Flavin Receptors. Effects of Melamine Derivatives Bearing Thiourea and Thiouronium Ion on the Binding and Reactivity of Flavin Mimics in Chloroform

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Melamine derivatives bearing thiourea and thiouronium ion were prepared as flavin receptors. The binding modes of the receptors for flavin mimics were evaluated from the binding constants and ^{1}H NMR data in chloroform. The effects of the receptors on the oxidation of *N*-benzyl-1,4-dihydronicotinamide (BNAH) and benzenethiol by flavin mimics were kinetically investigated in chloroform.

Synthetic receptors using hydrogen bonds have attracted considerable attention from the viewpoints of molecular recognition and supramolecular architecture.[1] Meanwhile, hydrogen bondings are known to play important roles in the regulation of diverse functions of flavoenzymes.[2] The importance of H-bondings in flavin chemistry has been discussed from theoretical[3] and experimental[4] viewpoints in model systems. Namely, the LUMO energy level of an N(5)-hydrogen bonded flavin is known to be lower than that of the N(1)-hydrogen bonded one,[3c] and intramolecularly N(5)-hydrogen bonded flavins are known to facilitate reactions involving a nucleophilic attack at the C(4a)-position.[4] To such H-bonding effects, the receptors which form H-bonds at specific positions of an isoalloxazine ring might be valid. Furthermore, artificial flavin receptors using H-bonds are useful not only to investigate H-bonding effects on redox properties of flavin mimics, but also for an apoprotein model in the sense that functionalized flavin receptors could provide such functionalities as a substrate-binding site and a ligand for a metal ion close to the bound flavin mimic via noncovalent bonds.[5]

We have previously reported that melamine derivatives bearing guanidinium ion(s) strongly bind 6-aza-10-dodecylisoalloxazine (6-AzaFl) via five or seven H-bonds in $CHCl_3$, as shown in Fig. 1.[6a] It should be noted that 6-AzaFl is an oxidation-active flavin mimic due to the electron-withdrawing N(6) atom, which also serves as an H-accepting site. By employing 6-AzaFl and the guanidinium receptors, we found that (i) the redox potential of 6-AzaFl is shifted to a positive direction, (ii) the anionic semiquinone radical is stabilized, and (iii) the oxidation reactivity is considerably enhanced.[6a] Namely, the oxidation of PhSH by 6-AzaFl is remarkably accelerated ($\sim 10^3$-fold) by forming H-bonded complexes, as shown in Fig. 1, and the oxidation of BNAH is accelerated by ~10-fold for the seven H-bonds, whereas there is almost no effect for the five H-bonds. This suggests that N(1)-hydrogen bonding is responsible for the rate acceleration of BNAH oxidation, and N(5)-hydrogen bonding is responsible for PhSH oxidation. We have also reported that three H-bonds at C(2)=O, N(3)–H, and C(4)=O only little affect the rate of BNAH oxidation in $CHCl_3$.[6c] However, to evaluate the role of the N(1)-hydrogen bonding, it is necessary to exclude the H-bond at the N(5)-position. Based on CPK model construction, we chose melamine derivatives bearing a thiourea or a thiouronium-ion moiety. It is also of interest to compare the binding abilities of guanidinium, thiourea, and thiouronium moieties because of different acidities of the H-donors. Namely, the pK_a values are 12–13 for guanidinium ion,[7] 21 for thiourea,[8] and 9.8 for thiouronium ion.[9] The following receptors and flavin mimics were employed (Chart 1).

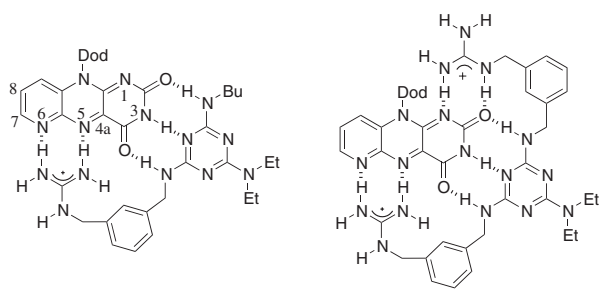

Fig. 1. Complex structures of 6-AzaFl and melamine derivatives bearing guanidinium ion(s).

