Functionalized Flavin Receptors. Regulation of Redox Properties of 6-Azaflavin via Hydrogen Bondings with Melamine Derivatives Bearing Guanidinium Ion(s) in Organic Solvents

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Melamine derivatives bearing guanidinium ion(s) were synthesized as a flavin receptor using hydrogen bonds. The receptors bind 6-aza-10-dodecylisoalloxazine (6-azaflavin, 6-AzaFl) quite strongly via five or seven hydrogen bonds in $CHCl_3$ or $CHCl_3$−MeCN. Redox potentials of 6-AzaFl were considerably affected by hydrogen bondings of the receptors (positive shift: $\Delta E_{1/2} = 220-317$ mV). Anionic semiquinone radical of 6-AzaFl was found to be stabilized by hydrogen bondings with a melamine derivative bearing an *N*-phenylguanidinium ion or two guanidinium ions. Effects of the hydrogen bondings on the oxidation activity of 6-AzaFl were kinetically investigated for the oxidation of *N*-benzyl-1,4-dihydronicotinamide (BNAH) and PhSH in $CHCl_3$−MeCN under anaerobic conditions. It was found that the N(1)−hydrogen bonding facilitates a slight BNAH oxidation (<10-fold) and the N(5)−hydrogen bonding remarkably accelerates the rate of PhSH oxidation (10^3-10^4-fold).

Flavin coenzymes such as FMN and FAD exhibit diverse redox functions through interactions with apoproteins. For construction of artificial enzymes,[1] it would be of importance to introduce apoprotein functions into the catalytic systems. Among them, hydrogen bonding to an isoalloxazine ring is undoubtedly one of the factors to regulate the functions of flavin coenzymes. In fact, Massey et al. have proposed that N(1)−hydrogen bonding activates the N(5) and C(10a) positions of an isoalloxazine ring and N(5)−hydrogen bonding activates the C(4a) position.[2] Nishimoto et al. have reported, on the basis of molecular orbital calculations, that hydrogen bondings involving N(1), C(2)═O, N(3)−H, C(4)═O, and N(5) positions are the most effective for lowering LUMO energy levels.[3] Furthermore, it is known that an intramolecularly hydrogen-bonded flavin at the N(5) position facilitates the reactions proceeding via a nucleophilic attack at the C(4a) position,[4] and the anionic semiquinone radical of a flavin-6-carboxylate is stabilized by intramolecular N(5)−hydrogen bonding even in aqueous solution.[4b] The other strategy to investigate hydrogen bondings on the redox properties of flavin may be use of a flavin receptor using hydrogen bondings. If functional groups are introduced into the receptor, it would be possible to assemble the functional groups in the vicinity of the flavin. In other words, such a receptor could be regarded to exhibit an apoprotein function. In addition, a noncovalent molecular assembly using hydrogen bondings is also of interest from the viewpoint of molecular recognition.[5]

Receptor molecules for coenzyme models have been little reported,[6] although many coenzyme models covalently connected with a substrate-binding site such as a cyclodextrin, a cyclophane, and a crown ether are known.[1a,7] We and others reported that 2,6-diamidopyridine derivatives, exploited as a thymine receptor by Hamilton,[8] are able to bind an isoalloxazine ring via three hydrogen bonds at C(2)═O, N(3)−H, and C(4)═O positions in $CHCl_3$.[9] However, the binding constant is quite small ($\sim 10^2$ M^{-1} in $CHCl_3$)[9d] due to three alternate hydrogen bonds proposed by Jorgensen.[10] Although the three hydrogen bonds are known to shift the redox potential of the flavin to a positive direction,[9c] the oxidation activity is little affected by these hydrogen bondings in $CHCl_3$.[9b] To design efficient functional flavin receptors, we considered the following: (i) the number and arrangement of hydrogen-bonding sites, (ii) acidity

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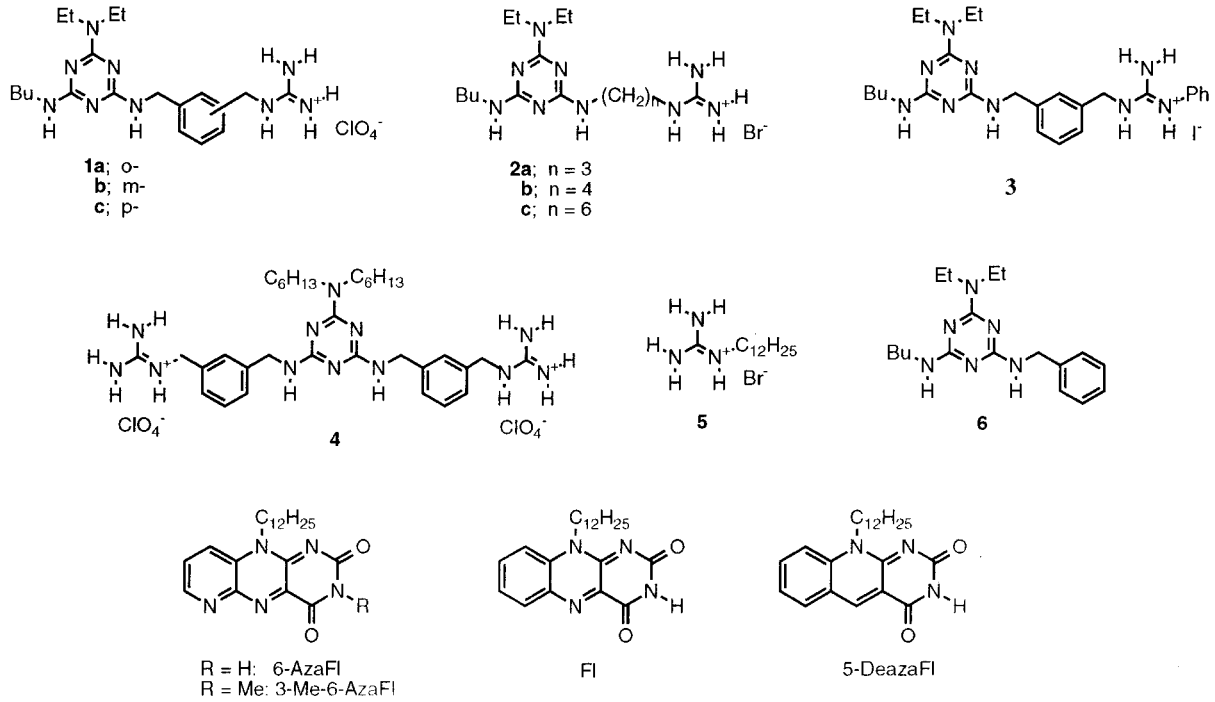
Chart 1



of H-donors, and (iii) synthetic simplicity for introduction of plural functional groups. In addition, an oxidation-active flavin model is required to examine its oxidation activity in organic solvents. We chose a combination of 6-AzaFl and melamine derivatives bearing guanidinium ion(s), since 6-AzaFl is an oxidation-active flavin model,[11] in which the N(6) atom can be used as another hydrogen-bonding site. In this paper, we report synthesis of receptors, their binding ability for flavins in $CHCl_3$ or $CHCl_3$−MeCN, and the effects of the hydrogen bondings on redox potentials, stability of anionic radical, and oxidation activity of 6-AzaFl.[12] The flavin models and receptors employed are shown in Chart 1.

Results and Discussion

Synthesis of Receptors. There is a drawback in 2,6-diamidopyridine derivatives as a flavin receptor. Namely, 2-alkanoylamido-6-benzoylamidopyridine shows a considerable steric hindrance between *o*-H of the phenyl group and the carbonyl oxygen of an isoalloxazine ring for cmplexation via three hydrogen bonds at C(2)═O, N(3)−H, and C(4)═O (K_a = 0−10 M^{-1} in $CHCl_3$) due to a planarity requirement of benzoyl amide moiety.[9b] This is a restriction on the design of functionalized receptors using a 2,6-aminopyridine moiety. Thus, we chose a melamine skeleton as the sites of three hydrogen bonds. To examine the effects of the hydrogen bondings involving N(1) and N(5) positions, we also chose guanidinium ion(s) as additional H-bonding groups. We considered that pK_a of a guanidinium ion (pK_a 12−13)[13] may allow us to examine H-bonding effects on flavin reactivity for base-promoted reactions. Melamine derivatives bearing guanidinium ion(s) employed were synthesized according to the routes outlined in Scheme 1. Namely, the melamine skeletons were prepared by stepwise substitution of cyanuric chloride with the corresponding amines,[14] and guanidinium ions were introduced by the reactions of the corresponding amines with S-alkylthiouronium halides.[15] Purification was performed by column chromatography and/or recrystallization.

Synthesis of Flavin Models. 6-Azaflavins were synthesized by condensation of 2-amino-3-dodecylaminopyridine[16] and alloxan monohydrate as shown in Scheme 2. 10-Dodecylisoalloxazine (Fl)[9b] and 5-deaza-10-dodecylisoalloxazine (5-DeazaFl) were synthesized by the methods of Yoneda.[17] Dodecyl groups were employed to increase solubility of the flavins in $CHCl_3$.

Binding Constants. Binding behavior of the receptors (**1b**, **2b**, and **3**) for 6-AzaFl was first examined by ^{1}H NMR spectroscopy in $CDCl_3$. As typical examples, ^{1}H NMR spectra of 6-AzaFl, **1b**, **3**, and respective 1:1 mixture of 6-AzaFl and **1b** or **3** were shown in Figure 1.

