195–197 °C; ^{1}H NMR (DMSO-d_6) δ 2.28 (s, 6 H), 3.06 (s, 3 H), 3.22 (s, 3 H), 4.24 (s, 1 H), 6.86 (s, 2 H), 6.96 (s, 1 H), 10.44 (s, 1 H); high resolution EI-MS calcd for $C_{14}H_{17}N_3O_2$ 259.1321, found 259.1319.

3,6,8,10-Tetramethylisoalloxazine-5-oxide. 6-(*N*-Methyl-3,5-xylidino)-3-methyluracil (1.0 g, 3.66 mmol) and $NaNO_2$ (760 mg, 11.0 mmol) in CH_3COOH (5 mL) was stirred at room temperature for 5 min, and the orange precipitate was filtered off and washed with ethanol. Recrystallization from ethanol gave an orange powder of 3,6,8,10-tetramethylisoalloxazine-5-oxide (780 mg, 74.3%): dec 270–278 °C; ^{1}H NMR ($CDCl_3$) δ 2.53 (s, 3 H), 2.94 (s, 3 H), 3.43 (s, 3 H), 7.11 (s, 1 H), 7.29 (s, 1 H); IR ($CHCl_3$) ν 3000, 1690, 1650, 1545 cm^{-1}. Anal. Calcd for $C_{14}H_{14}N_4O_3$: C, 58.72; H, 4.93; N, 19.57. Found: C, 58.89; H, 4.80; N, 19.60.

3,6,8,10-Tetramethylisoalloxazine. A suspension of 3,6,8,10-tetramethylisoalloxazine-5-oxide (750 mg, 2.86 mmol) and $Na_2S_2O_4$ (750 mg, 4.31 mmol) in 20 mL of H_2O was stirred at room temperature for 6 h. The yellow precipitate was collected by filtration, washed thoroughly with H_2O, and recrystallized from ethanol to give 527 mg of 3,6,8,10-tetramethylisoalloxazine as yellow needles: mp >300 °C; ^{1}H NMR ($CDCl_3$) δ 2.59 (s, 3 H), 2.83 (s, 3 H), 3.51 (s, 3 H), 4.11 (s, 3 H), 7.27 (s, 1 H), 7.31 (s, 1 H); IR ($CHCl_3$) ν 3000, 1700, 1655, 1560 cm^{-1}. Anal. Calcd for $C_{14}H_{14}N_4O_2$: C, 62.21; H, 5.22; N, 20.73. Found: C, 62.01; H, 5.12; N, 20.80.

3,10-Dimethylisoalloxazine-6,8-dicarboxylic Acid (6). A solution of 3,6,8,10-tetramethylisoalloxazine (500 mg, 1.85 mmol) and $KMnO_4$ (2.2 g, 13.9 mmol) in 15 mL of 20% H_2SO_4 was stirred at room temperature for 2 days. Ethanol (10 mL) was added to the reaction mixture and neutralized with $NaHCO_3$. After the reaction mixture was filtered, the filtrate was concentrated in vacuo to 1/3 volume, followed by addition with concentrated HCl to give an orange powder. Recrystallization from H_2O gave 3,10-dimethylisoalloxazine-6,8-dicarboxylic acid (**6**) (225 mg, 41.1%) as orange needles: dec 259 °C; ^{1}H NMR (DMSO-d_6) δ 3.28 (s, 3 H), 4.05 (s, 3 H), 8.16 (s, 1 H), 8.39 (s, 1 H), 4–5 (br, 2 H). Anal. Calcd for $C_{14}H_{10}N_4O_6 \cdot {}^2/_3H_2O$: C, 49.13; H, 3.34; N, 16.87. Found: C, 49.14; H, 3.23; N, 16.48.

5-Bromo-6-(*N*-ethylamino)-3-methyluracil. Bromine (2 mL) was gradually added to a suspension of 6-(*N*-ethylamino)-3-methyluracil (6.00 g, 38.7 mmol) in 30 mL of methanol. After the reaction mixture was stirred at room temperature for 1 h, the pale yellow crystals were collected by filtration. Recrystallization from methanol gave colorless needles of 5-bromo-6-(*N*-ethylamino)-3-methyluracil (5.90 g, 61.5%): mp 208–210 °C; ^{1}H NMR (DMSO-d_6) δ 1.08 (t, J = 7.1 Hz, 3 H), 3.13 (s, 3 H), 3.35 (m, J = 7.1 Hz, 2 H), 6.71 (t, 1 H). Anal. Calcd for $C_7H_9N_3O_2Br$: C, 34.07; H, 3.94; N, 17.13; Br, 32.03. Found: C, 33.89; H, 4.06; N, 16.94; Br, 32.21.