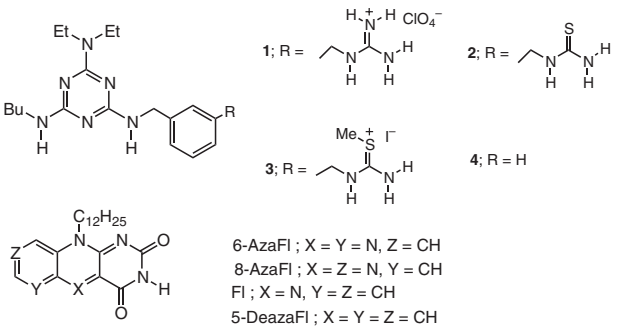

Chart 1. Receptors and flavin mimics.

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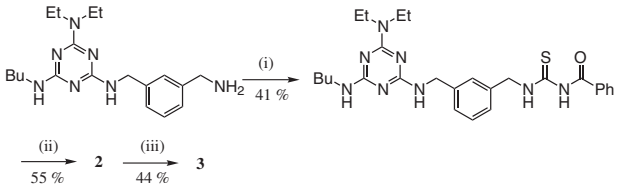
Results and Discussion

Synthesis of Receptors. Receptors were prepared from 2-[3-(aminomethyl)benzylamino]-4-butylamino-6-diethylamino-*s*-triazine, as shown in Scheme 1. Namely, **2** was prepared by the reaction with benzoyl chloride and ammonium thiocyanate, followed by alkaline hydrolysis, and **3** was obtained by the reaction of **2** with methyl iodide.[10] Purification was performed by column chromatography and recrystallization, and identification was performed by ^{1}H NMR and elemental analysis. Receptor **1** and flavin mimics were supplied from our previous studies.[6a,c]



Scheme 1. Synthetic routes of **2** and **3**. (i) PhCOCl, NH_4SCN, acetone, reflux, 3 h, (ii) 10% NaOH, 90 °C, 4 h, (iii) MeI, MeOH, r.t., 13 h.

Binding Constants. The binding constants of the receptors for flavin mimics were determined by UV–vis or fluorescence spectroscopy in $CHCl_3$ as described previously.[6a,c] A 1:1 complex formation of 6-AzaFl and **2** was confirmed by a Job's plot using the absorption changes of 6-AzaFl at 489 nm (data not shown). The binding constants were calculated from nonlinear curve fittings for UV–vis spectroscopic titration and the Stern–Volmer plots for fluorescence titration.[6a] The results are summarized in Table 1.

A survey of the K values allows us to estimate the complex structures based on the number and positions of the H-bondings. As shown in Figs. 2 and 3, at least two complex modes (A and B) are conceivable for each of flavin mimics (**3** was omitted because of similarity to **2**). A remarkably large K value of 6-AzaFl·**1** implies an A-mode structure, as shown in Fig. 1(a), which is unable to be formed with other flavin mimics. The slightly larger K values of 8-AzaFl·**1** and Fl·**1** compared

Table 1. Binding Constants (K) for Complexation of Flavins and Receptors in $CHCl_3$ at 25 °C

Receptor	K/M^{-1}			
	6-AzaFl[a)]	8-AzaFl[b)]	Fl[b)]	5-DeazaFl[b)]
1	$1.4 (\pm 0.1) \times 10^5$ [c)]	$1.6 (\pm 0.0) \times 10^3$	$2.0 (\pm 0.1) \times 10^3$	$8.4 (\pm 0.1) \times 10^2$
2	$3.0 (\pm 0.1) \times 10^3$	$6.6 (\pm 0.1) \times 10^2$	$5.9 (\pm 0.3) \times 10^2$	$6.1 (\pm 0.7) \times 10^2$
3	$3.4 (\pm 0.1) \times 10^3$	$3.9 (\pm 0.1) \times 10^2$	$3.1 (\pm 0.4) \times 10^2$	$2.4 (\pm 0.0) \times 10^2$
4[c)]	$1.4 (\pm 0.0) \times 10^2$	nd[d)]	$1.5 (\pm 0.2) \times 10^2$	$1.3 (\pm 0.0) \times 10^2$

