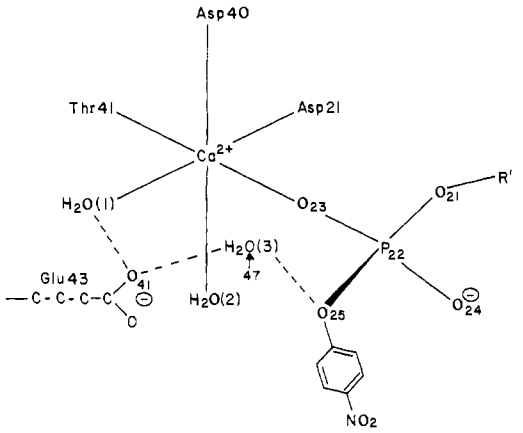


Figure 2. A portion of the system included in the staphylococcal nuclease–Ca^{2+}–*p*-NO_2Ph-pdTp calculation. Amino acid residues which were included in the calculation but not illustrated: Arg 35, Arg 87, Tyr 85, and Tyr 113. Oxygens 23 and 24 are bonded to the enzyme through Arg 35; oxygens 21 and 24 are bonded to Arg 87. R′ is defined in Figure 1. The oxygen atom of $H_2O(3)$ is O_{47}.

causes the calcium coordination sphere to be strained and also causes strain in the bonds and angles between the 5′-phosphorus and ribose. Since the α and β carbons of Glu 43 were held fixed throughout the calculation, movement of O_{41} toward P_{22} strains the bonds and angles of Glu 43.

The results obtained in this calculation might be compared to those obtained in a simulation of ribonuclease action on uridylyl-(3′–5′)-adenosine.[13] There it was shown (under similar restrictions) that the movement of Lys 41 could easily span a 4.8-Å distance to interact with a cyclized intermediate.

An additional calculation was performed to see if any steric interference is present hindering the movement of Glu 43 toward phosphorus. When the α and β carbons of Glu 43 were released from their fixed positions, thus allowing unrestricted movement, the energy of the system was calculated to be quite similar to the energy calculated for attack for $H_2O(3)$. The implication is that motion of Glu 43 is not impeded by steric blockage at the enzyme active site.

On the basis of these calculations, we conclude that initiating attack by Glu 43 is not a feasible mechanism for the hydrolysis by staphylococcal nuclease on *p*-NO_2Ph-pdTp. Only if there were severe modification of the active site in the presence of the latter substrate which allows much closer approach of Glu 43 to the 5′-phosphate, would this mechanism become feasible. Our previous results of ribonuclease action on uridylyl-(3′–5′)-adenosine (UpA) using this model program,[13] which were supported by low-temperature protein crystallographic studies,[14] indicate this possibility highly unlikely.[22] Hence, the favored mechanism involves attack by a water molecule, $H_2O(3)$, in line with the leaving group, thymidine 3′-phosphate (Figure 2).

Acknowledgment. This investigation was supported by a grant from the National Institutes of Health (GM 21466) and is gratefully acknowledged. Appreciation is expressed to the University of Massachusetts Computing Center for generous allocation of computer time.

Registry No. NO_2Ph-pdTp, 24418-11-9; glutamate, 56-86-0; staphylococcal nuclease, 9013-53-0.

(21) The interactions between Arg 35 and Arg 87 with the 5′-phosphate oxygens are not shown in Figure 2 but were included in the calculation. For a discussion of phosphate–arginine bonding, see ref 11 and 12.

(22) A copy of our program is available from R.R.H.

Photochemistry of Flavins with Sulfur-Activated Carboxylic Acids: Identification and Reactions of the Photoproducts

Gert A. Eberlein* and Michael F. Powell†

Contribution from the Institut fur Biologie, Universitaet Konstanz, Am Giesberg, D-7250 Konstanz, West Germany. *Received May 31, 1983*

Abstract: Photoreduction of 3-methyllumiflavin by α-sulfide- or α-disulfide-substituted carboxylic acids does not give dihydroflavin–4a-sulfur adducts or result in the sulfur–carbon bond scission as claimed previously[1,2] (eq 7 and 19). Instead decarboxylation of the acid accompanied by dihydroflavin–4a-carbon adduct formation (eq 8 and 10) was shown to occur. Several other substitution products were also isolated and characterized, including an example of the little known 6-substituted flavins. Isoalloxazine also gave similar products, including the 8-methyl-substituted derivatives, when dithiodiglycolic acid was employed. A primary electron-transfer mechanism between photoexcited lumiflavin and substituted carboxylic acid with consecutive radical coupling is supported. Reaction of 4a-(((carboxymethyl)dithio)methyl)-4a,5-dihydro-3-methyllumiflavin with formic acid and acetic anhydride gave 5-formyl-4a-(((carboxymethyl)dithio)methyl)-4a,5-dihydro-3-methyllumiflavin and 5,8,10,11-tetramethyl-8*H*-benzo[*g*]thiazolo[3,4-*e*]pteridine-4,6-dione (eq 17). The latter compound is a modified flavin containing four rings (ring closure over the 4a and 5 positions) and was found to be stable toward photoinduced oxidation. Dihydroflavin was found to convert sulfides to sulfoxides in the presence of oxygen; two sulfoxy diastereoisomers of 4a-(((carbomethoxymethyl)sulfinyl)methyl)-4a,5-dihydrolumiflavin are described herein (eq 11). Intramolecular reduction of the 6α-disulfide bond in 6-(((carboxymethyl)dithio)methyl)-1,5-dihydrolumiflavin was observed (eq 13). Scission of the disulfide bond in 4a-(((carboxymethyl)dithio)methyl)-4a,5-dihydro-3-methyllumiflavin by various nucleophiles gave 4a,5-dihydro-3-methyllumiflavin-4a-methyl mercaptan (eq 23, 28, 34) which rapidly decomposed to eliminate thioformaldehyde as indicated by the formation of thioformaldehyde polymers of flavin 4a-adducts.

