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Synthesis of Flavins Bearing a Sulfur Functional Group and Their Catalytic Activities for Reduction of Disulfides

Toshiyuki Nagata,[a] Ken Fujimori, and Shigeru Oae[b]*

Department of Chemistry, University of Tsukuba, Tsukuba, Ibaraki, 305 Japan

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ABSTRACT

Bis-flavins, each of which bears a disulfide linkage in the molecule, have been synthesized as model compounds for dihydronicotinamide dependent disulfide reductase. These flavins were found to be much better catalysts to promote the reduction of dibenzyl disulfide to α-toluenethiol than 3-methyllumiflavin.

INTRODUCTION

The thiol-disulfide redox process (Scheme 1) is very important in biological systems [1]. This reaction is catalyzed by pyridine nucleotide dependent disulfide reductases, such as lipoamide dehydrogenase, glutathione reductase, etc. Each of these enzymes uniformly possesses flavin adenine dinucleotide (FAD) as a prosthetic group and a disulfide group of apoenzyme due to a cystine residue in proximity to the active site [2].

Glutathione reductase isolated from human erythrocyte is the only example in which the three-dimensional structure has been elucidated by X-ray diffraction analysis [3]. Scheme 2 shows the catalytic mechanism proposed by Pai and Schulz [3] for this enzyme. Namely, the disulfide group at the enzyme active site is believed to mediate one electron transfer from the flavin to the oxidized form of glutathione (GSSG) to afford the reduced form of glutathione (GSH) through the apoprotein bound thiol-disulfide redox reaction.

In our previous article we demonstrated that the 3-methyllumiflavin/1-benzyl-1,2-dihydronicotinamide (BNAH) system promotes the reduction of diaryl disulfide under mild conditions [4]. However, this system is not reactive enough to reduce aliphatic disulfides, while the actual NADPH dependent disulfide reductase promotes smoothly the reduction of such aliphatic disulfides in living bodies [2]. Thus, flavin catalysts bearing the sulfur functional group, $[Fl\text{-}C_n\text{-}S\text{-}]_2$, have been designed as disulfide reductase models for the pyridine nucleotide dependent disulfide reductase based upon the working hypothesis shown in Scheme 3.

The initial step consists of the reduction of the flavin catalyst, $[Fl\text{-}C_n\text{-}S]_2$, by BNAH (a model compound of NADPH) to form $FlH^-\text{-}C_n\text{-}S\text{-}S\text{-}C_n\text{-}Fl$, since flavins are known to be reduced by dihydropyridines [5–7], such as BNAH, while dialkyl disulfides are not reduced at all by BNAH [8]. $FlH^-\text{-}C_n\text{-}S\text{-}S\text{-}C_n\text{-}Fl$ may undergo intramolecular reduction of the disulfide bond by the reduced form of flavin in the same catalyst molecule, generating $Fl^-\text{-}C_n\text{-}S$ and $Fl\text{-}C_n\text{-}SH$. The intramolecular redox reaction (Scheme 3, Initial Step) is expected to be reversible, and the equilibrium would be favored for the backward reaction. However, since $Fl^-\text{-}C_n\text{-}S$ is in an equilibrium with $Fl\text{-}C_n\text{-}S^-$ and a thiolate-disulfide interchange reaction is generally quite fast in an uncomplicated S_N2 process [9–11], $Fl^-\text{-}C_n\text{-}S$ formed by the intramolecular redox reaction reacts with RS-SR to liberate RS^-, as shown in the catalytic cycle in Scheme 3. The RS^- formed, then, picks up a proton to yield the thiol.

[a]Present address: Tsukuba Research Laboratory, Toagosei Chemical Industry Co. Ltd., Ohkubo 2, Tsukuba, Ibaraki, 300-33 Japan.
[b]Present address: Institute of Heteroatom Chemistry, 2509 Hirao, Mihara, Minamikawachi, Osaka, 587 Japan.
*To whom correspondence should be addressed.