a) UV–vis spectroscopy: [Flavin] = 5.0×10^{-5} M, [Receptor] = 0–1.0×10^{-3} M. b) Fluorescence spectroscopy: [Flavin] = 1.0×10^{-5} M, [Receptor] = 0–3.0×10^{-4} M. c) From our previous study (Ref. 6a). d) Not determined.

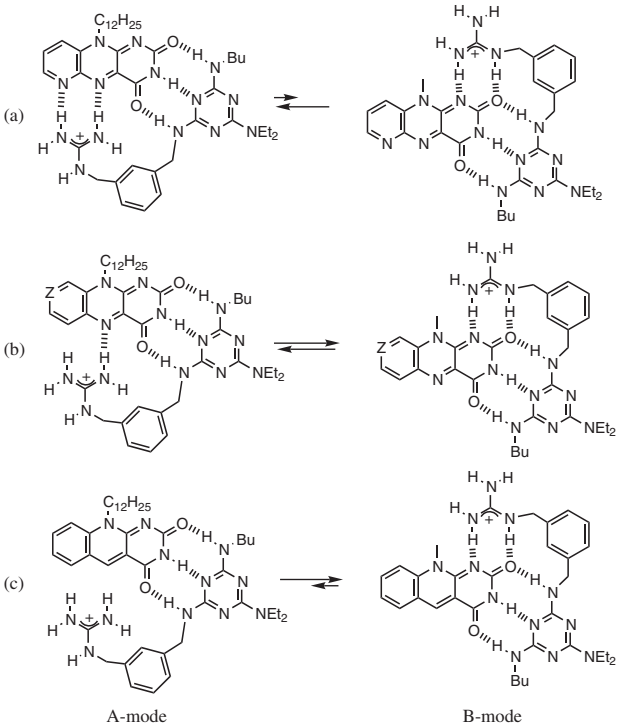


Fig. 2. Possible complex structures of the flavin mimics with **1**.

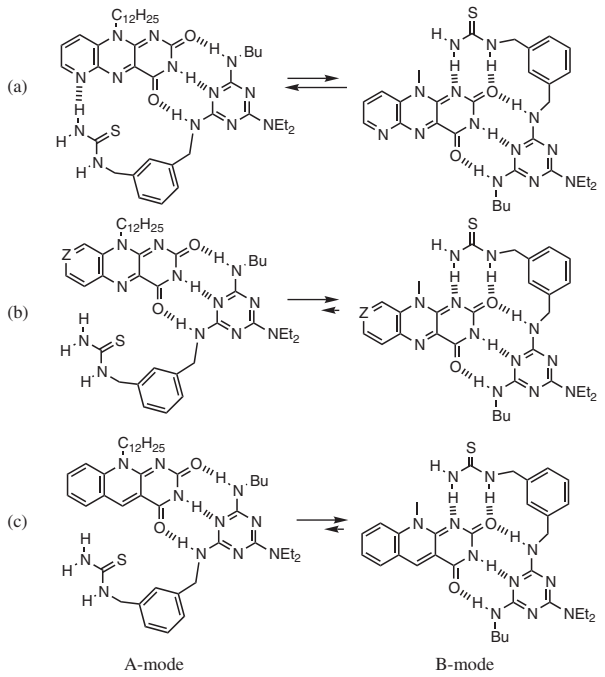


Fig. 3. Possible complex structures of the flavin mimics with **2**.