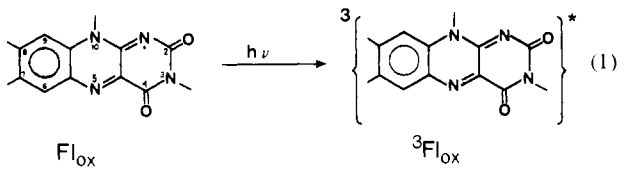
The flavin moiety is the active component of more than a hundred different flavoproteins which are able to undergo both electron- and group-transfer reactions.[3] Oxidized flavins are redox active in both the ground and excited states. The excited state

* Address correspondence to Department of Chemistry, University of California at Santa Barbara, Santa Barbara, CA 93106.
† Present address: Lawrence Berkeley Laboratories, University of California at Berkeley, Berkeley, CA 94720.

(1) Knappe, W.-R.; Hemmerich, P. *Z. Naturforsch., B* **1972**, *27B*, 1032–1034.

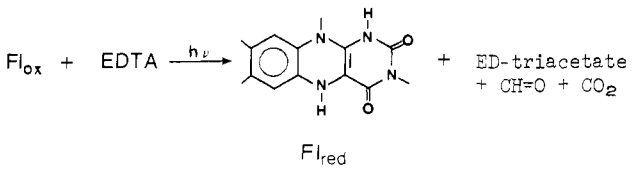
0002-7863/84/1506-3309$01.50/0

is a triplet ($^3Fl_{ox}$*) (eq 1) and reacts with various substrates by



Fl_{ox} $^3Fl_{ox}$ (1)

electron abstraction. The flavin triplet has been shown to react with ethylenediaminetetraacetic acid (EDTA) in aqueous solution[4] to form reduced 3-methyllumiflavin, ethylenediaminetriacetate (ED-triacetate), formaldehyde, and carbon dioxide[5] (eq 2). In

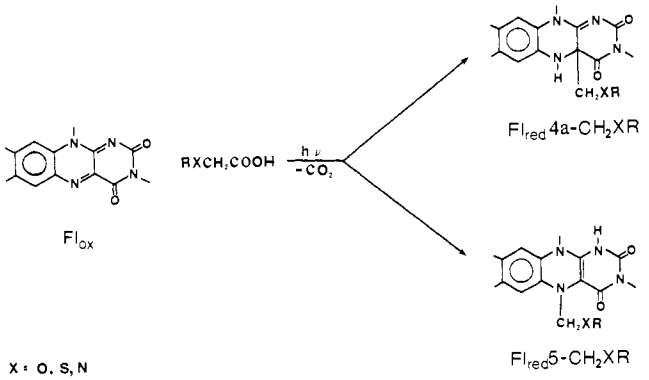


Fl_{red} (2)

nonaqueous solution the flavin triplet reacts with cyclohexadiene[6] (eq 3) to give reduced 3-methyllumiflavin (Fl_{red}) and benzene.

$$^3Fl_{ox}* + \text{cyclohexadiene} \rightarrow Fl_{red} + \text{benzene} \quad (3)$$

Various other substrates (such as α-activated carboxylic acids) have been shown to react with $^3Fl_{ox}$* to give 4a-substituted 4a,5-dihydrolumiflavin adducts and 5-substituted 1,5-dihydrolumiflavin adducts[7] (eq 4). In addition, numerous other substrates



X = O, S, N (4)

for the photoreduction of flavins have been reported,[1] and a review concerning the photoalkylation of the flavin moiety has been given by Heelis.[8]