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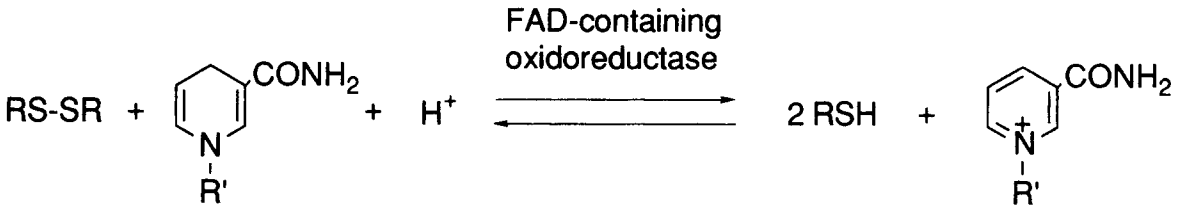
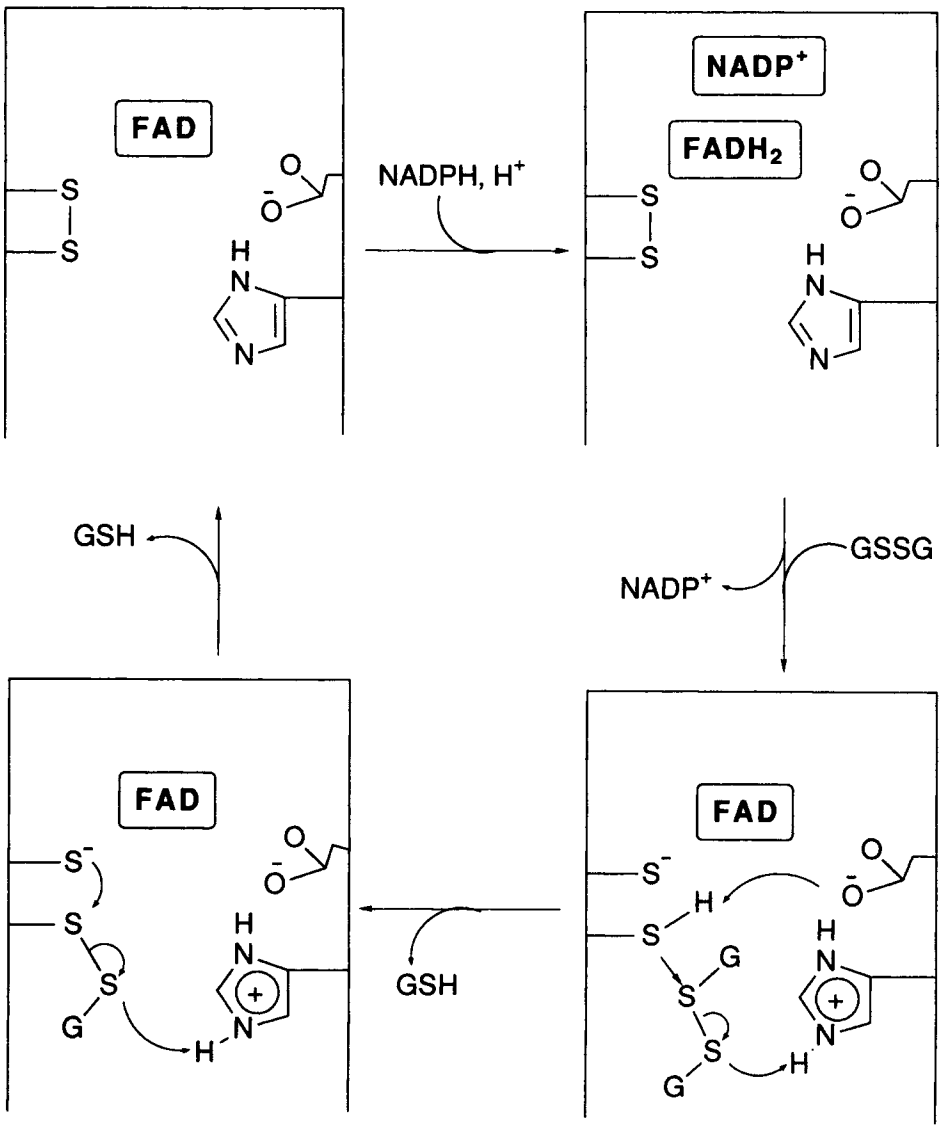
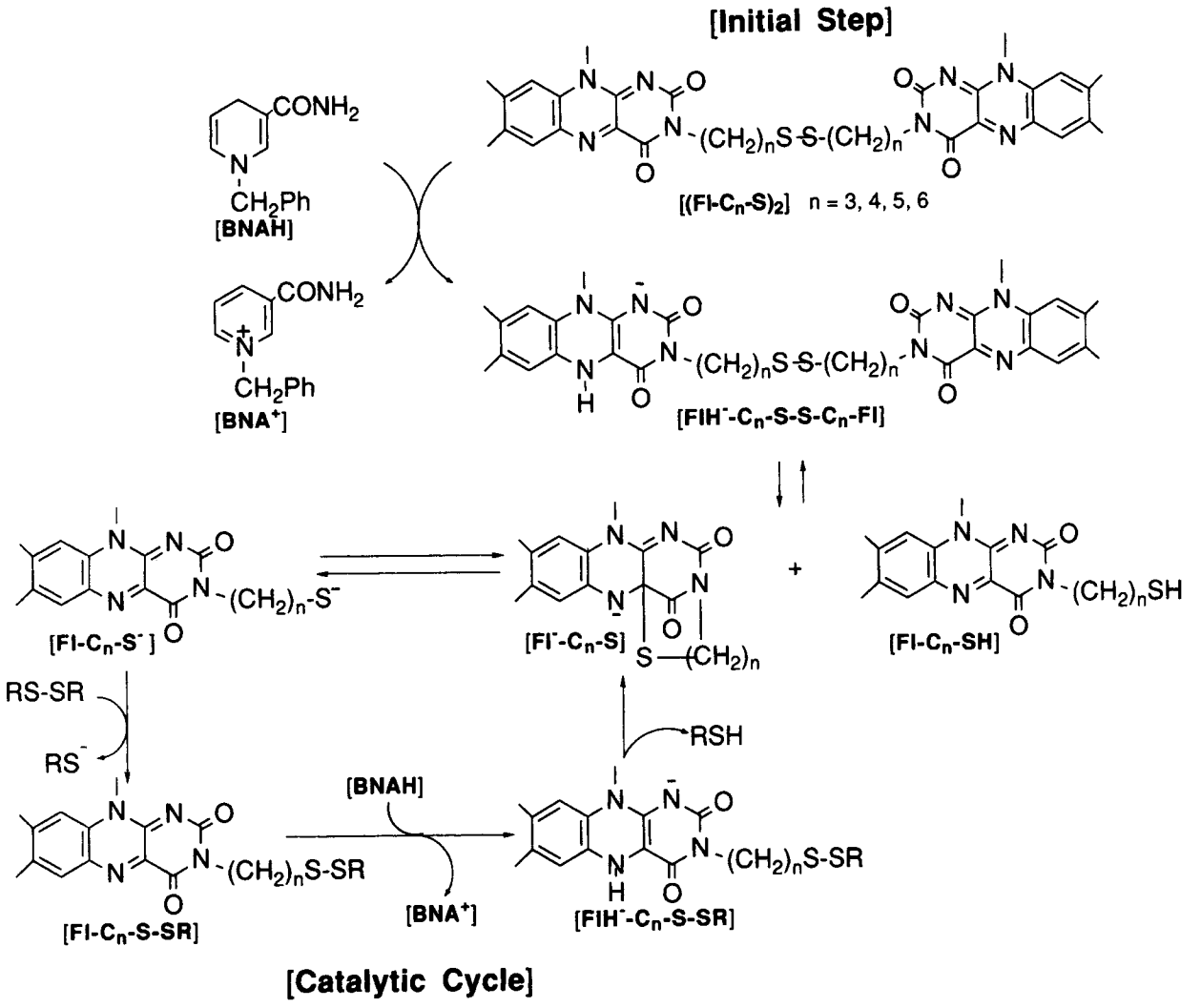