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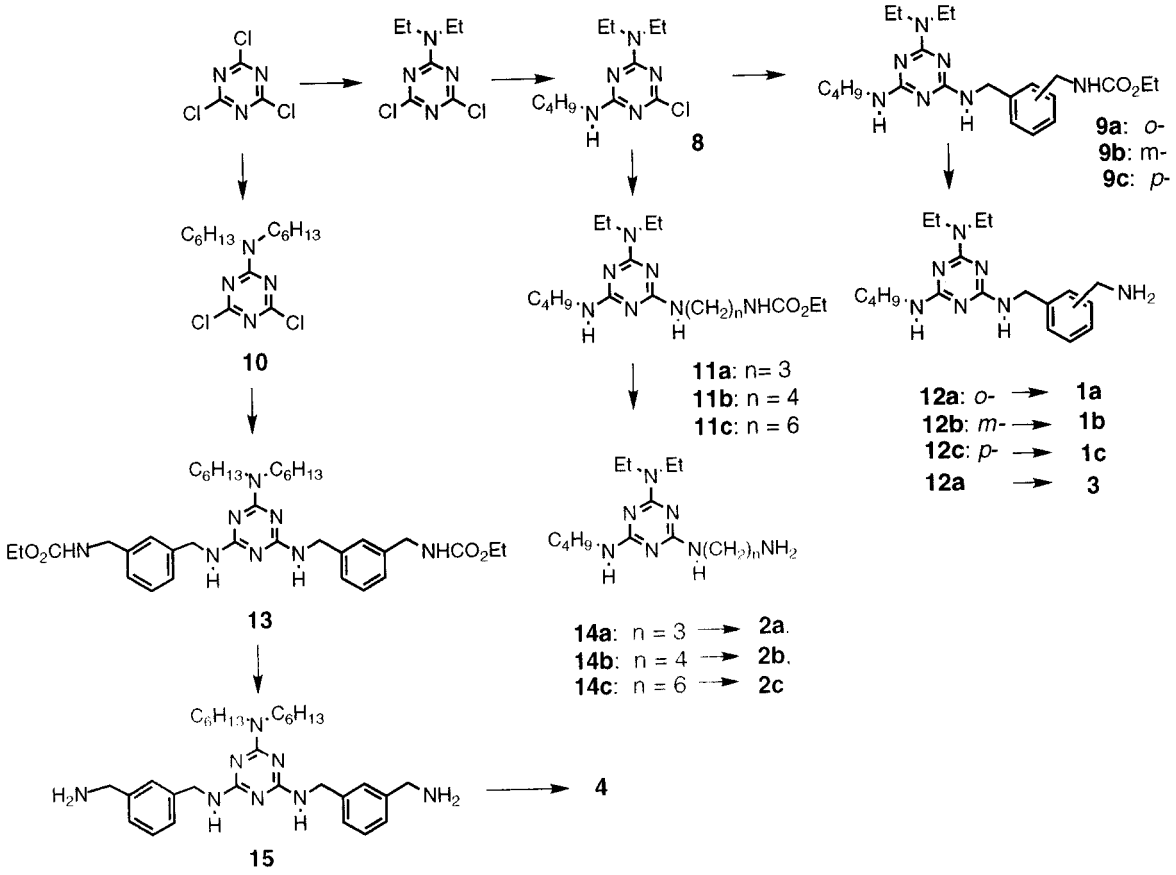
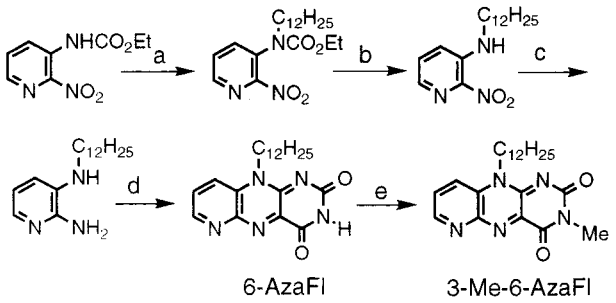
Scheme 1. Synthetic Routes of Receptors



Scheme 2. Synthetic Routes of 6-Azaflavins[a]



[a] Reagents and conditions: (a) $C_{12}H_{25}Br$, K_2CO_3, CH_3COCH_3, reflux, 79%; (b) KOH, H_2O-EtOH, reflux, 20%; (c) H_2, Pd/C, EtOH, 90%; (d) alloxan hydrate, H_3BO_3, AcOH, reflux, 43%; (e) CH_3I, Cs_2CO_3, DMF, rt, 40%.

Chemical shifts of all the N−H's of the receptors in the 1:1 mixtures shifted downfield, whereas such shifts were not observed when 3-Me-6-AzaFl was used, indicating that the N−H protons are involved in hydrogen bondings for complexation. It should be noted that C(7)−H of 6-AzaFl shows considerable upfield shift by complexation with **3**, which will be discussed later.

The complex formation of 6-azaflavins with the receptors was also examined by electronic absorption spectroscopy in $CHCl_3$. The absorption spectrum of 6-AzaFl was found to change through isosbestic points upon addition of the receptors except for **5** and **6**.[12a] It should be noted that such a spectral change was not observed for 3-Me-6-AzaFl under the same conditions. By employing **1b**, **2**, and **4**, the stoichiometry of the complex formation was examined by Job plots using the absorption changes of 6-AzaFl at 489 nm. As shown in Figure 2, all the Job plots showed maxima at a mole fraction of 0.5, indicating 1:1 complex formation. Structures of the complexes can be depicted as shown in Figure 3.

The binding constants (K_a) were spectrophotometrically determined by following the absorption changes at 489 nm upon addition of the receptors.[12a] Since the absorption spectrum of 6-AzaFl was not changed upon addition of **5** or **6**, the binding constants of 6-AzaFl·**5** and 6-AzaFl·**6** were determined by Stern−Volmer plots for fluorescence quenching of 6-AzaFl in the presence of the receptors.[9b] The binding constants and free energy changes except **4** were summarized in Table 1. The K_a values are dependent on the structures of the receptors and the flavins. The K_a values of **1** are in the order of 6-AzaFl > Fl > 5-DeazaFl, indicating that the guanidinium ion of **1** participates in hydrogen bondings at N(5) and N(6) positions. The K_a values of Fl·**1** and 5-DeazaFl·**1** are larger than those of Fl·**6** and 5-DeazaFl·**6**, suggesting that the guanidinium ion of **1** is involved in the hydrogen bonding at N(1) or N(5) for the former and N(1) for the latter. Furthermore, the larger K_a of 6-AzaFl·**5** than that of Fl·**5** implies that the guanidinium ion forms hydrogen bonds at N(5) and N(6) positions of 6-AzaFl. The K_a values of **6** for 6-AzaFl, Fl, and 5-DeazaFl are almost same because of the same mode of three hydrogen bonds at C(2)=O, N(3)−H, and C(4)=O. For binding of 6-AzaFl, the K_a values of **1** are in the order of **1b** > **1a** > **1c**, and those of **2** are in the order of **2b** > **2a** > **2c**. From CPK model construction, this may be explained by structure and/or entropy factors for the complexes formation. It is worthy to note that K_a of 6-AzaFl·**3** is much smaller than that of 6-AzaFl·**1b** (1/380). This order is unusual, since a more acidic H donor is known to form stronger

Table 1. Binding Constants (K_a) and Free Energy Changes (ΔG) for Complexation of Flavins and Receptors in $CHCl_3$[a]

	6-AzaFl		Fl		5-DeazaFl	
receptor	K_a (M^{-1})	ΔG (kcal mol^{-1})	K_a (M^{-1})	ΔG (kcal mol^{-1})	K_a (M^{-1})	ΔG (kcal mol^{-1})
1a	$2.8 (\pm 0.2) \times 10^4$ [a]	-6.1 ± 0.4	$1.9 (\pm 0.2) \times 10^3$ [c]	-4.4 ± 0.1	$1.2 (\pm 0.1) \times 10^3$ [c]	-4.1 ± 0.0
1b	$1.4 (\pm 0.1) \times 10^5$ [a]	-7.0 ± 0.0	$2.0 (\pm 0.1) \times 10^3$ [c]	-4.4 ± 0.0	$8.4 (\pm 0.1) \times 10^2$ [c]	-3.9 ± 0.0
1c	$1.8 (\pm 0.2) \times 10^4$ [a]	-5.8 ± 0.1	$4.0 (\pm 0.4) \times 10^2$ [c]	-3.5 ± 0.1	$3.0 (\pm 0.2) \times 10^2$ [c]	-3.3 ± 0.0
2a	$2.9 (\pm 0.1) \times 10^4$ [a]	-6.1 ± 0.0	*b*		*b*	
2b	$6.3 (\pm 0.3) \times 10^4$ [a]	-6.5 ± 0.0	*b*		*b*	
2c	$1.3 (\pm 0.1) \times 10^4$ [a]	-5.6 ± 0.0	*b*		*b*	
3	$5.3 (\pm 0.3) \times 10^3$ [a]	-5.1 ± 0.0	*b*		*b*	
5	$1.0 (\pm 0.2) \times 10^2$ [c]	-2.7 ± 0.1	$1.7 (\pm 0.1) \times 10$[c]	-1.6 ± 0.0	*b*	
6	$1.4 (\pm 0.0) \times 10^2$ [c]	-2.9 ± 0.0	$1.5 (\pm 0.2) \times 10^2$ [d]	-3.0 ± 0.0	$1.3 \pm 0.0 \times 10^2$ [c]	-2.8 ± 0.0

[a] By UV−vis spectroscopy, [flavin] = 5.0×10^{-5} M, 25 °C. [b] Not determined. [c] By fluorescence spectroscopy, [flavin] = 1.0×10^{-5} M, 20 °C. [d] By ^{1}H NMR spectroscopy, [**Fl**] = 2.5×10^{-3} M, $CDCl_3$, 25 °C.

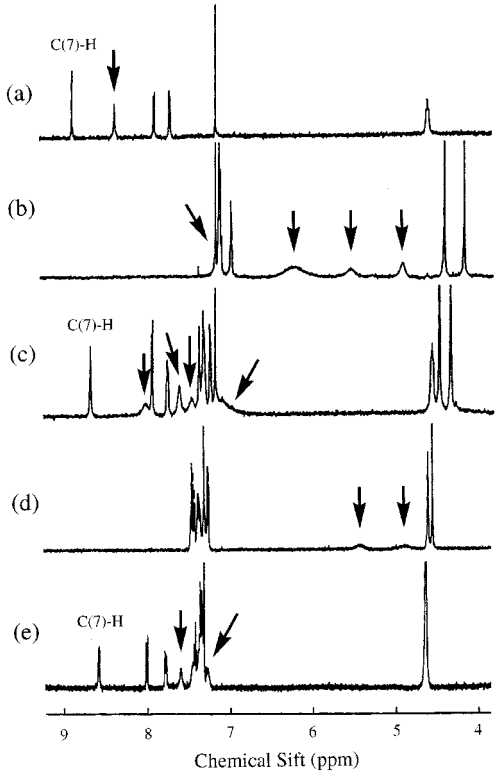


Figure 1. ^{1}H NMR spectra (500 MHz) of (a) 6-AzaFl, (b) **1b**, (c) a 1:1 mixture of 6-AzaFl and **1b**, (d) **3**, and (e) a 1:1 mixture of 6-AzaFl and **3** in $CDCl_3$ at 25 °C. [6-AzaFl] = [**1b** or **3**] = 2.5×10^{-3} M. Arrows indicate N−H protons.