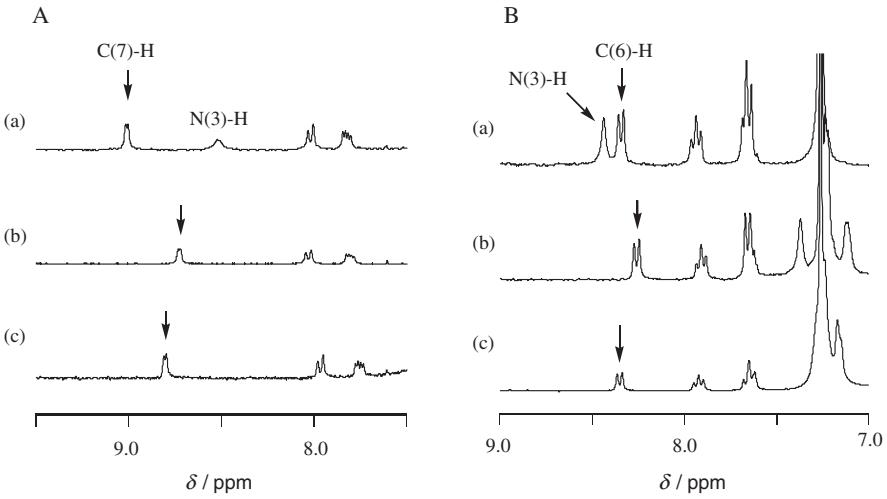


Fig. 4. ^{1}H NMR Spectra of 6-AzaFl (A) and Fl (B). (a) Flavin, (b) Flavin + **1**, (c) Flavin + **2**. [flavin] = [**1**] = 2.5×10^{-3} M, [**2**] = 5.0×10^{-3} M, $CDCl_3$, 25 °C.

to that of 5-DeazaFl·**1** suggest that N(5)-hydrogen bonded complexes (A-mode) are included in 8-AzaFl·**1** and Fl·**1**, as shown in Fig. 2(b). Almost similar K values for 8-AzaFl and Fl for each of the receptors indicate that the N(8) position of 8-AzaFl is not included as an H-bonding site. Larger K values of **2** and **3** than those of **4** indicate that the thiourea and thiouronium moieties participate in complex formation as H-donors. Similar K values of **2** for three flavin mimics imply that the additional H-bonding sites are N(1) and C(2)=O. A slightly larger K value of 6-AzaFl·**2** than those of 8-AzaFl·**2**, Fl·**2**, and 5-DeazaFl·**2** suggests that the N(6)-position is included as an H-bonding site, as shown in Fig. 3(a), although the ratio of the A and B modes is uncertain.

To obtain more information on the H-bonding sites, a ^{1}H NMR study was undertaken by employing 6-AzaFl and Fl in the presence of **1** and **2** in $CDCl_3$ (Fig. 4). For 6-AzaFl, the chemical shift of C(7)–H shifted to upfield upon the addition of **1** and **2** (Figs. 3(a), (b), and (c)). These results suggest that the N(6)-position of 6-AzaFl is included as an H-bonding site for **1** and **2**. In other words, the complexes of mode A are involved in some ratio for 6-AzaFl·**1** and 6-AzaFl·**2**. For Fl, however, the chemical shift of C(6)–H showed an upfield shift upon the addition of **1**, whereas no shift exists upon the addition of **2**, suggesting that the ratios of modes A and B are different for Fl·**1** and Fl·**2**.

The K values of 8-AzaFl·**2**, Fl·**2**, and 5-DeazaFl·**2** are almost the same, and are larger than those for **4**, indicating that the additional H-bonding sites are not the N(5)-position, but the N(1) and C(2)=O positions, as shown in Figs. 3(b) and 3(c). Similar tendencies were also observed for **3**. These observations may allow us to conclude that **2** and **3** bind 8-AzaFl, Fl, and 5-DeazaFl via H-bonds including the N(1) position but not the N(5) position.