SCHEME 1



SCHEME 2



SCHEME 3

Thus, the catalytic activities of [Fl-C_n-S-]$_2$ have been examined in the reduction of dibenzyl disulfide by BNAH.

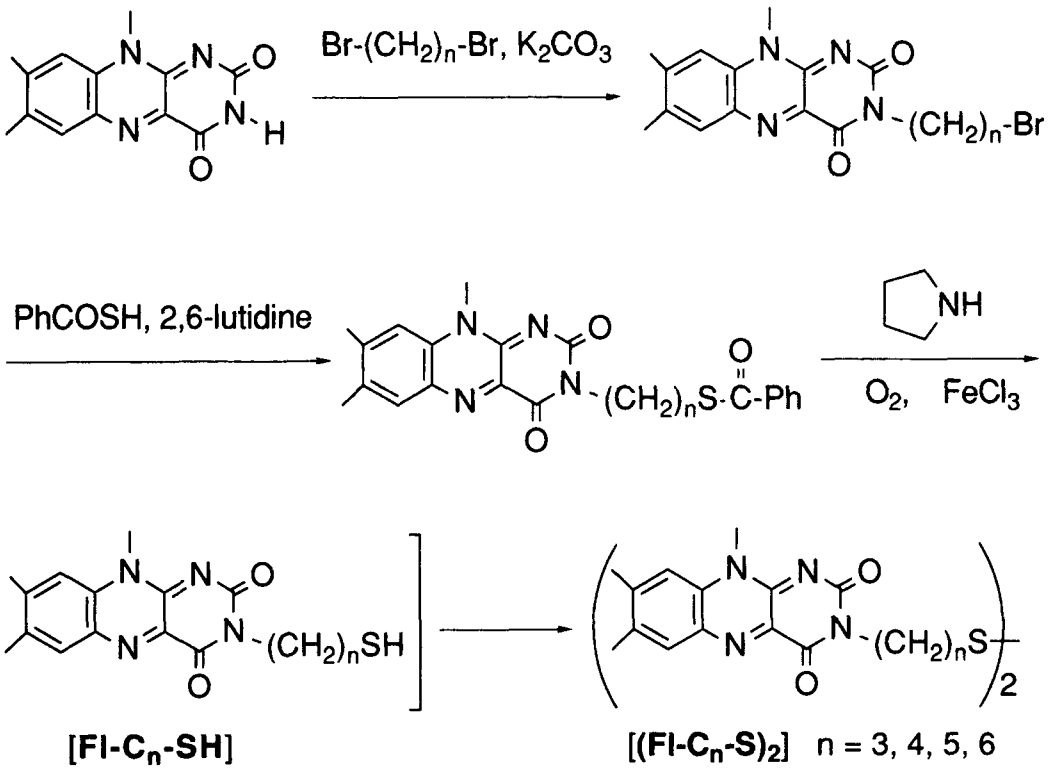
RESULTS AND DISCUSSION

The model compounds, [Fl-C_n-S-]$_2$ (n = 3, 4, 5, 6), have been synthesized by the path shown in Scheme 4. The 3-(ω-bromoalkyl)lumiflavins were prepared by treating lumiflavin with a large excess of ω,ω'-dibromoalkanes in the presence of potassium carbonate in dimethylformamide. Subsequent nucleophilic substitution of the bromide on the terminal carbon at the 3-position of Fl-C_n-Br with thiobenzoic acid proceeded quantitatively to give Fl-C_n-S-COPh.

All attempts to obtain Fl-C_n-SH by either acid or base catalyzed hydrolysis or aminolysis with pyrrolidine were unsuccessful. The treatment of Fl-C_n-S-COPh with pyrrolidine under argon gave labile products, and eventually, the disulfides, [Fl-C_n-S-]$_2$, were obtained as stable products in poor yields. The desired compounds, Fl-C_n-SH, would be formed, as shown in Scheme 4, but readily oxidized subsequently to the disulfides, [Fl-C_n-S-]$_2$, during the work-up procedure under air. Thus, attempts to isolate Fl-C_n-SH were abandoned.

However, these enzyme models, [Fl-C_n-S-]$_2$, have been found to be obtained by the aminolysis of Fl-C_n-S-COPh in the presence of a catalytic amount of ferric chloride under air. The idea behind this successful procedure is that the labile primary product, Fl-C_n-SH, may be autoxidized to the stable disulfide in the presence of an appropriate transition metal ion catalyst in a good yield under aerobic conditions.

The catalytic abilities of [Fl-C_n-S-]$_2$ to reduce an aliphatic disulfide with BNAH have been examined in acetonitrile in an evacuated sealed tube in the dark using dibenzyl disulfide as the sub-



SCHEME 4

TABLE 1 Flavin Catalyzed Reduction of Dibenzyl Disulfide with BNAH[a]

Falvin Catalyst	*Yield of $PhCH_2SCOPh$ (Mol%)*[b]
None	0
3-Methyllumiflavin	190
3-Butyllumiflavin	210
[Fl-C_3-S]$_2$	600
[Fl-C_4-S]$_2$	1400
[Fl-C_5-S]$_2$	880
[Fl-C_6-S]$_2$	950

[a]The reaction was carried out in acetonitrile under aerobic conditions.
[b]Based on one flavin unit.

strate because of the convenience of the analysis of the thiol formed. The results are listed in Table 1. The yield of reduced α-toluenethiol was determined as the thiol ester by acetylation to prevent the autoxidation of this thiol during the work-up procedure.

Inspection of the data in Table 1 reveals that these flavin catalysts, each bearing a sulfur functional group, are obviously better catalysts than such a simple flavin as 3-methyllumiflavin. The fact that the models, [Fl-C_n-S-]$_2$, are better catalysts than 3-methyllumiflavin indicates that the enhanced catalytic activities of these models are not due to the introduction of a carbon chain but to the presence of the ω-sulfide group attached to the methylene chain stemmed at the N^3 position of the flavin. Among these, [Fl-C_4-S-]$_2$ was found to be the best catalyst for the reduction of dibenzyl disulfide.