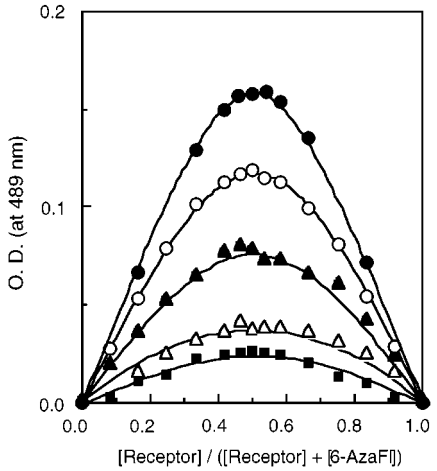


Figure 2. Job plots for complexation of 6-Azafl and receptors. [6-AzaFl] + [**1b**, **2** or **4**] = 1.0×10^{-4} M in $CHCl_3$. For **4**, $CHCl_3$−MeCN (10%) was used as the solvent. ●: **1b**. △: **2a**. ○: **2b**. △: **2c**. □: **4**.

hydrogen bond.[18] To clarify this, the following were studied. (a) pK_a of **3**: The pK_a of the guanidinium ion of **3** was spectrophotometrically determined by pH titration in aqueous buffer solution containing 20% MeCN. From the plot of absorption changes of **3** at 280 nm vs pH (Figure 4), pK_a was determined to be 10.7, which is smaller than that of conventional guanidinium ions such as **1** by 2−3 pK_a units.[13] (b) Thermodynamic parameters: The thermodynamic parameters (ΔH and $T\Delta S$) for the complex formation of 6-AzaFl·**1b** and 6-AzaFl·**3** were determined by temperature dependence on K_a values (Figure 5). The thermodynamic parameters are listed in Table 2, indicating that the complex formation is mainly controlled by the enthalpy term rather than the entropy term. (c) ^{1}H NMR study: As can be seen in Figure 1, the chemical shift of C(7)−H of 6-AzaFl shifts to upfield upon addition of the receptors. The chemical shifts of C(7)−H were plotted against the concentrations of the receptors (**1b** and **3**) (Figure 6), indicating that the change of the chemical shifts is much larger for **3** than **1b**. This suggests that C(7)−H is situated in a position close enough to feel the ring current of the *N*-phenyl ring of **3** due to the steric hindrance between C(7)−H and the *o*-H of *N*-phenyl ring. The smaller K_a of 6-AzaFl·**3** than that of 6-AzaFl·**1b** is explained by steric hindrance of the *N*-phenyl group of **3** for complexation. This information may be useful for design of the functionalized flavin receptors.

The binding constant of 6-AzaFl·**4** was determined in $CHCl_3$ containing MeCN because of insolubility of **4** in $CHCl_3$. The K_a values are listed in Table 3 together with those of **1b**. The larger K_a values of **4** than those of **1b** suggest that both guanidinium moieties of **4** are involved in the hydrogen bondings for complex formation as shown in Figure 3b. With increasing MeCN content, however, the K_a values of **1b** and **4** become close, suggesting that the hydrogen bonds at N(5) and N(6) are stronger than those at N(1) and C(2)═O.

Attempt To Isolate Hydrogen-Bonded Complexes. Isolation of hydrogen-bonded complexes (6-AzaFl·**1b** and 6-AzaFl·**3**) was attempted by spontaneous evaporation of solvents (CH_2Cl_2, $CHCl_3$, CH_3COCH_3, and THF) or vapor diffusion of hexane or ether into the mixture of 6-AzaFl and the receptors in the solvent in a closed box.

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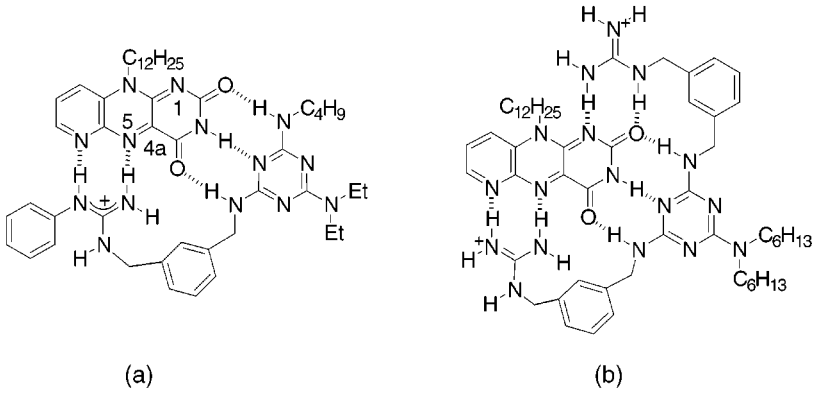


Figure 3. Hydrogen-bonded molecular complexes: (a) 6-AzaFl·**1b** and (6) 6-AzaFl·**4**.

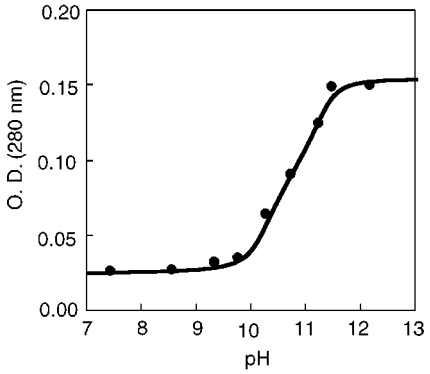


Figure 4. Spectroscopic pH titration of **3** at 280 nm. [**3**] = 1.0×10^{-4} M, buffer (0.1 M, $I = 0.3$ with KCl), MeCN (20%), 25 °C.

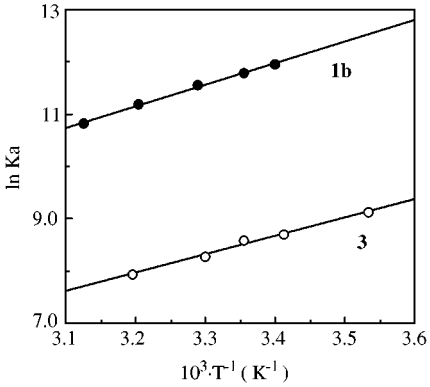


Figure 5. Temperature dependency of K_a values of 6-AzaFL·**1b** and 6-AzaFl·**3**.

Table 2. Thermodynamic Parameters for Formation of 6-AzaFl·1b and 6-AzaFl·3 in $CHCl_3$

receptor	ΔG_{298} (kcal mol^{-1})	ΔH (kcal mol^{-1})	$T\Delta S_{298}$ (kcal mol^{-1})
1b	−7.0	−8.2	−1.2
3	−5.1	−7.0	−1.9

In both the cases, the complexes were isolated as powders. The 1:1 stoichiometry was confirmed with MS (FAB and ESI) and elemental analysis for 6-AzaFl·**1b** and with ESI MS for 6-AzaFl·**3**. However, single crystals for X-ray crystallographic analysis have not yet been obtained.

Redox Potentials. Redox potentials of organic molecules such as quinones and 1,8-naphthalimide are known to shift to a positive direction by hydrogen bonding.[19] Rotello et al. reported that redox potentials

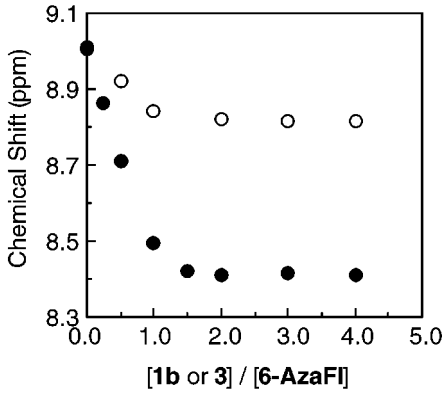


Figure 6. Plots of chemical shifts of C(7)-H of 6-AzaFl in the presence of **1b** or **3**. [6-AzaFl] = 2.5×10^{-3} M, $CHCl_3$, 25 °C. ○: **1b**. ●: **3**.

Table 3. K_a Values in 1b and 4 for 6-AzaFl in $CHCl_3$−MeCN

	K (M^{-1})	
$CHCl_3$ (%, v/v)	**1b**	**4**
100	$1.4 (\pm 0.1) \times 10^5$	*a*
90	$1.8 (\pm 0.5) \times 10^4$	$3.8 (\pm 0.6) \times 10^4$
80	$1.3 (\pm 0.3) \times 10^4$	$1.9 (\pm 0.2) \times 10^4$
50	$6.2 (\pm 0.2) \times 10^3$	$6.5 (\pm 0.8) \times 10^3$

[a] Not determined due to insolubility of **4**.

of the conventional flavin model shift to a positive direction by three hydrogen bonds at C(2)=O, N(3)−H, and C(4)=O of the isoalloxazine ring in CH_2Cl_2.[9d] Furthermore, they have reported that the redox waves are attributable to one-electron redox couple to form an anionic radical by spectroelectrochemical technique.[20]

Redox potentials of 6-AzaFl were determined by cyclic voltammometry (CV) in CH_2Cl_2 and CH_2Cl_2−MeCN (20%) in the presence of the receptors (**1b**, **3**, and **4**). Cyclic voltammograms of 6-AzaFl and 3-Me-6-AzaFl showed reversible one-electron couples [$E_{1/2} = -971$ mV (vs ferrocene) for 6-AzaFl, −1095 mV for 3-Me-6-AzaFl] in the absence of the receptor in CH_2Cl_2. Cyclic voltammograms of 6-AzaFl in the presence of receptors (**1b** and **3**) are shown in Figure 7. New peaks appeared in reduction and oxidation waves with decrease of original peak current, and the original waves disappeared to give

(19) Ge, Y.; Lilienthal,; Smith, D. K. *J. Am. Chem. Soc.* **1996**, *118*, 3976−3977. (b) Guota, N.; Linschitz. *J. Am. Chem. Soc.* **1997**, *119*, 6384−6391.

(20) Niemz, A.; Imbriglio, J.; Rotello, V. M.*J. Am. Chem. Soc.* **1997**, *119*, 887−892.

Table 4. Redox Potentials (vs Ferrocene) of 6-AzaFl in the Presence of Receptors

	in CH_2Cl_2			in CH_2Cl_2−MeCN (20% v/v)		
receptor	$E_{1/2}$[a] (mV)	$\Delta E_{1/2}$ (mV)	$\Delta\Delta G$ (kcal mol^{-1})	$E_{1/2}$[b] (mV)	$\Delta E_{1/2}$ (mV)	$\Delta\Delta G$ (kcal mol^{-1})
none	−971	0	0	−926	0	0
1b	−757	+204	−4.7	−706	+220	−5.2
3	−738	+233	−5.4	−674	+252	−5.8
4				−609	+317	−7.3

[a] [6-AzaFl] = 1.0×10^{-3} M, [**1b**] = 3.0×10^{-3} M, [**3**] = 5.0×10^{-3} M. [b] [6-AzaFl] = 5.0×10^{-4} M, [**1b**] = 2.5×10^{-3} M, [**3**] = 5.0×10^{-3} M, [**3**] = 1.5×10^{-3} M.