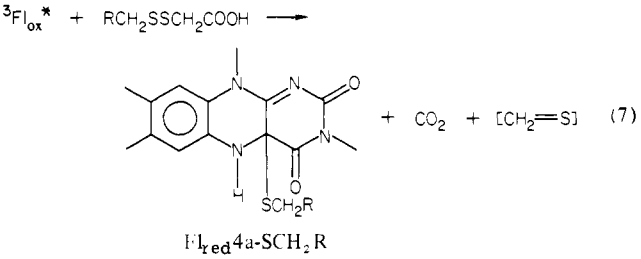
In 1976 Hemmerich proposed that $^3Fl_{ox}$* serves as a model for certain flavoenzymes due to the fact that the ground-state chemistry of the protein-bound flavin moiety and the excited-state chemistry of oxidized flavin may be similar.[9] Several flavoenzymes are known to be involved in mercaptide/disulfide interactions such as glutathione reductase,[10-12] lipoamide dehydrogenase,[13-15] thioredoxin reductase, and CoA-S-glutathione reductase.[16] Glutathione reductase catalyzes the reduction of glutathione (GSSG) by oxidation of NADPH (eq 5) whereas

$$GSSG + NADPH + H^+ \xrightarrow{\text{glutathione reductase}} 2GSH + NADP^+ \quad (5)$$

lipoamide dehydrogenase catalyzes the oxidation of dihydroliponamide [$Lip(SH)_2$] by reduction of NAD^+ (eq 6). The type

$$NAD^+ + Lip(SH)_2 \xrightarrow{\text{lipoamide dehydrogenase}} NADH + H^+ + LipS_2 \quad (6)$$

of flavin/dihydroflavin sulfide/disulfide interaction and the site of initial sulfide/disulfide attack on the flavin moiety were the subject of various studies.[18] The work of Thorpe and Williams,[17] Lochler and Hollocher,[18] Bruice,[19] and Radda[20] has largely resolved these questions by presenting several lines of evidence in favor of an electron-transfer mechanism via flavin–4a-sulfur adduct formation. Such adduct formation is also supported by a direct X-ray structure determination of the active site of glutathione reductase by Schultz and co-workers.[12] It was, therefore, not unusual when Hemmerich and Knappe[1] reported additional evidence in favor of a flavin–4a-sulfur adduct (Fl_{red}4a-SR) on the basis of its preparation from photoexcited lumiflavin and dithiodiglycolic acid (eq 7). A photogenerated adduct of this type



Fl_{red}4a-SCH_2R (7)

could provide a strong cross-link between enzymatic bioorganic reactions and photoinduced organic reactions. This paper reports our investigation of the aforementioned flavin–sulfur interaction and is pertinent to Knappe's earlier work.[1]

Experimental Section

Melting points were determined on a Koffler heating block and are uncorrected. Elemental analyses were performed by Hoffman-La Roche Co. with errors typically less than 0.30%. Spectra were recorded on the following instruments: IR, Perkin-Elmer 621; UV-visible, Varian SuperScan 3 and Cary 118 C; fluorescence, Perkin-Elmer MPF 3; ^1H NMR, 90-MHz Brucker; MS, Varian MAT CH-7. Thin-layer chromatography was carried out by using Silica 60 F 254 plates (Merck No. 5735) with the following solvent systems: A, ethyl acetate (100%); B, $CHCl_3$/MeOH/butanone (82:12:6); and C, ethyl acetate/acetic acid (80:20). Hydrogen ion activity was recorded by using a Metrohm E 366 pH meter and a Metrohm UX microglass electrode. Column chromatographic separations were performed on a 450 × 20 mm silica (Merck

(2) Knappe, W.-R.; Hemmerich, P. *Liebigs Ann. Chem.* **1976**, *1976*, 2037–2057.
(3) Registered and numbered enzymes containing flavins as cofactors: "Enzyme Nomenclature"; Academic Press: New York, 1978.
(4) Frisell, W. R.; Chung, C. W.; Mackenzie, C. G. *J. Biol. Chem.* **1959**, *234*, 1297–1302.
(5) Massey, V.; Stankovich, M.; Hemmerich, P. *Biochemistry* **1978**, *17*, 1–8.
(6) Knappe, W.-R. *Chem. Ber.* **1974**, *107*, 1614–1636.
(7) Walker, W. H.; Massey, V.; Hemmerich, P. *Helv. Chim. Acta* **1967**, *50*, 2269–2279.
(8) Heelis, P. F. *Chem. Soc. Rev.* **1982**, *11*, 15–41.
(9) Hemmerich, P. Prog. Chem. Org. Nat. Prod. **1976**, *33*, 452–527.
(10) Williams, D. H.; Burleigh, B. D.; Ronchi, S.; Arscott, L. D.; Jones, E. D. In "Flavins and Flavoproteins"; Kamin, H., Ed.; University Press: Baltimore, 1970.
(11) Yabroff, D. L. *Ind. Eng. Chem.* **1940**, *32*, 257.
(12) Schulz, G. E.; Schirmer, R. H.; Sachsenheimer, W.; Pai, E. F. *Nature (London)* **1978**, *273*, 120–124.
(13) Williams, C. H. In "The Enzymes", 3rd ed.; Boyer, P., Ed.; Academic Press: New York, 1976; Vol. 13, pp 90–173.
(14) Hemmerich, P.; Nagelschneider, G.; Veeger C. *FEBS Lett.* **1970**, *8*, 69–83.
(15) Massey, V.; Mueller, F.; Feldberg, R.; Schuman, M.; Sullivan, P. A.; Howell, L. G.; Mayhew, S. G.; Matthews, R. H.; Foust, G. P. *J. Biol. Chem.* **1969**, *244*, 3999–4006.
(16) Hemmerich, P.; Massey, V. In "Oxidases and Related Redoxsystems"; Proceedings of the Third International Symposium on Oxidases and Related Redox Systems, King, T. E., Mason, H. S., Morrison, M., Eds.; New York: 1979.
(17) Thorpe, C.; Williams, C. H., Jr. *J. Biol. Chem.* **1976**, *251*, 3553–3557.
(18) Loechler, E. L.; Hollocher, T. C. *J. Am. Chem. Soc.* **1975**, *97*, 3235–3237. Loechler, E. L.; Hollocher, T. C. *Ibid.* **1980**, *102*, 7312–7321.
(19) Yokoe, I.; Bruice, T. C. *J. Am. Chem. Soc.* **1975**, *97*, 450–451.
(20) Gascoigne, I. M.; Radda, G. K. *Biochim.* Biophys. *Acta* **1967**, *131*, 498–507.