The oxidation of thiols with the oxidized form of flavin is known to be initiated by a nucleophilic attack of a thiol to give a 4a-flavin thiol-adduct as an intermediate [12,13]. This suggests that the reductive cleavage of the disulfide bond with the conjugate base of the reduced form of flavin is initiated by the nucleophilic attack at the 4a-carbon of FlH-moiety in the catalyst to afford 4a-flavin-thiol adduct, as shown in Scheme 3.

Stereochemical analysis using a CPK model of FlH^--C_n-S-S-C_n-Fl reveals that such an intramolecular interaction between the flavin-4a-carbon and the disulfide group linked to the terminal carbon of the N^3-n-alkyl group affording the cyclic 4a-adduct, Fl^--C_n-S, is the most favorable when n is 4. Accordingly, the catalytic activity of [Fl-C_4-S-]$_2$ would decrease in the order of $n = 4 > 5 > 6 > 3$, which is the same order for the ease of formation of the intramolecular 4a-adduct, FlH-C_n-S, from the stereochemical examination with a CPK model. This prediction has been found to be in good accor-

dance with the actual experimental results, as shown in Table 1.

According to this hypothesis (Scheme 3), a mixed disulfide formed by coupling of one half of the catalyst and one half of the substrate disulfide, Fl-C_n-S-S-R, must be involved in the catalytic cycle. In order to verify this point, the reaction mixture of the [Fl-C_4-S-]$_2$ catalyzed reduction of dibenzyl disulfide with BNAH was carefully analyzed by HPLC. Indeed, the mixed disulfide, Fl-C_n-S-S-R (R = benzyl), was identified by comparison with an authentic sample that was prepared by the aminolysis of Fl-C_4-S-COPh with pyrrolidine in the presence of a large excess of dibenzyl disulfide under anaerobic conditions (Scheme 5).

During [Fl-C_n-S-]$_2$ catalyzed reduction of benzyl disulfide with BNAH, BNAH was converted to unidentified products, as observed in the meso-tetraphenylporphinatoiron catalyzed reduction of sulfoxides with NADPH [14]. In this case, the thiolate anions, i.e., Fl-C_n-S^- and α-toluenethiolate, formed in the course of the reaction, react with BNA^+. Undoubtedly, this undesired reaction not only consumed the thiol product but also inactivated the bifunctional flavin catalyst.

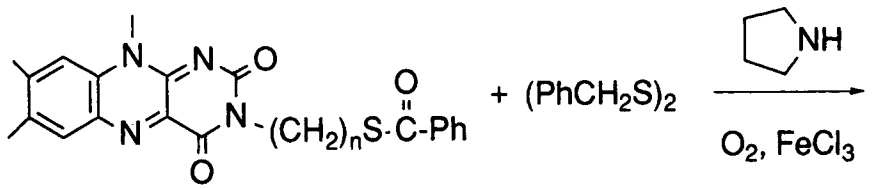
The catalytic activity of [Fl-C_n-S-]$_2$ has also been examined by using another reducing agent. Since flavins are well known to be reduced by amines upon irradiation with visible light [15,16], a solution of a flavin catalyst, dibenzyl disulfide, and N-methylpyrrolidine in acetonitrile-ethanol (1:1, v/v) contained in an evacuated sealed tube was irradiated with visible light. Under these conditions [Fl-C_n-S-]$_2$ compounds have again been found to be much better catalysts than simple 3-methyllumiflavin, as shown in Table 2.

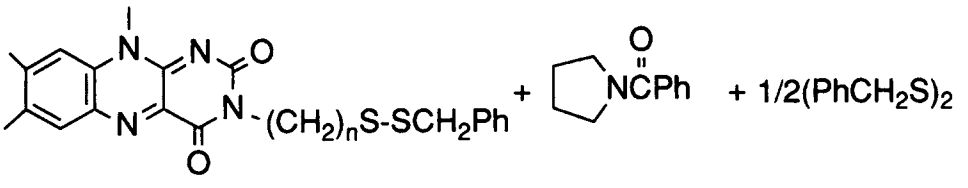
Intramolecular flavin-flavin interaction in bis-flavin derivatives shows a different shape of the visible spectrum from that of a simple mono-flavin [17]. The enzyme models [Fl-C_n-S-]$_2$, which are a kind of bis-flavin, exhibited the same electronic spectra as 3-methyllumiflavin, as shown in Table 3, suggesting that there is no special flavin-flavin interaction at the ground state of [Fl-C_n-S-]$_2$. This is in keeping with the facts that all flavins in Table 3 have apparently the same redox potentials. Thus, the intramolecular reductive cleavage of the S-S bond with the reduced form of the flavin moiety in FlH^--C_n-S-S-C_n-Fl seems to be the key step of the reduction rather than the initial step to reduce [Fl-C_n-S-]$_2$ with BNAH forming FlH^--C_n-S-S-C_n-Fl.