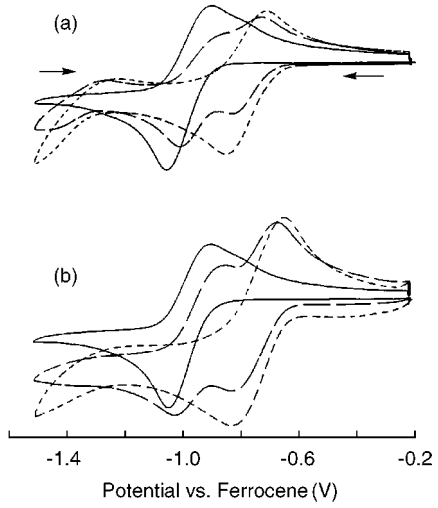
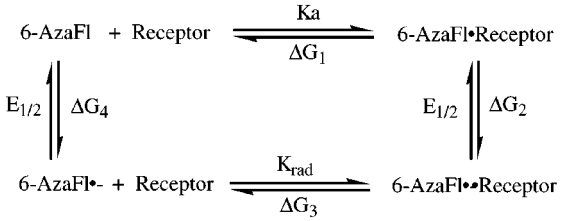


Figure 7. Cyclic voltammograms of 6-AzaFl (1.0×10^{-3} M) in CH_2Cl_2. Part a: —, [**1b**] = 0; — —, [**1b**] = 5.0×10^{-4} M; - - -, [**1b**] = 1.0×10^{-3} M. Part b: —, [**3**] = 0; — —, [**3**] = 1.0×10^{-3} M; ---, [**3**] = 2.0×10^{-3} M.

Scheme 3. Thermodynamic Cycle



new redox couples with increasing concentrations of the receptors. The new waves are attributable to redox couples of 6-AzaFl·**1b** and 6-AzaFl·**3**, respectively. Because of solubility problem of **4** in CH_2Cl_2, MeCN was used as a cosolvent. Similar experiments were carried out in CH_2Cl_2−MeCN (20% v/v). The redox potentials and free energy changes are summarized in Table 4. The redox potential of 6-AzaFl is considerably affected by hydrogen bondings of the receptors. Namely, the ability of the receptors to shift the redox potential to a positive direction is in the order of **4** > **3** > **1b**, and the anionic semiquinone radical (6-AzaFl$^{\bullet-}$) is stabilized by 7.3, 5.8, and 5.2 kcal mol^{-1}, respectively.

From the thermodynamic cycle as shown in Scheme 3, the binding constants (K_{rad}) of the receptors for the anionic semiquinone radical (6-AzaFl$^{\bullet-}$) can be calculated by using $\Delta G_1 + \Delta G_2 = \Delta G_3 + \Delta G_4$ and the Nernst equation. As shown in Table 5, the K_{rad} values are much larger than K_a values by factors of 5.4×10^3 for **1b**, 2.4×10^4 for **3**, and 2.5×10^5 for **4**. Such markedly different binding abilities due to an electron going in and out would be useful for design of electrochemically controlled molecular switches.[21] It should be noted that K_{rad} values

Table 5. Calculated ΔG_3 and K_{rad} in CH_2Cl_2−MeCN (20%)

receptor	K_a (M^{-1})	$E_{1/2}$ (mV)	ΔG_3 (kcal mol^{-1})	K_{rad} (M^{-1})	K_{rad}/K_a
none		−926			
1b (5 equiv)	$1.3 (\pm 0.3) \times 10^4$	−706	−10.7	7.0×10^7	5.4×10^3
3 (10 equiv)	$5.1 (\pm 0.5) \times 10^3$	−674	−11.0	1.2×10^8	2.4×10^4
4 (3 equiv)	$1.9 (\pm 0.2) \times 10^4$	−609	−13.2	4.8×10^9	2.5×10^5

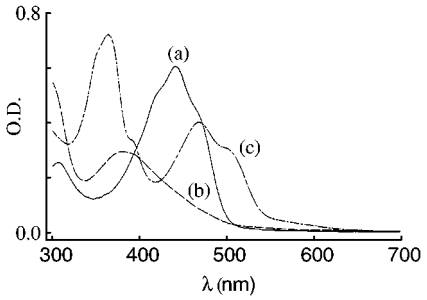


Figure 8. Absorption spectra of 6-AzaFl in the reaction with PhSH: [6-AzaFl] = 5.0×10^{-5} M [PhSH] = [Bu_3N] = 2.0×10^{-4} M, [**4**] = 1.0×10^{-4} M, $CHCl_3$−MeCN (20%), N_2, 25 °C. (a) Oxidized form. (b) Reduced form. (c) Anionic semiquinone radical.

are in the order of **4** > **3** > **1b**, whereas K_a values are in the order of **4** > **1b** > **3**, suggesting that the steric hindrance is compensated by the stronger hydrogen bonding.

Spectroscopic Detection of the Anionic Semiquinone Radical. A free flavin radical is known to be quite unstable to disproportionate to the oxidized and reduced flavins in aqueous solution.[22] As seen in flavodoxins, however, semiquinone radicals of flavins are generally stable when bound to apoproteins.[23] Hydrogen bonding is suggested to be responsible for stabilization of anionic semiquinone radicals in flavoproteins.[24]

Formation of a semiquinone radical of 6-AzaFl was examined spectrophotometrically by employing the oxidation of thiols [PhSH or dithiothreitol (DTT)] and BNAH in $CHCl_3$ for **1b** and **3** and in $CHCl_3$−MeCN (20%) for **4** under anaerobic conditions. As shown in Figure 8, in the presence of **1b** or a mixture of **5** and **6**, the absorption spectrum of 6-AzaFl (spectrum a) was changed to that of 2e-reduced 6-AzaFl (spectrum b). On the other hand, spectrum c was observed in the presence of **3**, suggesting formation of the anionic semiquinone radical of 6-AzaFl.[25] The stability of the radical was dependent on the amount

(21) Boal, A. K.; Rotello, V. M. *J. Am. Chem. Soc.* **1999**, *121*, 4914−4915.

(22) Ehrenberg, A.; Muller, F.; Hemmerich, P. *Eur. J. Biochem.* **1967**, *2*, 286−293.

(23) (a) Mayhew, S. G.; Ludwig, M. L. In *The Enzymes*, 3rd ed.; Boyer, P. D., Ed.; Academic Press: New York, 1975; Vol. XII, pp 57−109. (b) Edmondson, D. E.; Tollin, G. In *Topics in Current Chemistry*; Boschke, F. L., Ed.; Springer-Verlag: Berlin, 1983; Vol. 108, pp 109−138.

(24) Muller, F.; Hemmerich, P.; Ehrenberg, A.; Palmer. G.; Massey, V. *Eur. J. Biochem.* **1970**, *14*, 185−196.

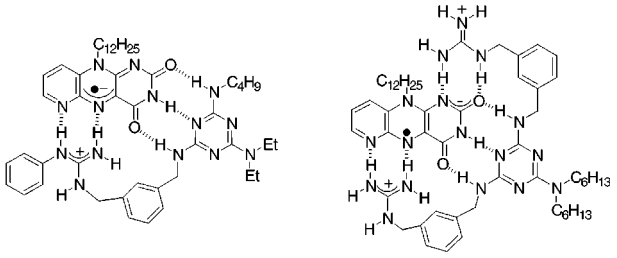


Figure 9. Anionic semiquinone radical stabilized by hydrogen bondings with **3** and **4**.

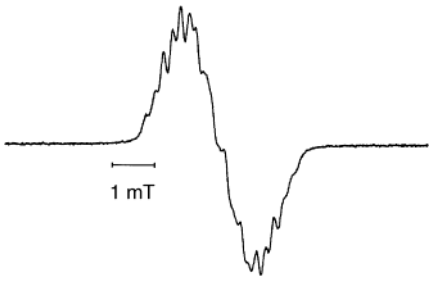


Figure 10. EPR spectrum of 6-AzaFl$^{\bullet-}$ generated by the reaction with DTT. [6-AzaFl] = 5.0×10^{-3} M, [DTT] = [Bu_3N] = 5.0×10^{-3} M, [**3**] = 5.0×10^{-5} M, $CHCl_3$, N_2, 25 °C.

of the substrate. With a large excess of thiols, spectrum c changed to that of the reduced 6-AzaFl (spectrum b). Spectrum b was found to give spectrum c by O_2 bubbling only in the presence of **3**. These observations suggest that the radical is formed by comproportionation of the oxidized 6-AzaFl and the reduced 6-AzaFl. Similar spectral changes were also observed in the presence of **4** in $CHCl_3$−MeCN (20%). However, when EtOH or MeCN was used as the solvent, such spectral behavior was not observed, giving the spectrum of the reduced 6-AzaFl (spectrum b). In the case of 3-Me-6-AzaFl, the spectrum of the radical was not observed in the presence of **3** or **4**, only giving the spectrum of the reduced 3-Me-6-AzaFl. These spectral observations suggest that an anionic radical of 6-AzaFl is stabilized by N(5)−hydrogen bonding of acidic H bond donor or by both N(1)− and N(5)− hydrogen bondings as shown in Figure 9.

Formation of radical species during the reaction of 6-AzaFl with DTT was examined by EPR spectroscopy. Namely the reaction mixture of 6-AzaFl and DTT with Bu_3N in $CHCl_3$ in an EPR tube at N_2 atmosphere gave the EPR spectrum as shown in Figure 10. Although hyperfine lines could not be obtained, a *g* value of 2.0040 is in reasonable agreement with those obtained for other flavin radicals.

Many flavoenzymes are known to give stable flavin radicals in dithionite reduction and EDTA photoreduction, and N(5)−hydrogen bonding has been proposed to stabilize the radicals.[23] It should be noted that the pK_a of the anionic radical of 6-AzaFl is assumed to be smaller than that of the anionic semiquinone radical of riboflavin (pK_a = 8.3 in H_2O)[26] due to the electron-withdrawing ability of the N(6) ring nitrogen. For the stabilization of the anionic semiquinone radical, the pK_a of the H donor would be important. It is the first example that intermolecular hydrogen bonds of receptor molecules are able

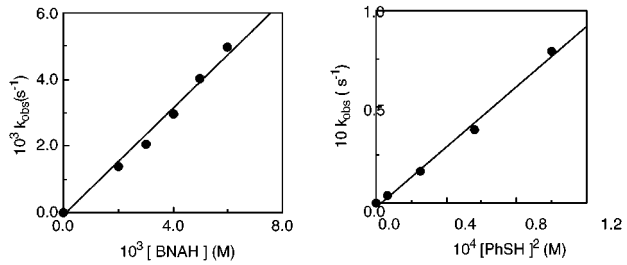


Figure 11. Concentration effects of substrates on the rates. [3-Me-6-AzaFl] = 5.0×10^{-5} M, $CHCl_3$−MeCN (20%), N_2, 25 °C. For PhSH oxidation, a base ([Bu_3N] = 1.5×10^{-2} M) was added.