7734) column (25 °C) using 1% EtOH in $CHCl_3$ (flow rate 350 mL/h); fractions were detected by UV spectroscopy and refractometry.

Apparatus. A 250-W 24-V tungsten/halogen lamp, equipped with a spherical condenser and heat filter, was used for the analytical photoreductions. Cuvettes were irradiated [420–490 nm, K 45 filter (Balzer, Lichtenstein)] approximately 2 cm from the lamp at 25.5 ± 1.5 °C. The light intensity was stabilized to within 1%. Reactants were deoxygenated before mixing by flushing with oxygen-free (<0.1 ppm) argon for 40 min (flow rate = 15 mL/min). Argon was deoxygenated by use of platinum catalyst (Heraeus Corp.) and contained 1% hydrogen. Solutions in Thunberg cuvettes remained oxygen-free for at least 20 h after mixing [e.g., only 3% of a 7×10^{-5} M Fl_{red} solution (phosphate buffer, pH 7) was oxidized in 25 h as shown by the small change in absorbance at 444 nm]. Perparative photoreactions were carried out in a radiation apparatus (Quickfit/Germany) equipped with a medium-pressure mercury light source (Orginal Hanau TQ 150). A 5% $NaNO_2$ solution was used to filter out light below 400 nm. The solutions were maintained oxygen-free by flushing with argon for 1 h prior to and during irradiation. The reaction temperature was held at approximately 50 °C in order to solubilize the reactants.

Chemicals. Acetonitrile was codistilled with MeOH, redistilled over CaH_2, and passed through a column of basic alumina (activity 1) before being stored over 4 Å molecular sieves.[21] Ethyl acetate was distilled before use. $CHCl_3$ (containing 1% EtOH) was dried over $CaCl_2$ before use. 3-Methyllumiflavin (Fl_{ox}) was donated by Hoffmann-La Roche Co. and was synthesized by known procedures.[22] 3,10-Dimethylisoalloxazine (Fl'_{ox}) was a gift from Dr. A. Wessiak and was synthesized by the method of Kuhn and Weygand.[23] Commercially available 0.1 M "Titrisol" buffer solutions (Merck) were used to span pH 1–11.

Syntheses. ***N*-(Benzylthio)phthalimide.** *N*-Bromophthalimide (11.3 g, 50.0 mmol) and 12.3 g (50.0 mmol) of dibenzyl disulfide were refluxed for 0.6 h in 50 mL of dry benzene. The red reaction mixture was allowed to cool to room temperature before addition of 150 mL of *n*-hexane. The crystals formed were filtered off and were recrystallized from hot EtOH: yield, 10.5 g (80%); mp 167–8 °C (EtOH) (lit. mp 168 °C[24]).

(Benzyldithio)glycolic Acid. *N*-(Benzylthio)phthalimide (26.9 g, 100 mmol) and 9.2 g (100 mmol) of mercaptoacetic acid were refluxed for 24 h in 200 mL of dry benzene. The reaction mixture was filtered and the filtrate was treated with 20 mL of $CHCl_3$ and 20 mL of saturated aqueous $NaHCO_3$ solution before acidifying the aqueous phase with HCl. The aqueous layer was extracted again with $CHCl_3$, the organic extracts were dried, and the solvent was removed by rotary evaporation to yield an oil which crystallized after several days: yield, 9.7 g (50%); mp 68–90 °C; ^1H NMR ($CDCl_3$) δ 10.73 (s, COOH), 7.24 (s, C_6H_5), 3.91 (s, CH_2COO–), 3.14 (s, CH_2Ph).

Anal. Calcd for $C_9H_{10}O_2S_2$: C, 50.45; H, 4.70; S, 29.92. Found: C, 49.48; H, 4.71; S, 30.35.