EXPERIMENTAL

General

All melting points were taken on a Yanaco Instrument and were uncorrected. HPLC analysis was done by the Japan Analytical Industry LC-09 instrument equipped with a JAIGEL 1H column by the Hitachi 638-50 instrument equipped with Hitachi gel #3011 or the UNISIL NQ-C18 column, or by the JASCO Familic-100N instrument with Finepack-sill-C18 or Finepack-gel 110 columns. Electronic spectra were recorded on a Hitachi 200-20 spectrophotometer. NMR spectra were recorded on a Hitachi R-600 spectrometer using TMS as an internal standard. Gas chromatography was effected with a Hitachi 163 instrument using a 1 m OV-1 or a 1 m SE 30 column.





SCHEME 5

TABLE 2 Flavin Catalyzed Reduction of Dibenzyl Disulfide in the Presence of N-methylpyrrolidine under Irradiation with Visible Light[a]

Flavin Catalyst	*Yield of $PhCH_2SH$ (Mol%)*[b]
3-Methyllumiflavin	330
[Fl-C_4-S]$_2$	800
[Fl-C_6-S]$_2$	720

[a]The reaction was carried out in CH_3CN and EtOH under unaerobic conditions.
[b]Based on one flavin unit.

Materials

3-Methyllumiflavin was Prepared by the Method of Yoneda et al. [18]

3-(4-bromobutyl)lumiflavin (Fl-C4-Br). 3-Methyllumiflavin (2.38 g, 9.30 mmol) and 1,4-dibromobutane (25.1 g, 0.116 mol) were allowed to react in dry DMF (350 mL) in the presence of anhydrous potassium carbonate at 65–70°C with mechanical stirring. The progress of the reaction was followed by TLC (silica gel, $CHCl_3$-CH_3OH(5:1)) After 2 hours, the reaction mixture was cooled to room temperature. Solids were filtered off. The filtrate was concentrated in vacuo and extracted with $CHCl_3$ three times. The $CHCl_3$ layer was washed with water, dried over Na_2SO_4, and evaporated in vacuo. The residue was column chromatographed with alumina. The column was washed first with hexane, and then eluted with CH_2Cl_2. The CH_2Cl_2 eluent was concentrated, and the residue was recrystallized from ethyl acetate-hexane to obtain orange needles. Yield 78%; mp 210°; UV: λ_{max} = 341, 445 nm in ethanol; NMR ($CDCl_3$): δ = 1.8–2.05 (m, 4 H, -CH_2-), 2.46 (s, 3 H, C-CH_3), 2.57 (s, 3 H, C-CH_3), 3.46 (t, J = 6.4 Hz, 2 H, CH_2Br), 4.12 (s, 3 H, N^{10}-CH_3), 4.13 (t, J = 6.6 Hz, 2 H, N^3-CH_2-), 7.45 (s, 1 H, aromatic H), 8.01 (s, 1 H, aromatic H). Anal. Calcd. for $C_{17}H_{19}N_4O_2Br$: C, 52.18; H, 4.89; N, 14.31%. Found: C, 52.04; H, 4.87; N, 14.30%.

3-(4-Benzoylthiobutyl)lumiflavin (Fl-C_4-S-COPh). 1.65 g of thiobenzoic acid (12.0 mmol) and 0.69 g of 2,6-lutidine (5.9 mmol) were added to the solution of Fl-C_4-Br (1.51 g, 3.86 mmol) in 10 mL of $CHCl_3$. The reaction mixture was then extracted with $CHCl_3$. The $CHCl_3$ layer was washed with $NaHCO_3$ solution, dil HCl solution, again with water, and dried over $MgSO_4$. The solvent was removed in vacuo. The residue was chromatographed on silica gel, eluting with benzene-$CHCl_3$. Further purification was carried out by recrystallization of the solid product from ethyl acetate-hexane. Yield 93%; mp 221–222°; UV: λ_{max} = 444, 340 nm in ethanol; NMR ($CDCl_3$) δ = 1.8–2.08 (m, 4 H, -CH_2-), 2.44 (s, 3 H, C-CH_3), 2.55 (S, 3 H, CCH_3), 3.12 (t, J = 6.5 Hz, 2 H, S-CH_2), 4.10 (s, 3 H, N^{10}-CH_3), 4.15 (t, J = 6.9 Hz, 2 H, N^3-CH_2-), 7.40–4.60, 7.87–8.00 (m, 7 H, aromatic H). Anal. Calcd. for $C_{24}H_{24}N_4O_3S$: C, 64.26; H, 5.39; N, 12.49. Found: C, 64.65; H, 5.36; N, 12.44.