A more acidic H-donor is known to form a stronger H-bond.[11] In fact, we have reported that the log K values of the melamine derivatives bearing the 2-arylguanidinium ion for 6-AzaFl are nicely correlated with the Hammett σ.[6b] However, the more acidic H-donor of the thiouronium moiety of **3** showed a slightly smaller K values than those of **2**. This could be explained by a steric hindrance of methyl group of the thiouronium moiety. Such a steric hindrance is also observed for the 2-arylguanidinium ion of **1**.[6b]

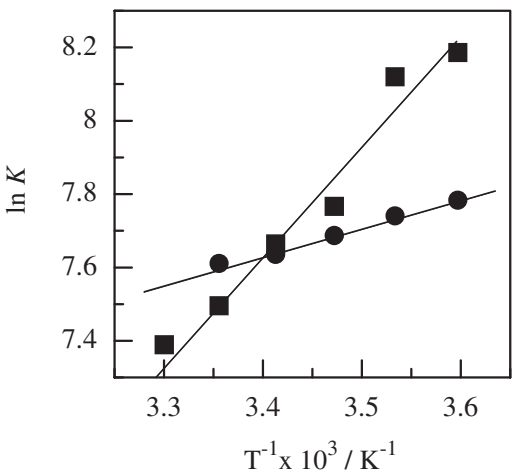


Fig. 5. Temperature dependency of K values of 6-AzaFl·**2** (●) and 6-AzaFl·**3** (■).

Table 2. Thermodynamic Parameters for Complex Formation with 6-AzaFl in $CHCl_3$

Receptor	ΔG_{298}/kJ mol^{-1}	ΔH/kJ mol^{-1}	$T\Delta S_{298}$/kJ mol^{-1}
1[a)]	−29.3	−34.3	−5.0
2	−18.9	−6.2	12.7
3	−18.6	−23.9	−5.3

a) From Ref. 6a.

Thermodynamic Parameters. The thermodynamic parameters (ΔH and $T\Delta S$) for the complex formation of 6-AzaFl·**2** and 6-AzaFl·**3** were determined by the temperature dependence on the K values (Fig. 5). The thermodynamic parameters are listed in Table 2 together with those of **1**.[6a] The complex formation of ionic receptors **1** and **3** is controlled by the enthalpy, indicating negative entropy terms due to the loss of translational and rotational motion inherent in bimolecular association. However, the complex formation of nonionic **2** is

controlled by the entropy. Namely, the binding takes place due to the entropic gain resulting from the liberation of solvent molecules from the nonionic receptor **2**. Such a positive entropy association is often observed in cases of ionic receptors and polar solvents.[12] The larger negative enthalpy of **3** than that of **2** suggests that the complex of 6-AzaFl·**3** is stabilized by the H-bondings accompanying with partial charge neutralization in $CHCl_3$.

Effects of Hydrogen Bondings on Oxidation Activity. We have reported that the rate of the oxidation of PhSH by 6-AzaFl is remarkably accelerated ($\sim 10^3$-fold) in the presence of **1** in $CHCl_3$–CH_3CN, whereas that of BNAH is little affected.[6a] It seems to be accepted that the reactions proceeding via a nucleophilic attack at the C(4a)-position are facilitated by N(5)-hydrogen bonding, whereas almost no effect for BNAH oxidation, which proceeds via a hydride (or its equivalent) attack at the N(5) position. However, the effects of N(1)-hydrogen bonding on the redox properties of a flavin has not yet been settled. To evaluate the N(1)-hydrogen bonding by using receptors, it is required to employ systems that mainly involve a 'B-mode' complex with an oxidation-active flavin mimic. This requirement may be achieved by employing a combination of 8-AzaFl and receptors, as shown in Figs. 2(b) and 3(b).

(a) BNAH Oxidation: Pseudo-first-order rate constants (k_{obs}) were spectrophotometrically determined by following the absorption decreases of 8-AzaFl at 440 nm in $CHCl_3$ under anaerobic conditions. The concentration effects of the receptors on k_{obs} are shown in Fig. 6. The second-order rate constant (k_o) in the absence of the receptor was determined to be 0.16 $M^{-1}\,s^{-1}$. A kinetic analysis was performed in the same way as that for 6-AzaFl, as described previously.[6a] The computed rate constants, derived from the rate equation,[13] are given in Table 3. In contrast to 6-AzaFl,[6a] large rate accelerations were observed. The rate enhancements were 30-fold for **1**, 6.9-fold for **2**, and 49-fold for **3**, which could be explained by the acidity of the H-donors.