Dithiodiglycolic Acid Monoethyl Ester. Dithiodiglycolic acid (91.0 g, 500 mmol), 23.0 g (500 mmol) of dry EtOH, and 0.96 g (10.0 mmol) of methanesulfonic acid were refluxed in 100 mL of $CHCl_3$ for 15 h by using a Dean-Stark apparatus. The reaction mixture was extracted with 100 mL of saturated $NaHCO_3$ solution; the aqueous phase was then back-extracted four times with 20-mL aliquots of $CHCl_3$. After treatment of the aqueous phase with decolorizing charcoal, the pH was adjusted to 4 with HCl, resulting in the formation of a colorless oil: yield, 16 g (15%); ^1H NMR ($CDCl_3$) δ 9.34 (s, COOH), 4.20 (q, J = 7.0 Hz, CH_2), 3.61 (s, CH_2), 3.60 (s, CH_2), 1.27 (t, J = 7.0 Hz, CH_3). Note: This compound is susceptible to rearrangement to form the diacid and diester.

4a-((Benzyldithio)methyl)-4a,5-dihydro-3-methyllumiflavin (Fl_{red}4a-CH_2SSCH_2Ph). Procedure 1. Fl_{ox} (270 mg, 1.00 mmol) and 10.7 g (50.0 mmol) of benzyldithioglycolic acid were dissolved in 150 mL of CH_3CN and 100 mL of water. The solution was flushed with argon for 30 min and was then irradiated anaerobically at 50 °C for 18 h. The solvent was evaporated under reduced pressure and the residue was suspended in 50 mL of $CHCl_3$ before being washed three times with 25-mL aliquots of water. The $CHCl_3$ solution was dried ($MgSO_4$) and then reduced to 2.5 mL in volume before 12.5 mL of diethyl ether was added. This resulted in the precipitation of light yellow crystals which were washed with a small amount of ether and dried under vacuum (1×10^{-2} torr) at 50 °C for 10 h: yield, 198 mg (45%); R_f 0.52 (A); mp 179 °C ($CHCl_3$/ether); IR (KBr), 3330 (N(5)—H), 1725 (C(4)=O),[25] 1675 (C(2)=O), 1570 cm^{-1} (C—C aromatic); UV (CH_3CN) λ_{max} [ϵ (M^{-1} cm^{-1})] 359 (6200), 295 (sh), 274 (12 600), 222 nm (27 500); UV (6 N HCl) λ_{max} [ϵ (M^{-1} cm^{-1})] 406 (2600), 300 (sh), 272 (10 700), 219 nm (23 400); ^1H NMR ($CDCl_3$) δ 7.13 (s, C_6H_5), 6.82 (s, 6-H), 6.56 (s, 9-H), 4.86 (s, 5-H), 3.55 (s, 10-CH_3), 3.37 (s, CH_2), 3.29 (s, 3-CH_3), 2.50 (q, J = 14.0 Hz, a = 7.4 Hz, 4a-CH_2), 2.25 (s, 8-CH_3), 2.20 (s, 7-CH_3); MS (70 eV, 250 °C), m/e 440 (16%, M^+), 271 (100%, $M^+ - CH_2S_2Bz$).

Anal. Calcd for $C_{22}H_{24}N_4O_2S_2$ (M_r 440.6): C, 59.98; H, 5.49; N, 12.72; S, 14.55. Found: C, 59.97; H, 5.40; N, 12.47; S, 14.95. Note: The dihydroflavin–4a-adduct solutions were very light sensitive in the presence of oxygen and were thus kept in the dark during workup. Approximately 30% of the Fl_{ox} was reduced to Fl_{red} in this preparation.

Procedure 2. Fl_{red}4a-CH_2SSCH_2Ph could also be prepared by the addition of a suspension of 45 mg (0.10 mmol) of Fl_{red}4a-CH_2SSCH_2COOEt (vide infra) in 3 mL of CH_3CN to 12.4 mg (0.10 mmol) of benzyl mercaptan in 1 mL of saturated $NaHCO_3$ solution. After vigorous shaking for 30 s, 20 mL of water was added, and the solution was extracted several times with $CHCl_3$. The $CHCl_3$ was dried and evaporated to yield the product which could be further purified by column chromatography (silica/$CHCl_3$): yield, 4.5 mg (10%) of a mixture of different 4a-substituted 4a,5-dihydroflavins; MS (70 eV, 250 °C), m/e 440 (2%, Fl_{red}4a-CH_2SSCH_2Ph), 486 (4%, Fl_{red}4a-$(CH_2S)_2SCH_2Ph$), 482 (4%, Fl_{red}4a-$(CH_2S)_2SCH_2COOEt$), 532 (0.5%, Fl_{red}4a-$(CH_2S)_3SCH_2Ph$), 526 (0.3%, Fl_{red}4a-$(CH_2S)_3SCH_2COOEt$).