Bis[4-(3-lumiflavinyl)butyl] Disulfide, [Fl-C_4-S-]$_2$. 0.5 mL of pyrrolidine and 1 mg of $FeCl_3$ were added to a solution of Fl-C_4-S-COPh (230 mg, 0.513 mmol) in 5 mL of $CHCl_3$. The reaction mixture was stirred with a magnetic stirrer under an air or oxygen atmosphere in the dark at room temperature. The reaction was followed by HPLC. When the starting Fl-C_4-S-COPh was consumed, the mixture was diluted with $CHCl_3$ and washed with dil HCl solution and water. The organic layer was dried over Na_2SO_4, and the solvent was evaporated in vacuo. The residue was chromatograped twice on silica gel, eluting with $CHCl_3$, followed by alumina chromatography, eluting with $CHCl_3$. The fractions containing flavin were combined and concentrated. Since separation of [Fl-C_4-S-]$_2$ from the remaining Fl-C_4-S-COPh could not be achieved even by preparative TLC, the crude product was subjected to LC-separation (LC-09 equipped with JAIGEL 1H). Recrystallization of the isolated [Fl-C_4-S-]$_2$ from ethyl acetate gave yellow needles; mp 227–228°; UV: λ_{max} (ϵ) = 444 nm (25,900), 342 nm (19,200), 271 nm (80,600), 222.5 nm (71,100); δ = 1.76–1.87 (m, 8 H, -CH_2-), 2.45 (s, 6 H, C-CH3), 2.55 (s, 6 H, CCH_3), 2.73 (t, J = 6.2 Hz, 4 H, S-CH_2-), 4.11 (s, 6 H, N^{10}-CH_3), 4.11 (t, 6.3 Hz, 4 H, N^3-CH_2-), 7.42 (s, 2 H, aromatic H), 8.01 (s, 2 H, aromatic H). Anal. Calcd. for $C_{34}H_{38}N_8O_4S_2$: C, 59.45; H, 5.57; N, 16.31. Found: C, 58.96; H, 5.53; N, 16.19. Absence of $FeCl_3$ or use of a prolonged reaction time reduced the yield of [Fl-C_4-S-]$_2$.

Other [Fl-C_n-S-]$_2$ (n = 3, 5, 6) Compounds Were Prepared in a Manner Similar to [Fl-C_4-S-]$_2$

Bis[(3-lumiflavinyl)propyl] Disulfide, [Fl-C_3-S-]$_2$. mp > 300°; UV λ_{max} (ϵ) = 445 nm (25,000), 348 nm (18,000), 273 nm (63,000); NMR ($CDCl_3$) δ = 2.12–2.40 (m, 4 H, -CH_2-), 2.44 (s, 6 H, C-CH_3), 2.54 (s, 6 H, C-CH_3), 2.81 (t, J = 7.0 Hz, 4 H, S-CH_2-), 4.10 (s, 6 H, N^{10}-CH_3), 4.22 (t, 6.0 Hz, 4 H, N^3-CH_2-), 7.40 (s, 2 H, aromatic H), 8.05 (s, 2 H, aromatic H). Anal. Calcd. for $C_{32}H_{34}N_8O_4S_2$: C, 58.34; H, 5.20; N, 17.00. Found: C, 60.09; H, 5.95; N, 15.22.

Bis[(3-lumiflavinyl)pentyl)] Disulfide [Fl-C_5-S-]$_2$. mp 252–253°. UV: λ_{max} (ϵ) = 444 nm (25,800), 342 nm (19,400), 271 nm (81,400), 222 nm (69,000). NMR ($CDCL_3$) d = 1.50–1.90 (m, 12 H, -CH_2-), 2.44 (s, 6 H, C-CH_3), 2.54 (s, 6 H, C-CH_3), 2.68 (t, J = 6.0

Hz, 4 H, CH_2-S), 4.09 (s, 6 H, N^{10}-CH_3), 4.17 (t, J = 6.1 Hz, 4 H, N^3-CH_2-), 7.41 (s, 2 H, aromatic H); 8.02 (s, 2 H, aromatic H). Anal. Calcd. for $C_{36}H_{42}N_8O_4S_2$: C, 60.48; H, 5.92; N, 15.67%. Found: C, 60.09; H, 5.95; N, 15.22%.