6-(*N*-Ethylamino)-5-*N*-(*o*-toluidino)-3-methyluracil. *o*-Toluidine (17 mL) was added in a degassed dimethyl sulfoxide solution (65 mL) containing 5-bromo-6-(*N*-ethylamino)-3-methyluracil (3.97 g, 16 mmol), and the mixture was stirred at room temperature under argon for 7 days. A reaction mixture was poured into 0.05 N HCl, and the resulting precipitate was collected by filtration. The precipitate was a mixture (Scheme I). These products were used for the next step without further purification.

3,6-Dimethyl-10-ethylisoalloxazine. A solution of crude 6-(*N*-ethylamino-5-*o*-toluidyl-3-methyluracil (1.50 g) in DMF (5 mL) was heated at 100 °C under O_2 overnight. A reaction mixture was cooled to room temperature, and the resulting precipitate was collected by filtration. The filtrate was evaporated in vacuo to dryness, followed by addition of ethanol to give further products. Recrystallization from ethanol/chloroform gave 576 mg (38.3%) of 3,6-dimethyl-10-ethylisoalloxazine as yellow needles: mp >300 °C; ^{1}H NMR ($CDCl_3$) δ 1.50 (t, J = 7.1 Hz, 3 H), 2.89 (s, 3 H), 3.53 (s, 3 H), 4.80 (q, J = 7.1 Hz, 2 H), 7.47 (d,

J = 5.6 Hz, 1 H), 7.51 (d, J = 4.2 Hz, 1 H), 7.79 (dd, J = 5.6, 4.2 Hz, 1 H); IR ($CHCl_3$) ν 3000, 1705, 1650, 1560, 1350, 1180 cm^{-1}. Anal. Calcd for $C_{14}H_{14}N_4O_2$: C, 62.21; H, 5.22; N, 20.73. Found: C, 61.94; H, 5.20; N, 20.61.

10-Ethyl-3-methylisoalloxazine-6-carboxylic Acid (1). $KMnO_4$ (439 mg, 2.78 mmol) was added to a solution of 3,6-dimethyl-10-ethylisoalloxazine (250 mg, 0.926 mmol) in 20% H_2SO_4 (4 mL), and the reaction mixture was stirred under argon atmosphere for 2 days. After the reaction mixture was neutralized by adding $NaHCO_3$ and filtrated, the filtrate was concentrated in vacuo to 10 mL and concentrated HCl was added to give yellow solid. Recrystallization from CH_3COOH/H_2O gave 10-ethyl-3-methylisoalloxazine-6-carboxylic acid (**1**) as yellow powder: dec 289–291 °C; ^1H NMR (DMSO-d_6) δ 1.39 (t, J = 8.2 Hz, 3 H), 3.33 (s, 3 H), 4.76 (q, J = 8.2 Hz, 2 H), 7.7–8.2 (m, 4 H); high resolution EI-MS calcd for $C_{14}H_{12}N_4O_4$ 300.0840, found 300.0849. Anal. Calcd for $C_{14}H_{12}N_4O_4 \cdot {}^1/_2H_2O$: C, 54.37; H, 4.24; N, 18.72. Found: C, 54.57; H, 4.36; N, 18.07.

6-(*N*-Ethyl-3-toluidino)-3-methyluracil. A mixture of 6-chloro-3-methyluracil (3.0 g, 8.75 mmol) and *N*-ethyl-*m*-toluidine (6.34 g, 46.9 mmol) was heated at 150 °C. After 6-chloro-3-methyluracil melted completely, the reaction mixture was cooled to room temperature to give brownish gum. The gum was crystallized by addition with ethanol/ether, and the powder was collected by filtration. The collected powder was suspended in H_2O and filtrated again. Recrystallization from ethanol gave 2.43 g (50.0%) of 6-(*N*-ethyl-3-toluidino)-3-methyluracil as colorless plates: mp 142–145 °C; ^1H NMR (DMSO-d_6) δ 1.07 (t, J = 7.0 Hz, 3 H), 2.33 (s, 3 H), 3.05 (s, 3 H), 3.28 (q, J = 7.0 Hz, 2 H), 4.34 (s, 1 H), 7.04 (d, J = 8.8 Hz, 1 H), 7.18 (t, 1 H), 7.31 (s, 1 H), 7.37 (d, J = 8.8 Hz, 1 H), 10.46 (s, 1 H). Anal. Calcd for $C_{14}H_{17}N_3O_2$: C, 64.85; H, 6.61; N, 16.20. Found: C, 64.74; H, 6.59; N, 16.16.