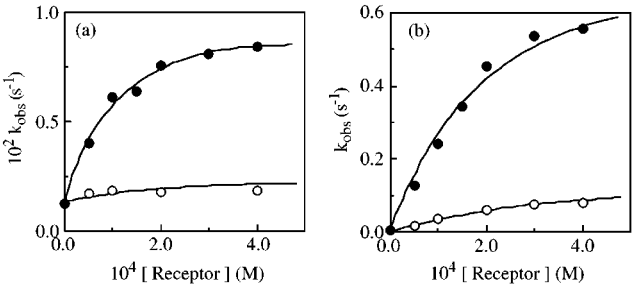


Figure 12. Concentration effects of receptors on the rates of the oxidation of BNAH and PhSH: [6-AzaFl] = 5.0×10^{-5} M, $CHCl_3$−MeCN (20%), N_2, 25 °C; (a) BNAH] = 2.0×10^{-3} M; (b) [PhSH] = 2.0×10^{-3} M, [Bu_3N] = 1.0×10^{-2} M. ○: **1b**. ●: **4**.

to stabilize the anionic semiquinone radical of a flavin model in the presence of reducing reagents such as BNAH and thiols. More importantly, the present results give information for understanding of the amino acid residues at the active sites of flavodoxins.

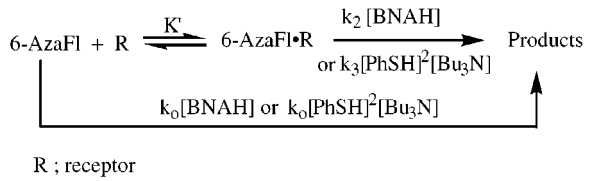
Effects of Hydrogen Bonding on the Oxidation Activity. It is known that intramolecular hydrogen bonding at the N(5) position of an isoalloxazine ring facilitates the reaction involving a nucleophilic attack at the C(4a) position.[4] Thus, the effect of the receptors (**1b** and **4**) on the rates of the oxidation of BNAH and PhSH by 6-AzaFl was kinetically examined in $CHCl_3$−MeCN (20%), since the oxidation mechanisms are well established in aqueous solutions.[27] Namely, the former oxidation proceeds via a hydride (or its equivalent) attack at N(5), and the latter proceeds through a nucleophilic attack of a thiol anion at C(4a) to form C(4a) adduct, followed by a nucleophilic attack of the second thiol anion to give 2e-reduced flavin and disulfide. Pseudo-first-order rate constants (k_{obs}) were spectrophotometrically determined by following the absorption decreases of 6-AzaFl at 440 nm under anaerobic conditions. First, the concentration effect of the substrates was examined by employing 3-Me-6-AzaFl. The rates were confirmed to be first order with respect to [BNAH], second order with [PhSH] as shown in Figure 11, and first-order with [Bu_3N] (not shown), suggesting that the oxidation proceeds via the mechanisms established in aqueous solutions.

Effects of the concentrations of the receptors on the rates were shown in Figure 12. For BNAH oxidation,

(25) (a) Massey, V.; Palmer, G. *Biochemistry* **1966**, *10*, 3181−3189. (b) Steenkamp, D. J.; Gallup, M. *J. Biol. Chem*. **1978**, *253*, 4086−4089.
(26) Meisel, D.; Neta, O. *J. Phys. Chem*. **1975**, *79*, 2459−2461.

(27) For NADH model oxidation: (a) Powell, M. F.; Wong, W. H.; Bruice, T. C. *Proc. Natl. Acad. Sci. U.S.A*. **1982**, *79*, 4604−4608. (b) Powell, M. F.; Bruice, T. C. *J. Am. Chem. Soc*. **1983**, *105*, 1014−1021. For thiol oxidation: (c) Loechler, E. L.; Hollocher, T. C. *J. Am. Chem. Soc*. **1980**, *102*, 7312−7321, 7322−7327.

Scheme 4. Reaction Scheme



R ; receptor

Table 6. Computed Binding Constants (*K*′) and Rate Constants

substrate		1b	4
BNAH	K' (M^{-1})	1.0×10^4	1.7×10^4
	k_2 ($M^{-1}\cdot s^{-1}$)	1.3	6.4
	k_2/k_o[a]	1.6	8.0
PhSH	K' (M^{-1})	3.7×10^3	4.5×10^3
	k_3 ($M^{-3}\cdot s^{-1}$)	1.0×10^8	4.7×10^8
	k_3/k_o[b]	1.7×10^3	7.8×10^3

[a] $k_o = 0.08\ M^{-1}\ s^{-1}$ for BNAH. [b] $k_o = 6.0 \times 10^4\ M^{-3}\ s^{-1}$ for PhSH.

severalfold rate enhancements (k_2/k_o) were observed in the presence of **4**, whereas almost no rate acceleration was observed for **1b**. This indicates that the N(1) hydrogen bonding is responsible for the rate acceleration. On the other hand, considerably large rate accelerations were observed for PhSH oxidation in the presence of the receptors, although a little larger for **4**. The kinetic analysis was performed by assuming the reaction scheme as shown in Scheme 4, which allows us to derive the rate equation as shown eq 1.

$$\text{rate} = k_{obs}[\text{6-AzaFl}]_o = k_o([\text{6-AzaFl}]_o - [\text{6-AzaFl}\cdot\text{R}])[\text{S}]_o + k_2[\text{6-AzaFl}\cdot\text{R}][\text{S}]_o$$

$$k_{obs} = [[k_o[\text{6-AzaFl}]_o + (k_2 - k_o)[\text{6-AzaFl}\cdot\text{R}]][\text{S}]_o]/[\text{6-AzaFl}]_o \quad (1)$$

where

$$[\text{6-AzaFl}\cdot\text{R}] = (K'[\text{6-AzaFl}]_o + K'[\text{R}]_o + 1)/2K' - [[([\text{6-AzaFl}]_o^2 - 2[\text{6-AzaFl}]_o[\text{R}]_o)K'^2 + 2K'([\text{6-AzaFl}]_o + [\text{R}]_o) + 1]^{1/2}]/2K'$$

$[\text{6-AzaFl}]_o$ and $[\text{R}]_o$ represent the initial concentrations of 6-AzaFl and receptor and $[\text{S}]_o$ corresponds to $[\text{BNAH}]_o$ or $[\text{PhSH}]_o^2[\text{Bu}_3\text{N}]_o$. For PhSH oxidation, k_2 is replaced by k_3.

The nonlinear curve fitting of the experimental data with eq 1 gave the computed binding constants (K') and rate constants (k_2 and k_3) as shown in Table 6. The binding constants (K') kinetically obtained for BNAH oxidation ($1.0 \times 10^4\ M^{-1}$ for **1b** and $1.7 \times 10^4\ M^{-1}$ for **4**) are in fairly good agreement with those determined spectrophotometrically ($1.3 \times 10^4\ M^{-1}$ for **1b** and $1.9 \times 10^4\ M^{-1}$ for **4**). Meanwhile, the binding constant of 6-AzaFl•**4** was found to decrease considerably in the presence of Bu_3N. Namely, the K_a values were $1.9 \times 10^4\ M^{-1}$ ($[Bu_3N] = 0$), $7.4 \times 10^3\ M^{-1}$ ($[Bu_3N] = 5.0 \times 10^{-3}$ M), 4.1×10^3 M ($[Bu_3N] = 1.0 \times 10^{-2}$ M), and 2.3×10^3 M ($[Bu_3N] = 1.5 \times 10^{-2}$ M). Thus, the smaller computed K' values for PhSH oxidation could be explained by the presence of Bu_3N. The rate accelerations (k_2/k_o) for BNAH oxidation are 1.6-fold for **1b** and 8-fold for **4**, indicating that N(5)− and N(6)−hydrogen bonding affects little the rate but N(1)−hydrogen bonding is more responsible for BNAH oxidation, although the effect is small. It should be noted that three hydrogen bonds at C(2)═O, N(3)−H, and C(4)═O do not affect the rate for BNAH oxidation.[9b] For PhSH oxidation, the rate accelerations (k_3/k_o) are 1.7 $\times 10^3$-fold for **1b** and 7.8 $\times 10^3$-fold for **4**, respectively. The larger rate acceleration for **4** can be explained by the N(5)−hydrogen bonding, which facilitates a nucleophilic attack of PhS^- at the C(4a) position and N(1)−hydrogen bonding facilitates a nucleophilic attack of second PhS^- at the sulfur atom of the C(4a) adduct. These results would be useful for understanding of the roles of H bonds seen in X-ray crystallographic data of flavoenzymes.[28]

Conclusions

Melamine derivatives bearing guanidinium ion(s) were found to act as flavin receptors that strongly bind 6-azaflavin through five or seven hydrogen bonds in $CHCl_3$ or $CHCl_3$−MeCN. The redox properties of 6-azaflavin were found to be remarkably affected with the receptors. Namely, redox potential of 6-azaflavin is shifted to a positive direction (220−317 mV) with the receptors. An anionic semiquinone radical of 6-azaflavin is stabilized by N(5)−hydrogen bonding of acidic H-donor or by both N(1)− and N(5)−hydrogen bondings of the receptors. The N(1)−hydrogen bonding facilitates the reaction at the N(5) position, and N(5)−hydrogen bonding remarkably accelerates the rate of the oxidation involving a nucleophilic attack at the C(4a) position. Moreover, since functional groups such as substrate-binding and metal-binding moieties can be introduced into the melamine derivatives, a more sophisticated flavin model system may be constructed. The present study provides fundamental experimental data for understanding roles of hydrogen bondings in redox properties of a flavin, and roles of amino acids residues in active sites of flavoenzymes. Furthermore, the present study demonstrates that apoprotein functions would be introduced into flavin model systems by employing functionalized receptor molecules via noncovalent bonds. The investigations in this line are in progress in our laboratory.

Experimental Section

^{1}H NMR (200 or 500 MHz) spectra were recorded in $CDCl_3$ with tetramethylsilane as internal standard. Melting points are uncorrected. Flash column chromatography was performed by using Wakogel C-200 (silica gel, 70−150 μm, Wako). Elemental analyses were performed at the Center of Instrumental Analysis of Gunma University. Special grade chloroform (Kanto Chemicals) was used without further purification. Acetonitrile and DMF were purified by distillation from calcium hydride.