(b) PhSH Oxidation: Pseudo-first-order rate constants were determined, as described previously.[6a] The concentration effects of the receptors showed that **2** and **3** do not affect the rates, whereas **1** shows a large rate enhancement (Fig. 7). From the reaction scheme and rate equation as described above,[13] the computed binding and rate constants are: $K' = 2.0 \times 10^3\ M^{-1}$, $k_3 = 1.9 \times 10^6\ M^{-3}\,s^{-1}$. Since the separately determined k_o value is $2.6 \times 10^4\ M^{-3}\,s^{-1}$, the rate acceleration is 74-fold (k_3/k_o). Because the binding constant of 8-AzaFl·**1** suggests the involvement of both the A and B modes, the rate enhancement could be explained by the presence of the N(5)-hydrogen bonded complex. No rate enhancements of **2** and **3** indicate that N(5)-hydrogen bonded complexes are little involved in 8-AzaFl·**2** and 8-AzaFl·**3**, as expected.

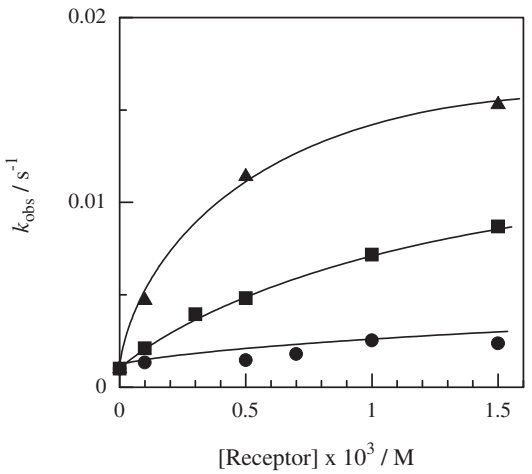


Fig. 6. Concentration effects of receptors on the rate of the oxidation of BNAH: [8-AzaFl] = 5.0×10^{-5} M, [BNAH] = 4.0×10^{-3} M, $CHCl_3$, N_2, 25 °C. ▲; **1**, ●; **2**, ■; **3**.

Table 3. Computed Binding Constants (K') and Rate Constants for BNAH Oxidation

	1	**2**	**3**
K'/M^{-1}	2.3×10^3	7.2×10^2	2.3×10^2
$k_2/M^{-1}\,s^{-1}$	4.7	1.1	7.9
k_2/k_0	30	6.9	49

$k_0 = 0.16\ M^{-1}\,s^{-1}$.

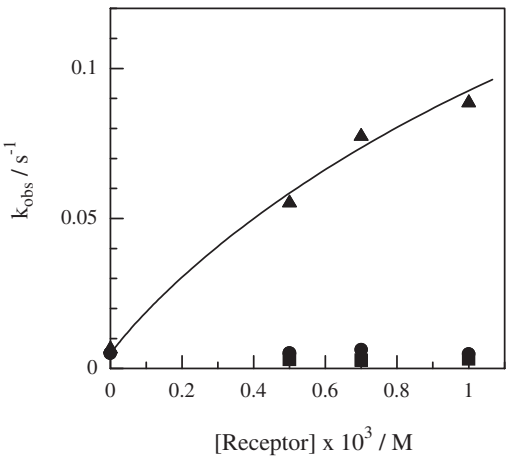


Fig. 7. Concentration effects of receptors on the rate of the oxidation of PhSH: [8-AzaFl] = 5.0×10^{-5} M, [PhSH] = 5.0×10^{-3} M, [Bu_3N] = 1.0×10^{-2} M, $CHCl_3$:MeCN = 4:1, N_2, 25 °C. ▲; **1**, ●; **2**, ■; **3**.