4a-(((Carboxymethyl)dithio)methyl)-4a,5-dihydro-3-methyllumiflavin (Fl_{red}4a-CH_2SSCH_2COOH). Fl_{ox} (270 mg, 1.00 mmol) and 9.10 g (50.0 mmol) of dithiodiglycolic acid were dissolved in 150 mL of water and 100 mL of CH_3CN. (Reactions carried out in pure CH_3CN afforded identical reaction products in similar yields.) The solution was flushed with argon for 30 min and was then irradiated under anaerobic conditions at 50 °C for 18 h. The solvent was evaporated under reduced pressure and the residue was suspended in 50 mL of $CHCl_3$, which was then washed with water (5 × 25 mL) and reduced to 20 mL in volume. The precipitate was collected by suction filtration and was washed with ether before drying under vacuum at 50 °C. The product was purified by dissolving in 20 mL of saturated $NaHCO_3$, washing with $CHCl_3$, and, after adjustment of the pH of the aqueous phase to 2 (HCl), extracting into small portions of $CHCl_3$. After the solution was dried ($MgSO_4$) and concentrated to 2 mL it afforded small, light-yellow crystals: yield, 150 mg (37%); R_f 0.14 (B); mp 198 °C dec; IR (KBr), 3290 (N(5)—H and —COOH), 1730 (—C=OOH), 1716 (C(4)=O), 1665 (C(2)=O), 1558 cm^{-1} (C—C aromatic); UV (CH_3CN) λ_{max} [ϵ (M^{-1} cm^{-1})] 358 (6500), 300 (sh), 273 (15 100), 222 nm (49 000); UV (6 N HCl) λ_{max} [ϵ (M^{-1} cm^{-1})] 395 (2600), 297 (sh), 265 nm (11 500); ^1H NMR (Me_2SO-d_6) δ 8.14 (s, COOH), 7.02 (s, 6-H), 6.91 (s, 9-H), 6.63 (s, N(3)–CH_3), 3.18 (q, J = 14.9 Hz, a = 17.8 Hz, 4a-CH_2), 2.18 (s, 8-CH_3), 2.13 (s, 7-CH_3); MS (70 eV, 200 °C), m/e 408 (1%, M^+), 271 (100, $M^+ - CH_2S_2CH_2COOH$).

Anal. Calcd for $C_{17}H_{20}N_4O_4S_2$ (M_r 408.5): C, 49.99; H, 4.93; N, 13.72; S, 15.70. Found: C, 49.50; H, 5.00; N, 13.12; S, 15.46.

4a-(((Carbomethoxymethyl)dithio)methyl)-4a,5-dihydro-3-methyllumiflavin (Fl_{red}4a-CH_2SSCH_2COOMe). Fl_{red}4a-CH_2SSCH_2COOH (405 mg, 1.00 mmol) was suspended in 10 mL of $CHCl_3$ and 10 mL of MeOH and then treated with small portions of an ethereal diazomethane solution until nitrogen evolution ceased. The $CHCl_3$ and MeOH were removed by rotary evaporation and the residue was taken up into $CHCl_3$. The crude product was purified by column chromatography and recrystallized from 5 mL of $CHCl_3$ and 25 mL of ether to give pale yellow crystals: yield, 405 mg (96%); R_f 0.44 (A); mp 172 °C ($CHCl_3$/ether); IR (KBr), 3315 (N(5)—H), 1730 (C=OOR), 1710 (C(4)=O), 1663 (C(2)=O), 1565 (C—C aromatic), 1320 sy, 1285 cm^{-1} as (OCOR); UV (CH_3CN) λ_{max} [ϵ (M^{-1} cm^{-1})] 363 (6500), 300 (sh), 275 (13 800), 218 nm (33 100); ^1H NMR ($CDCl_3$) δ 6.87 (s, 6-H), 6.64 (s, 9-H), 4.91 (s, 5-H), 3.63 (s, OCH_3), 3.62 (s, 10-CH_3), 3.38 (s, CH_2), 3.33 (s, 3-CH_3), 3.19 (q, J = 12.2 Hz, a = 3.5 Hz, 4a-CH_2), 2.23 (s, 8-CH_3), 2.21 (s, 7-CH_3); MS (70 eV, 150 °C), m/e 424 (11%, $M^+ + 2$), 423 (25, $M^+ + 1$), 422 (100, M^+).

Anal. Calcd for $C_{18}H_{22}N_4O_4S_2$ (M_r 422.5): C, 51.17; H, 5.25; N, 13.26; S, 15.18. Found: C, 51.27; H, 5.29; N, 13.29; S, 14.86.

4a-(((Carboethoxymethyl)dithio)methyl)-4a,5-dihydro-3-methyllumiflavin (Fl_{red}4a-CH_2SSCH_2COOEt). Fl_{ox} (270 mg, 1.00 mmol) and 2.10 g (10.0 mmol) of dithiodiglycolic acid monoethyl ester were dissolved in 150 mL of CH_3CN and 100 mL of water. The solution was irradiated at 50 °C for 18 h under anaerobic conditions. The solvent was then evaporated under vacuum and the residue dissolved in 50 mL of $CHCl_3$. The $CHCl_3$ solution was washed twice with 25-mL aliquots of water and dried ($MgSO_4$), and the solvent was removed. The crude product was purified by column chromatography. The second, slightly yellow fraction was reduced to 2.5 mL in volume, and 13 mL of ether was added, causing light yellow crystals to form after several hours: yield, 210 mg (48%); R_f 0.46 (A); mp 152 °C ($CHCl_3$/ether); IR (KBr) 3340 (N(5)—H), 1735 (C=OOR), 1720 (C(4)=O), 1660 (C(2)=O), 1560 (C—C aro-