Synthesis of Receptors. The receptors were prepared according to the routes outlined in Scheme 1. 2,4-Dichloro-6-diethylamino-*s*-triazine was prepared according to literature procedures.[14a]

2-Butylamino-4-chloro-6-diethylamino-*s*-triazine (8). A mixture of the above triazine (7.0 g, 31.7 mmol), butylamine (3.2 mL, 32 mmol), and K_2CO_3 (4.36 g, 31.7 mmol) in dioxane (50 mL) was stirred at 40 °C for 8 h. After evaporation of solvent in vacuo, water was added and the mixture extracted

(28) (a) Burnett, R. M.; Darling, G. D.; Kendall, D. S.; LeQuesune, M. E.; Mayhew, S. S.; Smith, W. W.; Ludwig, M. L. *J. Biol. Chem.* **1974**, *249*, 4383−4392. (b) Watenpaugh, K. D.; Sieker, L. C.; Jensen, L. H. *Proc. Natl. Acad. Sci. U.S.A.* **1973**, *69*, 3857−3860. (c) Vrielink, A.; Lloyd, L. F.; Blow, D. M. *J. Med. Biol.* **1991**, *219*, 533−554. (d) Hecht, H. J.; Kalisz, H. M.; Hendle, J.; Schmid, R. D.; Schornburg, D. *J. Med. Biol.* **1993**, *229*, 153−172.

with CH_2Cl_2. After the residue was dried over $MgSO_4$, CH_2Cl_2 was evaporated, and the residue was recrystallized from EtOH to give colorless needle crystals: yield 7.9 g (97%); mp 99−101 °C; ^{1}H NMR (200 MHz) δ (ppm) 0.93 (t, J = 7.0 Hz, 3H), 1.18 (t, J = 7.0 Hz, 6H), 1.30−1.60 (m, 4H), 3.38 (q, J = 6.2 Hz, 2H), 3.57 (q, J = 7.0 Hz, 4H), 5.15−5.27 (br s, 1H).

2-Butylamino-4-diethylamino-6-(2-ethoxycarbonylaminomethylbenzylamino)-*s*-triazine (9a). A mixture of **8** (3.10 g, 12 mmol), 2-ethoxycarbonylaminomethylbenzylamine (2.70 g, 12 mmol), and K_2CO_3 (1.82 g, 12 mmol) in dioxane (70 mL) was refluxed overnight. After the solvent was evaporated in vacuo, water was added and the mixture extracted with CH_2Cl_2. After the residue was dried over $MgSO_4$, CH_2Cl_2 was evaporated to dryness. The residue was purified by dry column chromatography (SiO_2, CH_2Cl_2/CH_3COCH_3= 7:1): oil; yield 3.5 g (69%); ^{1}H NMR (200 MHz) δ (ppm) 0.92 (t, J = 7.04 Hz, 3H), 1.12 (t, J = 7.24 Hz, 6H), 1.23 (t, J = 7.20 Hz, 3H), 1.30−1.60 (m, 4H), 3.32 (q, J = 6.20 Hz, 2H), 3.50 (q, J = 6.96, 4H), 4.15 (q, J = 7.20 Hz, 2H), 4.44 (d, J = 5.78 Hz, 2H), 4.63 (d, J = 5.86 Hz, 2H), 5.14 (br s, 3H), 7.22−7.37 (m, 4H).

2-Butylamino-4-diethylamino-6-(3-ethoxycarbonylaminomethylbenzylamino)-s-triazine (9b): colorless powders (88%); ^{1}H NMR (500 Hz) δ (ppm) 0.92 (t, J = 7.3 Hz, 3H), 1.05−1.09 (m, 6H), 1.25 (t, J = 7.05 Hz, 3H), 1.33−1.40, 1.49−1.55 (m, 4H), 3.34 (q, J = 6.40 Hz, 2H), 3.43−3.57 (m, 4H), 4.14 (q, J = 7.35 Hz, 2H), 4.33 (d, J = 5.8 Hz, 2H), 4.54 (d, J = 4.3 Hz, 2H), 4.74, 5.04, 5.13 (br s, 3H), 7.14−7.29 (m, 4H). Anal. Calcd for $C_{22}H_{35}N_7O_2$: C, 61.51; H, 8.21; N, 22.82. Found: C, 61.34; H, 8.22; N, 22.63. 3-Ethoxycarbonylaminomethylbenzylamine was prepared by reaction with *m*-xylylenediamine and ethyl chloroformate in diethyl ether. Purification was performed by column chromatography (CH_2Cl_2−MeOH = 15:1): ^{1}H NMR (200 MHz) δ 1.26 (t, J = 7.20 Hz, 3H), 3.87 (s, 2H), 4.14 (q, J = 7.20 MHz, 2H), 4.39 (d, J = 6.0 Hz,2H), 4.85−5.05 (br s, 1H), 7.16−7.36 (m, 4H).

2-Butylamino-4-diethylamino-6-(4-ethoxycarbonylaminomethylbenzylamino)-*s*-triazine (9c): oil (58%); ^{1}H NMR (200 MHz) δ (ppm) 0.88 (t, J = 7.16 Hz, 3H), 1.11 (t, J = 6.96 Hz, 6H), 1.25 (t, J = 7.12 Hz, 3H), 1.30−1.60 (m, 4H), 3.36 (q, J = 6.20 Hz, 2H), 3.50 (q, J = 6.96 Hz, 4H), 4.13 (q, J = 7.12 Hz, 2H), 4.36 (d, J = 5.94 Hz, 2H), 4.57 (d, J = 5.86 Hz, 2H), 4.72, 5.05 (br s, 3H), 7.25−7.28 (m, 4H).

2-Butylamino-4-diethylamino-6-(2-aminomethylbenzylamino)-*s*-triazine (12a) was obtained by alkaline hydrolysis of **9a**. A solution of EtOH (150 mL)−3 M KOH (100 mL) containing **12a** (4.5 g, 10.5 mmol) was refluxed for 48 h. After the solvent was evaporated in vacuo, water was added and the mixture extracted with CH_2Cl_2. After the residue was dried over $MgSO_4$, CH_2Cl_2 was evaporated to dryness to afford an oily product (TLC one spot): oil; yield 3.98 g (100%); ^{1}H NMR (200 MHz) δ (ppm) 0.92 (t, J = 7.28 Hz, 3H), 1.14 (t, J = 7.08 Hz, 6H), 1.30−1.60 (m, 4H), 3.36 (q, J = 6,40 Hz, 2H), 3.52 (q, J = 7.08 Hz, 4H), 3.95 (s, 2H), 4, 65 (d, J = 5.74 Hz, 2H), 4.67, 5.52 (br s, 2H), 7.22−7.40 (m, 4H).

2-Butylamino-4-diethylamino-6-(3-aminomethylbenzylamino)-s-triazine (12b) was obtained similarly: oil (100%); ^{1}H NMR (200 MHz$_3$) δ (ppm) 0.92 (t, J = 7.12 Hz, 3H), 1.12 (t, J = 7.0 Hz, 6H), 1.30−1.60 (m, 4H), 3.33 (q, J = 6.5 Hz, 2H), 3.51 (q, J = 7.0 Hz, 4H), 3.85 (s, 2H), 4.59 (d, J = 4.3 Hz, 2H), 4.67, 4.97 (br s, 2H), 7.16−7.32 (m, 4H).

2-Butylamino-4-diethylamino-6-(4-aminomethylbenzylamino)-*s*-triazine (12c): oil (94%); ^{1}H NMR (200 MHz) δ (ppm) 0.92 (t, J = 7.26 Hz, 3H), 1.12 (t, J = 6.96 Hz, 6H), 1.30−1.60 (m, 4H), 3.33 (q, J = 6.50 Hz, 2H), 3.51 (q, J = 6.96 Hz, 4H), 3.85 (s, 2H), 4.57 (d, J = 5.86 Hz, 2H), 4.77, 5.08 (br s, 2H), 7.27−7.29 (m, 4H). Compounds **12** were used without purification.

Compounds **1** were prepared by the reaction of **12** with *S*-ethylthiouronium bromide[15c] in ethanol.

1a. A solution of **12a** (0.80 g, 1.9 mmol) and *S*-ethylthiouronium bromide (0.35 g, 1.9 mmol) in EtOH (20 mL) was stirred under reflux for 3 d. After EtOH was evaporated, the residue was washed with diethyl ether, dissolved in water (10 mL), and filtered. To the solution was added excess sodium perchlorate. The precipitate was collected by filtration and recrystallized from $CHCl_3$−diethyl ether: yield 0.47 g (50%); mp 150−153 °C; ^{1}H NMR (500 MHz) δ (ppm) 0.91 (t, J = 7.00 Hz, 3H), 1.16 (t, J = 7.00 Hz, 6H), 1.28−1.36, 1.43−1.54 (m, 4H), 3.29 (t, J = 7.20 Hz, 2H), 3.50 (q, J = 6.70 Hz, 4H), 4.44 (s, 2H), 4.51 (s, 2H), 5.25 (br s, 2H), 6.52 (br s, 4H), 7.20−7.35 (m, 5H). Anal. Calcd for $C_{30}H_{20}N_9O_4Cl$: C, 48.04; H, 6.85; N, 25.21. Found: C, 47.82; H, 6.72; N, 24.90.

1b: yield 75%; mp 128−130 °C (H_2O); ^{1}H NMR (500 MHz) δ (ppm) 0.88 (t, J = 7.35 Hz, 3H), 1.10 (t, J = 6.70 Hz, 6H), 1.26−1.32, 1.43−1.69 (m, 4H), 3.26 (t, J = 7.30 Hz, 2H), 3.50 (q, J = 6.70 Hz, 4H), 4.22 (s, 2H), 4.47 (s, 2H), 4.97, 5.61 (br s, 2H), 6.32 (br s, 4H), 7.06−7.26 (m, 5H). Anal. Calcd for $C_{30}H_{20}N_9O_4Cl$: C, 48.07; H, 6.86; N, 25.24. Found: C, 48.29; H, 6.74; N, 25.05.

1c: yield 53%; mp 117−120 °C ($CHCl_3$−diethyl ether); ^{1}H NMR (500 MHz) δ (ppm) 0.87 (t, J = 7.35 Hz, 3H), 1.12 (t, J = 6.70 Hz, 6H),1.26−1.34, 1.43−1.73 (m, 4H), 3.27 (t, J = 7.30 Hz, 2H), 3,49 (q, J = 6.70 Hz, 4H), 4.19 (s, 2H), 4.41 (s, 2H), 5.46, 5.93 (br s, 2H), 6.17 (br s, 4H), 7.13−7.18 (m, 5H). Anal. Calcd for $C_{30}H_{20}N_9O_4Cl\cdot 3H_2O$: C, 43.36; H, 7.28; N, 22.75. Found: C, 43.38; H, 7.24; N, 22.94.