In conclusion, the present results demonstrate that the N(1)-hydrogen bonding of an isoalloxazine ring facilitates the reactions proceeding via an N(5) attack, like BNAH oxidation, and only little affects the oxidation proceeding via a C(4a)-attack, like PhSH oxidation. Together with our previous study[6a,15] and others,[4] we consider that Massey's proposal, where N(1)-hydrogen bonding activates the N(5)-position and N(5)-hydrogen bonding activates the C(4a)-position, is supported through model systems using artificial receptors with H-bonds. We believe that the present results are important for designing flavin model systems possessing apoprotein functions.

Experimental

^{1}H NMR spectra were recorded on a JEOL AL-300 (300 MHz) instrument with chemical shifts from tetramethylsilane. Electronic

absorption spectra were recorded on a JASCO Ubset-560 or Shimadzu UV-2500PC spectrophotometer. Fluorescence spectra were recorded on a Hitachi F4500 fluorescence spectrophotometer. Flash column chromatography was performed by using a Wakogel C-200 (70–150 μm, Wako Pure Chemical Co.). Elemental analyses were performed at the Center of Instrumental Analysis of Gunma University. Melting points are uncorrected. Special grade chloroform and acetonitrile (Kanto Chemicals) were used without further purification.

Synthesis of 2-[3-(benzoylthioureidomethyl)benzylamino]-4-butylamino-6-diethylamino]-*s*-triazine. To a solution of ammonium isothiocyanate (0.60 g, 8.2 mmol) in acetone (50 mL), benzyl chloride (0.65 g, 8.2 mmol) was slowly added (ca. 5 min), and the solution was refluxed for 20 min. After the solution was cooled at room temperature, 2-(3-aminomethylbenzylamino-4-butylamino-6-diethylamino-*s*-triazine[6a] (1.60 g, 4.47 mmol) in acetone (20 mL) was slowly added. After being refluxed for 3 h, the solution was cooled at 0 °C, and poured into water (200 mL), and extracted with chloroform (30 mL × 3). After the $CHCl_3$ layer was dried over Na_2SO_4, $CHCl_3$ was evaporated and the residue was purified by column chromatography (CH_2Cl_2–AcOEt = 5:1). Yield 0.95 g (41%, yellow oil). ^{1}H NMR ($CDCl_3$) δ 0.90 (3H, t, $J = 7.3$ Hz, $NHCH_2CH_2CH_2C\underline{H}_3$), 1.10 (6H, t, $J = 6.2$ Hz, $N(CH_2C\underline{H}_3)_2$), 1.34 (2H, m, $NHCH_2CH_2C\underline{H}_2CH_3$), 1.52 (2H, m, $NHCH_2C\underline{H}_2CH_2CH_3$), 3.32 (2H, q, $J = 6.2$ Hz, $NHC\underline{H}_2CH_2CH_2CH_3$), 3.50 (4H, $J = 7.0$ Hz, $N(C\underline{H}_2CH_3)_2$), 4.56 (2H, d, $J = 5.9$ Hz, $C_6H_4C\underline{H}_2NHC(=S)NH$–), 4.88 (2H, m, $C_6H_4C\underline{H}_2NH$–), 5.26 (1H, bs, NH), 7.26–7.32 (4H, m, C_6H_4), 7.45–7.80 (5H, m, C_6H_5), 11.0 (1H, bs, $N\underline{H}COC_6H_5$).