(21) Kiesele, H. *Anal. Chem.* **1980**, 2230–2232.
(22) Hemmerich, P. *Helv. Chim. Acta* **1964**, *47*, 464–475.
(23) Kuhn, R.; Weygand, F. *Ber. Dtsch. Ges.* **1934**, *67*, 1409–1413.
(24) Buechel K. H.; Conte, A. *Chem. Ber.* **1967**, *100*, 1248–1251.
(25) Hemmerich, P.; Prijs B.; Erlenmeyer E. *Helv. Chim. Acta* **1960**, *48*, 372–394.

matic), 1308 cm^{-1} as (COOR); UV (CH_3CN) λ_{max} [ϵ (M^{-1} cm^{-1})] 358 (6500), 300 (sh), 275 (13 500), 221 nm (29 500); ^1H NMR ($CDCl_3$) δ 6.86 (s, 6-H), 6.64 (s, 9-H), 4.93 (s, 5-H), 4.08 (q, J = 7.0 Hz, $-OCH_2$), 3.62 (s, 10-CH_3), 3.37 (s, SCH_2), 3.33 (s, 3-CH_3), 3.20 (q, J = 14.2 Hz, a = 4.0 Hz, 4a-CH_2), 2.22 (s, 8-CH_3), 2.20 (s, 7-CH_3), 1.21 (t, J = 7.0, CH_2CH_3); MS (70 eV, 225 °C), m/e 436 (16%, M^+), 271 (100, M^+ - CH_2SSCH_2COOEt).

Anal. Calcd for $C_{19}H_{24}N_4O_4S_2$ (M_r 436.5): C, 52.28; H, 5.54; N, 12.83; S, 14.69. Found: C, 52.01; H, 5.46; N, 12.72; S, 14.63.

5-Formyl-4a-(((Carbomethoxymethyl)dithio)methyl)-4a,5-dihydro-3-methyllumiflavin (Fl_{red}4a-CH_2SSCH_2COOMe-5-CHO). Fl_{red}4a-CH_2SSCH_2COOMe (42.2 mg, 0.10 mmol), 2 mL of acetic anhydride, and 8 mL of formic acid were heated at 50 °C for 2 h. The solution was evaporated to dryness and the product purified by column chromatography (ethyl acetate). The third fraction was evaporated to dryness and dissolved in a small amount of $CHCl_3$, and ether was added until slight turbitity occurred. After several hours, small colorless crystals formed: yield, 39 mg (87%); R_f 0.17 (A), 0.68 (B); mp 238 °C ($CHCl_3$/ether); IR (KBr) 1690 (5-CH=O), 1750 (C=OOR), 1730 (C(4)=O), 1670 (C(2)=O), 1325 sy, 1300 cm^{-1} as (COOR); UV (CH_3CN) λ_{max} [ϵ (M^{-1} cm^{-1})] 320 (9100), 260 (sh), 218 nm (17 400); ^1H NMR ($CDCl_3$) δ 8.30 (s, CHO), 7.30 (s, 6-H), 7.05 (s, 9-H), 3.70 (s, 10-CH_3), 3.58 (s, OCH_3), 3.43 (s, 3-CH_3), 3.36 (s, CH_2), 3.25 (q, J = 14.2 Hz, a = 2.7 Hz, 4a-CH_2), 2.38 (s, 8-CH_3), 2.35 (s, 7-CH_3); MS (70 eV, 175 °C), m/e 450 (2%, M^+), 422 (67, M^+ - CO), 395 (metastable), 271 (100, M^+ - CO - $CH_2SSCH_2COOCH_3$).

5-Formyl-4a-(((Carboxymethyl)dithio)methyl)-4a,5-dihydro-3-methyllumiflavin (Fl_{red}4a-CH_2SSCH_2COOH-5-CHO). This was prepared from Fl_{red}4a-CH_2SSCH_2COOH under similar reaction conditions used for the preparation of Fl_{red}4a-CH_2SSCH_2COOMe-5-CHO from Fl_{red}4a-CH_2SSCH_2COOMe: yield, 41 mg (94%); R_f 0.14 (B); mp 271 °C dec, DMF/MeOH/ether; IR (KBr) 1730 (C=OOR), 1720 (C(4)=O), 1690 (N(5)CH=O), 1670 (C(2)=O), 1565 (C—C aromatic), 1325 sy, 1300 cm^{-1} as (COOR); UV (CH_3CN) λ_{max} [ϵ (M^{-1} cm^{-1})] 321 (9500), 260 (sh), 242 nm (10 200); ^1H NMR (DMF-d_7) δ 8.50 (s, 5-CHO), 7.42 (s, 6-H, 9-H), 3.64 (s, 10-CH_3), 3.52 (s, CH_2COO), 3.33 (q, J = 14.4 Hz, a = 6.3 Hz, 4a-CH_2), 3.29 (s, 3-CH_3), 2.34 (s, 7-CH_3), 2.31 (s, 8-CH_3); MS (70 eV, 325 °C), m/e 436 (17%, M^+), 271 (100, M^+ - CO - CH_2SSCH_2COOH).