Compounds **2** were synthesized from **8** and α,ω-alkanediamines (propane, butane, hexane), followed by the reaction of *S*-ethylisothiuoronium bromide as described for **1**. Since purification of **14** was difficult, **14** was reacted with ethyl chloroformate and purified by flash column chromatography (CH_2Cl_2/EtOAc = 1:1). Hydrolysis and introduction of a guanidinium ion were performed in an essentially same manner as described for **1**. Purification of **2** was performed by repeated precipitation from $CHCl_3$ and diethyl ether.

2-Butylamino-4-(3-*N*-ethoxycarbonyaminopropylamino)-6-diethylamino-*s*-triazine (11a). A mixture of **8** (3.00 g, 11.7 mmol), 1,3-diaminopropane (1.0 mL, 11.5 mmol), and K_2CO_3 (1.50 g, 11.7 mmol) in dioxane (30 mL)−H_2O (20 mL) was refluxed overnight. After evaporation of the solvent, H_2O was added and the mixture extracted with CH_2Cl_2. After the residue was dried over $MgSO_4$, the solvent was evaporated. To the residue were added diethyl ether (50 mL), K_2CO_3 (1.23 g, 8.88 mmol), and ethyl chloroformate (0.9 mL, 9.4 mmol) and the mixture stirred at 0 °C for 1 h and at room temperature for 6 h. After evaporation of the solvent, H_2O was added and the mixture extracted with CH_2Cl_2. After the CH_2Cl_2 layer was dried over $MgSO_4$, the residue was purified by dry column chromatography (SiO_2, CH_2Cl_2−EtOAc = 1:1) to give a colorless oil: yield 1.52 g (46%); ^{1}H NMR (200 MHz) δ (ppm) 0.93 (t, J = 7.24 Hz, 3H), 1.15 (t,, J = 7.16 Hz, 6H), 1.24 (t, J = 7.20 Hz, 3H), 1.30−1.59 (m, 4H), 1.60−1.73 (m, 2H), 3.21 (m. 2H), 3.30−3.48 (m, 4H), 3.52 (t, J = 7.16 Hz, 4H), 4.13 (q, J = 7.20 Hz, 2H), 4.83 (br s, 3H).

2-Butylamino-4-(4-*N*-ethoxycarbonylaminobutylamino)-6-diethylamino-*s*-triazine (11b): yield 42%; ^{1}H NMR (200 MHz) δ (ppm) 0.92 (t, J = 7.24 Hz, 3H), 1.14 (t, J = 7.04 Hz, 6H,), 1.24 (t, J = 7.22 Hz, 3H),1.30−1.60 (m, 8H), 3.20 (m, 2H), 3.29−3.43 (m, 4H), 3.51 (t, J = 7.04 Hz, 4H), 4.14 (q, J = 7.22 Hz, 2H), 4.70 (br s, 3H).

2-Butylamino-4-(6-*N*-ethoxycarbonylaminohexylamino)-6-diethylamino-*s*-triazine (11c): yield 14%; ^{1}H NMR (200 MHz) δ (ppm) 0.93 (t,, J = 7.5 Hz, 1.16 (t, J = 7.25 Hz, 6H), 1.25 (m, 3H), 1.39 (m, 6H), 1.46−1.57 (m, 6H), 3.17−3.38 (m, 6H), 3.48−3.58 (m, 4H), 4.11 (m, 2H), 4.77 (br s, 3H).

Compounds **14** were obtained by hydrolysis of **11** and used as crude products. Namely, a solution of EtOH (50 mL)−3 M KOH (30 mL) containing **11a** (1.52 g, 4.08 mmol) was refluxed for 4 days. After evaporation of the solvent, H_2O was added to the residue and the mixture extracted with CH_2Cl_2. After the residue was dried over $MgSO_4$, the solvent was distilled in vacuo. To this crude product (**11a**) was added *S*-ethylthiouronium bromide (0.77 g, 4.15 mmol) in EtOH (15 mL), and the solution was refluxed for 2 days. The solvent was distilled in vacuo to give a white solid. This was purified by repeated reprecipitation from $CHCl_3$ and diethyl ether.

2a: yield 0.23 g (13%); mp 141−142 °C; ^{1}H NMR (500 MHz) δ (ppm) 0.89 (t, J = 7.5 Hz, 3H), 1.16 (t, J = 8.75 Hz, 6H), 1.35 (m, 2H), 1.50 (m, 2H), 1.84 (br s, 2H), 3.38 (br s, 6H), 3.47 (m, 4H), 5.49−5.82 (br s, 2H), 6.92−7.07 (br s, 4H), 7.62

(br s, 1H). Anal. Calcd for $C_{15}H_{32}BrN_9 \cdot H_2O$: C, 41.28; H, 7.85; N, 28.89. Found: C, 41.25; H, 7.51; N, 29.10

2b: yield 26%; mp 138−141 °C; ^{1}H NMR (500 MHz) δ (ppm) 0.90 (t, J = 7.5 Hz, 3H), 1.14 (t, J = 7.5 Hz, 6H), 1.34 (m, 2H), 1.51 (m, 2H), 1.64 (br s, 4H), 3.30 (br s, 6H), 3.51 (m, 4H), 5.41−5.79 (br s, 2H), 6.78−7.09 (br s, 4H), 7.58 (br s, 1H). Anal. Calcd for $C_{16}H_{34}BrN_9 \cdot 0.5H_2O$; C, 43.53; H. 7.99; N, 28.55. Found: C, 43.80; H, 7.72; N, 28.40.

2c: yield 22%; white wax; ^{1}H NMR (500 MHz) δ (ppm) 0.89 (t, J = 7.5 Hz, 3H), 1.16 (t, J = 8.75 Hz, 6H), 1.35 (m, 2H), 1.50 (m, 2H), 1.84 (br s, 8H), 3.38 (br s, 6H), 3.47 (m, 4H), 5.49−5.82 (br s, 2H), 6.92−7.07 (br s, 4H), 7.62 (br s, 1H). Anal. Calcd for $C_{18}H_{38}BrN_9 \cdot 2H_2O$: C, 43.54; H, 8.53; N, 25.39. Found: C, 43.40; H, 8.28; N, 25.50.

Receptor **3** was prepared as follows. A stirring solution of **12a** (1.67 g, 4. 66 mmol) and *S*-methyl-*N*-phenylthiouronium iodide[15] (1.37 g, 4.66 mmol) in *t*-BuOH (50 mL) was refluxed for 3 days. After evaporation of *t*-BuOH in vacuo, the residue was washed with diethyl ether and recrystallized from MeOH−diethyl ether: yield 1.52 g (54%); mp 178−9 °C; ^{1}H NMR (500 MHz) δ (ppm) 0.91 (t, J = 7.30 Hz, 3H), 1.10 (m, 6H), 1.33−1.38 (m, 2H), 1.50−1.53 (m, 2H), 3.31 (t, J = 7.00 Hz, 2H), 3.50 (m, 4H), 4.51 (s, 2H), 4.57 (s, 2H), 7.20−7.41 (m, 13H). Anal. Calcd for $C_{26}H_{38}IN_9$: C, 51.74; H, 6.35; N, 20.89. Found: C, 51.69; H, 6.10; N, 21.24

Receptor **4** was prepared in a manner essentially similar to that of **1**.

2,4-Dichloro-6-dihexyl-amino-*s*-triazine (10): To a solution of cyanuric chloride (10.3 g, 55.8 mmol) and K_2CO_3 (7.70 g, 55. 8 mmol) in dioxane (100 mL)−H_2O (50 mL) was added dihexylamine (11.6 mL, 55.8 mmol) dropwise at 0 °C, and the solution was stirred at 0 °C for 1 h and at room temperature for 2 h. After workup, purification was performed by dry column chromatography (CH_2Cl_2): yield 18.0 g (97%); colorless oil; ^{1}H NMR (500 MHz) δ (ppm) 0.90 (t, J = 7.50 Hz, 6H), 1.26 (m, 12H), 1.51 (br s, 4H), 3.42 (t, J = 6.25 Hz, 4H). Anal. Calcd for $C_{15}H_{26}N_4Cl_2$: C, 54.05; H, 7.86; N, 16.81. Found: C, 54.18; H, 7.88; N, 16.73.

2-Dihexylamino-2,4-bis(3-*N*-ethoxycarbonyamino-methyl)benzylamino-*s*-triazine (13) was obtained by reacting **10** (2.47 g, 7.41 mmol) with *N*-ethoxycarbonyl-*m*-xylylenediamine (3.09 g, 14.8 mmol) in dioxane (50 mL) under reflux. After workup, crude product was purified by flash column chromatography (CH_2Cl_2−acetone = 8:1) to give colorless crystals, which were recrystallized from EtOAc−hexane: yield 3.51 g (70%); mp 118 °C; ^{1}H NMR (200 MHz) δ (ppm) 0.85 (t, J = 6.76 Hz, 6H), 1.20−1.30 (m, 12H), 1.25 (t, J = 7.16 Hz), 1.40−1.60 (m, 4H), 3.42 (t, J = 7.40 Hz, 4H), 4.12 (q, J = 7.16 Hz, 4H), 4.34 (d, J = 6.02 Hz, 4H), 4.57 (d, J = 5.94 Hz, 4H), 4.95, 5.07 (br s, 4H), 7.12−7.32 (m, 8H).

2-Dihexylamino-4,6-bis(3-aminomethyl)benzylamino-*s*-triazine (15) was obtained by alkaline hydrolysis of **13** (2.0 g, 2.96 mmol) in 3 M KOH (20 mL)−EtOH (30 mL) under reflux for 2 days. After workup, the crude product was purified by recrystallization from EtOAc−hexane: yield 1.54 g (98%); mp 85−8 °C; ^{1}H NMR (200 MHz) δ (ppm) 0.86 (t, J = 6.72 Hz, 6H), 1.20−1.30 (m, 12H), 1.45−1.70 (m, 4H), 3.43 (t, J = 7.32 Hz, 4H), 3.84 (d, J = 6.02 Hz, 4H), 4.59 (d, J = 5.90 Hz, 4H), 5.04 (br s, 4H), 7.15−7.32 (m, 8H).

Receptor **4** was obtained by reaction of **15** (1.543 g, 2.87 mmol) with *S*-ethylthiouronium bromide (1.13 g, 5.74 mmol) in EtOH (30 mL) under reflux for 2 days. After evaporation of the solvent, the residue was washed with diethyl ether, dissolved in H_2O (10 mL), and filtered. To this solution was added excess $NaClO_4$. The precipitates formed were collected by filtration, washed well with H_2O, and recrystallized from $CHCl_3$−acetone: yield 1.40 g (82%); mp 124−127 °C; ^{1}H NMR (200 MHz, $CDCl_3$−DMSO-d_6) δ (ppm) 0.87 (t, J = 6.74 Hz, 6H), 1.20−1.30 (m, 12H), 1.40−1.60 (m, 4H), 3.42 (t, J = 7.44 Hz, 4H), 4.34 (d, J = 4.6 Hz, 4H), 4.55 (d, J = 5.12 Hz, 4H), 6.04−7.10 (br s, 2H), 6.78 (br s, 8H), 7.22−7.33 (m, 8H), 7.60 (br s, 8H). 7.10−7.55 (m, 10H). Anal. Calcd for $C_{33}H_{54}Cl_2N_{12}O_8 \cdot H_2O$: C, 47.42; H, 6.75; N, 20.11. Found: C, 47.44; H, 6.57; N, 19.62.