Receptor 2. A mixture of the above triazine (0.8 g, 1.54 mmol) in 1,4-dioxane (10 mL) and 10% NaOH (5 mL) was refluxed at 90 °C for 4 h. After cooling, the solution was extracted with $CHCl_3$ (30 mL × 3). The $CHCl_3$ layer was washed with water and dried over Na_2SO_4. After evaporating the solvent in vacuo, the residue was recrystallized from EtOH. Yield 0.35 g (55%), mp 151–152 °C. ^{1}H NMR ($CDCl_3$) δ 0.90 (3H, t, $J = 7.2$ Hz, $NHCH_2CH_2CH_2C\underline{H}_3$), 1.10 (6H, t, $J = 6.6$ Hz, $N(CH_2C\underline{H}_3)_2$), 1.34 (2H, m, $NHCH_2CH_2C\underline{H}_2CH_3$), 1.47 (2H, q, $J = 7.5$ Hz, $NHCH_2C\underline{H}_2CH_2CH_3$), 3.27 (2H, q, $J = 6.3$ Hz, $NHC\underline{H}_2CH_2CH_2CH_3$), 3.47 (4H, q, $J = 6.9$ Hz, $N(C\underline{H}_2CH_3)_2$), 4.59 (2H, m, $C\underline{H}_2C_6H_4C\underline{H}_2$), 5.05 (1H, bs, NH), 5.63 (1H, bs, NH), 6.22 (2H, bs, $C(=S)NH_2$), 7.12–7.26 (4H, m, C_6H_4). Found: C, 57.66; H, 7.80; N, 26.70%. Calcd for $C_{20}H_{32}N_8S$: C, 57.66; H, 7.74; N, 26.90%.

Receptor 3. A mixture of **2** (0.22 g, 0.53 mmol) and MeI (0.032 mL, 0.60 mmol) in MeOH (30 mL) was stirred overnight at room temperature. After evaporating the solvent, the residue was washed with diethyl ether (10 mL × 2) and recrystallized from diethyl ether–acetone. Yield 0.13 g (44%), mp 130–131 °C. ^{1}H NMR ($CDCl_3$) δ 0.92 (3H, t, $J = 7.3$ Hz, $NHCH_2CH_2CH_2C\underline{H}_3$), 1.15 (6H, t, $J = 6.6$ Hz, $N(CH_2C\underline{H}_3)_2$), 1.38 (2H, m, $NHCH_2CH_2C\underline{H}_2CH_3$), 1.53 (2H, q, $J = 7.5$ Hz, $NHCH_2C\underline{H}_2CH_2CH_3$), 2.61 (3H, s, SCH_3), 3.35 (2H, q, $J = 6.3$ Hz, $NHC\underline{H}_2CH_2CH_2CH_3$), 3.55 (4H, q, $J = 6.9$ Hz, $N(C\underline{H}_2CH_3)_2$), 4.56 (4H, d, $J = 5.6$ Hz, $C\underline{H}_2C_6H_4C\underline{H}_2$), 7.23–7.34 (4H, m, C_6H_4). Found: C, 45.00; H, 6.28; N, 19.73%. Calcd for $C_{21}H_{35}IN_8S$: C, 45.16; H, 6.32; N, 20.06%.

Flavin Mimics. 10-Dodecylisoalloxazine (Fl), 5-deaza-10-dodecylisoalloxazine (5-DeazaFl), 6-AzaFl, and 8-aza-10-dodecylisoalloxazine (8-AzaFl) were supplied from our previous study.[6,14]

Determination of Binding and Rate Constants. Experimental procedures were the same as described previously.[6a]

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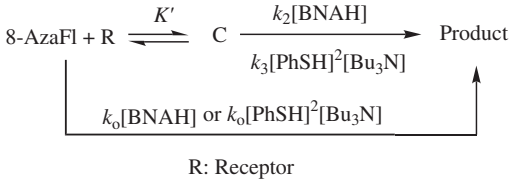

$$\text{Rate} = k_{obs}[\text{8-AzaFl}]_o = k_o([\text{8-AzaFl}]_o - [C])[S]_o + k_2[C][S]_o$$

$$k_{obs} = (k_o[\text{8-AzaFl}]_o + (k_2 - k_o)[C])[S]_o/[\text{8-AzaFl}]_o$$

$$\text{where } [C] = (K'[\text{8-AzaFl}]_o + K'[R]_o + 1)/2K'$$

$$- [([\text{8-AzaFl}]_o{}^2 + [R]_o{}^2 - 2[\text{8-AzaFl}]_o[R]_o)K'^2$$

$$+ 2K'([\text{8-AzaFl}]_o + [R]_o) + 1]^{1/2}/2K'$$

$$[S] = [\text{BNAH}] \text{ or } [\text{PhSH}]^2[Bu_3N]$$

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