3,8-Dimethyl-10-ethylisoalloxazine-5-oxide. A solution of 6-(*N*-ethyl-3-toluidino)-3-methyluracil (1.29 g, 4.98 mmol) in 10 mL of CH_3COOH was stirred at room temperature, and $NaNO_2$ (687 mg, 9.79 mmol) was added gradually. After the reaction mixture was stirred at room temperature for 20 min, the orange precipitate was filtered off, washed with ethanol. Recrystallization from ethanol/chloroform gave 936 mg (65.7%) of 3,8-dimethylisoalloxazine-5-oxide as orange plates: dec 214–215 °C; ^1H NMR ($CDCl_3$) δ 1.50 (t, J = 7.2 Hz, 3 H), 2.62 (s, 3 H), 3.44 (s, 3 H), 4.73 (q, J = 7.2 Hz, 2 H), 7.35 (d, J = 8.9 Hz, 1 H), 7.43 (s, 1 H), 8.40 (d, J = 8.9 Hz, 1 H); IR ($CHCl_3$) ν 3000, 1695, 1650, 1540, 1405, 1250, 1150 cm^{-1}. Anal. Calcd for $C_{14}H_{14}N_4O_3$: C, 58.73; H, 4.93; N, 19.57. Found: C, 58.80; H, 4.81; N, 19.56.

3,8-Dimethyl-10-ethylisoalloxazine. A suspension of 3,8-dimethylisoalloxazine-5-oxide (850 mg, 2.97 mmol) and $Na_2S_2O_4$ (776 mg, 4.46 mmol) in 10 mL of H_2O was stirred at room temperature for 30 min. The yellow precipitate was collected by filtration and recrystallized from ethanol/chloroform to give 800 mg (quantitative) of 3,8-dimethylisoalloxazine as orange needles: dec 258 °C; ^1H NMR ($CDCl_3$) δ 1.53 (t, J = 7.2 Hz, 3 H), 2.66 (s, 3 H), 3.53 (s, 3 H), 4.79 (q, J = 7.2 Hz, 2 H), 7.44 (s, 1 H), 7.46 (d, J = 7.1 Hz, 2 H), 8.22 (d, J = 7.1 Hz, 1 H); IR ($CHCl_3$) ν 3000, 1705, 1655, 1550, 1460, 1440, 1290, 1155, 1130 cm^{-1}; high resolution EI-MS calcd for $C_{14}H_{17}N_4O_2$ 270.1171, found 270.1121. Anal. Calcd for $C_{14}H_{17}N_4O_2$: C, 62.21; H, 5.22; N, 20.73. Found: C, 62.13; H, 5.26; N, 20.71.

10-Ethyl-3-methylisoalloxazine-8-carboxylic Acid (5). A solution of 3,8-dimethyl-10-ethylisoalloxazine (700 mg, 2.6 mmol) and $KMnO_4$ (1.64 g, 10.4 mmol) in 20% H_2SO_4 (10 mL) was stirred at room temperature under argon overnight. Ethanol (2 mL) was added to the reaction mixture and neutralized with $NaHCO_3$. After the reaction mixture was filtrated, the filtrate was concentrated in vacuo to 1/5 volume. Addition with concentrated HCl gave yellow powder, which was recrystallized from CH_3COOH/H_2O to give 601 mg (77.2%) of **5** as yellow powder: dec 280–288 °C; ^1H NMR (DMSO-d_6) δ 1.40 (t, J = 7.1 Hz, 3 H), 3.33 (s, 3 H), 4.74 (q, J = 7.3 Hz, 2 H), 8.11 (dd, J = 9.3, 1.0 Hz, 1 H), 8.24 (s, J = 9.3 Hz, 1 H), 8.34 (d, J = 1.0 Hz, 1 H); high resolution EI-MS calcd for $C_{14}H_{12}N_4O_4$ 300.0846, found 300.0852. Anal. Calcd for $C_{14}H_{12}N_4O_4 \cdot H_2O$: C, 52.83; H, 4.43; N, 17.60. Found: C, 52.83; H, 4.44; N, 17.41.