Bis[(3-lumiflavinyl)hexyl] Disulfide, [Fl-C_6-S-]$_2$. mp 229–231°; UV λ_{max} (ϵ) = 444 nm (23,100), 340 nm (17,300), 272.5 nm (72,900), 222 nm (64,300). NMR ($CDCl_3$) δ = 1.50–1.85 (m, 16 H, -CH_2-), 2.44 (s, 6 H, C-CH_3), 2.55 (s, 6 H, C-CH_3), 2.66 (t, J = 6.5 Hz, 4 H, S-CH2-), 4.10 (s, 6 H, N^{10}-CH_3), 4.10 (t, J = 6.0 Hz, 4 H, N^3-CH_2-), 7.42 (s, 2 H, aromatic H), 7.99 (s, 2 H, aromatic H). Anal. Calcd. for $C_{38}H_{46}N_8O_4S_2$: C, 61.43; H, 6.22; N, 15.08. Found: C, 61.19; H, 6.22; N, 14.58.

4-(3-lumiflavinyl)butyl Benzyl Disulfide, Fl-C_4-S-S-CH_2Ph. Fl-C_4-S-COPh (154 mg, 0.344 mmol), 0.3 mL of pyrrolidine (0.35 mmol), and 1.32 g of dibenzyl disulfide (0.54 mmol) were allowed to react in 6 mL of $CHCl_3$ in a degassed sealed tube in the dark at 40° for 6 hours. The tube was opened and the contents diluted with $CHCl_3$, washed with dil HCl solution and water, and dried over $MgSO_4$. The solvent was evaporated in vacuo, and the residue was chromatographed on alumina, eluting with CH_2Cl_2. The eluent was subjected to LC-separation (LC-09 equipped with JAIGEL 1H), followed by recrystallization. Yield 60%; mp 78–80°; NMR ($CDCl_3$) δ = 1.65–1.90 (m, 4 H, -CH_2-), 2.43 (s, 3 H, C-CH_3), 2.55 (s, 3 H, C-CH_3), 2.45–2.50 (br 2 H, S-CH_2-), 3.88 (s, 2 H, benzyl H), 4.08 (s, 3 H, N^{10}-CH_3), 4.05–4.20 (br 2 H, N^3-CH_2-), 7.29 (s, 5 H, aromatic H), 7.40 (s, 1 H, aromatic H), 8.03 (s, 1 H, aromatic H). Anal. Calcd. for $C_{24}H_{26}N_4O_2S_2$: C, 61.77; H, 5.61; N, 12.00. Found: C, 61.10; H, 5.56; N, 11.34.

BNAH Dependent Reduction of Dibenzyl Disulfide Catalyzed by [Fl-C_n-S-]$_2$

Dibenzyl disulfide (220 mg, 0.9 mmol) and BNAH (255 mg, 1.2 mmol) were placed in a side arm of the Pyrex tube, while [Fl-C_3-S-]$_2$ (7.2 × 10–4 mmol) in 6 mL of acetonitrile was placed in the bottom of the tube. The acetonitrile solution was carefully degassed by freeze-thaw cycles, and sealed and the components mixed. The tube was heated for 12 hours at 45°C in the dark. After opening of the tube, acetic anhydride (0.5 mL) and pyridine (0.5 mL) were added. The tube was flashed by argon, stoppered, and allowed to stand for 3 hours. The reaction mixture was concentrated in vacuo and chromatographed on silica gel, eluting with hexane. The eluent contained dibenzyl disulfide and S-benzyl thioacetate. The yield of S-benzyl thioacetate was determined by GC.

[Fl-C_4-S-]$_2$ Catalyzed Reduction of Dibenzyl Disulfide in the Presence of 1-methylpyrrolidine under Irradiation with Visible Light

A solution of dibenzyl disulfide (123 mg, 0.5 mmol), 1-methylpyrrolidine (425 mg, 5 mmol), and 0.5 μmol of [Fl-C_n-S-]$_2$ in a mixed solvent (3 mL of ethanol and 3 mL of acetonitrile) was placed in a Pyrex tube. The tube was connected to a vacuum line, degassed by freeze-pumping thaw cycles, and sealed. The evacuated tube was irradiated with a 150 W tungsten lamp for 20 hours at 20–25°C and then opened. After the reaction mixture was acidified with dil HCl, 40μl of 1,3,5-trimethylbenzene was added as an internal standard for GC analysis and extracted with $CHCl_3$. The organic layer was washed with water, dried over Na_2SO_4, and subjected to GC analysis.

Measurement of Cyclic Voltammograms

Cyclic voltammograms were obtained in argon saturated 0.1 M *n*-Bu_4NClO_4/CH_3CN solution. Scan rates were 200, 100, and 50 mVs^{-1}.

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