10-Dodecylisoalloxazine (**Fl**) was supplied from our previous study.[9b]

5-Deaza-10-dodecylisoalloxazine (5-DeazaFl). A mixture of 6-(*N*-dodecylanilino)uracil (1.0 g, 2.9 mmol) and phosphorus oxychloride (0.8 mL, 8.7 mmol) in DMF (3.3 mL) was stirred at 90 °C for 3 h.[29] After cooling, water (50 mL) was added and the mixture neutralized with $NaHCO_3$. The yellow crystals were collected by filtration, washed with water, and recrystallized from EtOH: yield 0.84 g (76%); mp 201−202 °C; ^{1}H NMR (500 MHz) δ (ppm) 0.88 (t, J = 6.7 Hz, 3H), 1.09−1.18 (m, 18H), 1.86 (m, 2H), 4.88 (m, 2H), 7.51 (t, J = 7.95 Hz, 1H), 7.68 (d, J = 8.85 Hz, 1H), 7.89−7.94 (m, 2H), 8.37 (br s, 1H), 8.92 (s, 1H); UV−vis ($CHCl_3$) λ_{max} 400.9 nm (log ϵ; 4.1). Anal. Calcd for $C_{23}H_{31}N_3O_2 \cdot 0.5H_2O$: C, 70.74; H, 8.26; N, 10.76. Found: C, 70.91; H, 8.05; N; 10.64.

6-Aza-10-dodecylisoalloxazine was prepared by condensation of 2-amino-3-dodecylaminopyridine and alloxan monohydrate. 2-Amino-3-dodecylaminopyridine was prepared according to the literature procedures described for 2-amino-3-methylaminopyridine.[14] Namely, 3-dodecylamino-2-nitropyridine (0.8 g, 2.6 mmol) in EtOH (5 mL) was reduced with an H_2 balloon in a catalytic amount of 10% Pd/C at room temperature. After filtration of the catalyst, the solvent was evaporated to dryness. The residue was purified by flash column chromatography (CH_2Cl_2−MeOH = 20:1) to give colorless crystals: yield 0.65 g (90%); mp 75−76 °C;. ^{1}H NMR (200 MHz); δ (ppm) 0.88 (t, J = 6.9 Hz, 3H), 1.2−1.8 (m, 20H), 3.07 (t, J = 7.1 Hz, 2H), 4.17 (br s, 2H), 6.6−6.7 (m, 1H), 6.81 (dd, 1H, J = 7.7, 1.7 Hz), 7.59 (dd, 1H, J = 4.9, 1.6 Hz).

6-Aza-10-dodecylisoalloxazine (6-AzaFl). A stirred solution of 2-amino-3-dodecylaminopyridine (0.60 g, 2.1 mmol), alloxan monohydrate (0.31 g, 2.1 mmol), and H_3BO_3 (0.13 g, 2.1 mmol) in acetic acid (2 mL) was refluxed for 2 h. The solvent was removed in vacuo, and the residue was dissolved in CH_2Cl_2 (50 mL) and washed with water. The CH_2Cl_2 layer was dried over $MgSO_4$ and removed in vacuo. The crude product was recrystallized from EtOH to give yellow crystals: yield 0.35 g (43%); mp > 250 °C dec; ^{1}H NMR (500 MHz) δ (ppm) 0.88 (t, J = 5.0 Hz, 3H), 1.2−1.4 (m, 18H), 1.86 (m, 2H), 4.67 (m, 2H), 7.81−7.83 (m, 1H), 8.01−8.02 (m, 1H), 8.49 (br s, 1H), 9.01 (m, 1H); UV−vis ($CHCl_3$) λ_{max} 442 nm (log ϵ, 3.9), 443 (4.1), 466 (3.9). Anal. Calcd for $C_{21}H_{29}N_5O_2 \cdot 0.5H_2O$; C, 64.26; H, 7.70; N, 17.84. Found: C, 64.52; H, 7.70; N, 17.71.

6-Aza-3-methylisoalloxazine (3-Me-6-AzaFl). A mixture of 6-AzaFl (100 mg, 0.26 mmol), MeI (21 μL, 0.34 mmol), and Cs_2CO_3 (85 mg, 2.6 mmol) in DMF (5 mL) was stirred at room temperature for 2 days. The solvent was removed in vacuo, and CH_2Cl_2 (100 mL) was added. The CH_2Cl_2 layer was washed with 3% sodium thiosulfate solution (50 mL) to remove I_2 and water. After the solution was dried over $MgSO_4$, the solvent was removed in vacuo. The crude product was recrystallized from EtOH to give yellow crystals: yield 42 mg (40%); mp 196−7 °C; ^{1}H NMR (500 MHz) δ (ppm) 0.88 (t, J = 6.70 Hz, 3H), 1.23−1.60 (m, 18H), 1.82−1.89 (m, 2H), 3.55 (s, 3H), 4.64−4.74 (m, 2H), 7.77−7.82 (m, 1H), 7.97−8.01 (m, 1H), 8.97−8.99 (m, 1H); UV−vis ($CHCl_3$) λ_{max} 425.5 nm (log ϵ, 4.0), 444.5 (4.1), 468 (3.9). Anal. Calcd for $C_{22}H_{31}N_5O_2 \cdot 1H_2O$: C, 63.59; H, 8.00; N, 16.85. Found: C, 63.89; H, 7.73; N, 16.66.

Isolation of the Receptor−Flavin Complexes. All the attempts to obtain crystals for X-ray structural analysis have been unsuccessful, giving powders. Namely, the procedures were as follows: (i) Recrystallization or spontaneous vaporization of a solvent dissolving equal amounts (ca. 0.2 M) of 6-AzaFl and **1b**. Solvents examined were CH_2Cl_2, $CHCl_3$, MeCOMe, MeCN, and THF. (ii) Vapor diffusion of hexane or diethyl ether into the above solvents dissolving equal amounts (ca. 0.2 M) of 6-AzaFl and **1b**. Powders obtained by recrystallization from $CHCl_3$ gave satisfactory elemental analysis as 1:1 complex: FAB-MS m/z 783 ($[M - ClO_4]^+$, M; calcd for 6-AzaFl·**1b**); ESI-MS m/z 883.5 ($[M + H]^+$), 783.6 ($[M - ClO_4]^+$). Anal. Calcd for $C_{41}H_{63}N_{14}ClO_4 \cdot {}^2/_3H_2O$: C, 54.99; H,

(29) Yoneda, F.; Sakuma, Y.; Mizumoto, S.; Ito, R. *J. Chem. Soc., Perkin Trans. 1* **1976**, 1805−1808.

7.24; N, 21.90. Found: C, 54.96; H, 6.87; N, 21.79. The isotope patterns were in good accordance with the theoretical ones. Fast atom bombardment (FAB) mass spectra were recorded with *m*-nitrobenzyl alcohol as a matrix. Electron spray ionization (ESI) mass spectra were recoded by using a sample (1.0×10^{-4}M) in MeCN.

6-AzaFl·**4** was obtained in a manner similar to that for the powders: ESI-MS *m*/*z* 1100.7 ($[M - ClO_4]^+$, M; calcd for 6-AzaFl·**4**).

Determination of Binding Constants. (a) From ^{1}H NMR Titration. The procedures were described previously.[9b] **(b) From UV−vis Titration.** Into a quartz cuvette containing the flavin (5.0×10^{-5} M) in $CHCl_3$ (3 mL) was added a stock solution of receptor (5.0×10^{-3} M in $CHCl_3$) in 5 μL increments, and the absorption at 489 nm was recorded. Binding constants were calculated by nonlinear curve-fitting. **(c) From Fluorescence Titration.** Into a cell containing a flavin (1.0×10^{-5} M) in $CHCl_3$ (3 mL), the decrease of fluorescence intensity at 530 nm (excitation at 440 nm) was recorded. Binding constants (K_a) were calculated by $I_o/I = K_a$[receptor] + 1, where I_o and I are the fluorescence intensity of flavin without receptor and that with receptor, respectively. The concentration of the receptor was chosen as to form the complex in a range of 0.2−0.8.

Determination of Redox Potentials. Cyclic voltammograms were recorded with an X−Y recorder using a platinum plate (3 mm in diameter), an Ag/Ag^+ (0.1 M TBAP in MeCN), and platinum wire as working, reference, and counter electrodes in CH_2Cl_2 or CH_2Cl_2−MeCN. The supporting electrolyte was tetrabutylammonium perchlorate (TBAP) (0.1 M). A solution of 6-AzaFl (1.0×10^{-3} M in CH_2Cl_2 containing 0.1 M TBAP) and the receptor (0.5 equiv each) was degassed by bubbling N_2 presaturated with CH_2Cl_2 for 20 min. All potentials were measured with scan rate 100 mV/s (0−1300 mV) and corrected with ferrocene/ferrocenium couple (244 mV), which was determined under the same conditions. The solvents (CH_2Cl_2 and MeCN) used were purified by distillation from calcium hydride, and TABP was recrystallized from EtOH−H_2O two times and dried overnight in a vacuum at 100 °C.

Measurement of EPR Spectrum. In an EPR tube (∅ = 3 mm) containing N_2-degassed $CHCl_3$ (1 mL), with N_2-bubbling, 6-AzaFl (2.0 mg) and **3** (3.0 mg) were added. After 5 min, DTT (5 mg) and Bu_3N (2.5 μL) were added quickly and sealed with septum cap and measured.

Rate Measurements. The rate constants for slow reactions were determined spectrophotometrically by using a Thunberg cuvette as described previously.[9b] Those for the faster reactions were determined by stopped-flow technique. In one reservoir, 120 μL of the flavin (2.5×10^{-3} M in $CHCl_3$) was placed with the solvent ($CHCl_3$−MeCN = 4:1, 3 mL), and the suitable amounts of the receptor (1.0×10^{-2} M in $CHCl_3$−MeCN = 4:1) and the substrate (1.25×10^{-3} M in $CHCl_3$−MeCN = 4:1) and Bu_3N (1.25×10^{-3}M in $CHCl_3$−MeCN = 4:1) were placed with the solvent in another reservoir. After quick mixing, the absorption decreases at 440 or 450 nm were followed.

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