Anal. Calcd for $C_{18}H_{20}N_4O_5S_2$ (M_r 436.5): C, 49.53; H, 4.62; N, 12.84; S, 14.69. Found: C, 49.77; H, 4.68; N, 12.53; S, 14.20.

4a-(((Carboxymethyl)thio)methyl)-4a,5-dihydro-3-methyllumiflavin (Fl_{red}4a-CH_2SCH_2COOH). This was prepared from Fl_{ox} and thiodiglycolic acid under similar reaction conditions used for the preparation of Fl_{red}4a-CH_2SSCH_2COOH: yield, 150 mg (40%); R_f 0.83 (D); mp 235 °C dec; UV (phosphate buffer, pH 7.0) λ_{max} [ϵ (M^{-1} cm^{-1})] 366 (6300), 300 (sh), 268 (14 800), 224 nm (25 700); MS (70 eV, 225 °C), m/e 376 (10%, M^+), 213 (100).

6-(((Carboxymethyl)dithio)methyl)-3-methyllumiflavin (Fl_{ox}6-CH_2SSCH_2COOH). The remaining $CHCl_3$ solution from the preparation and recrystallization of Fl_{red}4a-CH_2SSCH_2COOH was subjected to column chromatography (silica/ethyl acetate). The desired product was eluted from the column after the Fl_{ox} was removed. The solvent was evaporated to dryness and the residue dissolved in a small amount of $CHCl_3$. Addition of ether caused yellow crystals to precipitate: yield, 9 mg (2%); mp 320 °C dec; IR (KBr) 3400 (COOH), 1715 (C=OOR), 1700 (C(4)=O), 1645 cm^{-1} (C(2)=O); UV (CH_3CN) λ_{max} [ϵ (M^{-1} cm^{-1})] 451 (12 600), 375 (10 500), 271 (46 800), 229 nm (33 100); ^1H NMR (Me_2SO-d_6) δ 7.78 (s, 9-H), 4.84 (s, 6-CH_2), 4.07 (s, 10-CH_3), 3.65 (s, SCH_2-), 3.36 (s, 3-CH_3), 2.62 (s, 8-CH_3), 2.51 (s, 7-CH_3).

6-(((Carbomethoxymethyl)dithio)methyl)-3-methyllumiflavin (Fl_{ox}6-CH_2SSCH_2COOMe). Fl_{ox}6-CH_2SSCH_2COOH (4.05 mg 0.01 mmol), 2 mL of MeOH, 0.02 mL of methanesulfonic acid, and 4 mL of $CHCl_3$ were refluxed for 1 h. The solution was then evaporated to dryness, and the product was purified by column chromatography: yield, 3.5 mg (83%); R_f 0.52 (B); mp 212 °C dec ($CHCl_3$/ether); IR (KBr) 1730 (C=OOR), 1700 (C(4)=O), 1650 (C(2)=O), 1540 (C—C aromatic), 1285 sy (COOR), 1256 cm^{-1} as (COOR); UV (CH_3CN) λ_{max} [ϵ (M^{-1} cm^{-1})] 448 (12 600), 363 (10 500), 272 (43 700), 227 nm (30 900); UV (6 N HCl) λ_{max} [ϵ (M^{-1} cm^{-1})] 394 (20 900), 273 (26 300), 224 nm (30 200); ^1H NMR ($CDCl_3$) δ 7.39 (s, 9-H), 4.74 (s, 6-CH_2), 4.11 (s, 10-CH_3), 3.73 (s, CH_2COO), 3.71 (s, OCH_3), 3.47 (s, 3-CH_3), 2.58 (s, 8-CH_3), 2.47 (s, 7-CH_3); MS (70 eV, 350 °C), m/e 420 (5%, M^+), 315 (100, M^+ - SCH_2COOCH_3).

Anal. Calcd for $C_{18}H_{20}N_4O_4S_2$ (M_r 420.5): C, 51.41; H, 4.79; N, 13.32; S, 15.22. Found: C, 51.33; H, 4.72; N, 12.89; S, 13.07; fluorescence spectra (phosphate buffer, pH 7.0), λ_{max} 518 nm (emission, uncorrected). The fluorescence intensity was 3% of the fluorescence of a solution of Fl_{ox} of equivalent concentration ±10%.

4a-(((Carboxymethyl)dithio)methyl)-4a,5-dihydro-3,10-dimethylisoalloxazine (Fl'_{red}4a-CH_2SSCH_2COOH). 3,10-Dimethylisoalloxazine (Fl'_{ox}) (242 mg, 1.00 mmol) and 9.10 g (50.0 mmol) of dithiodiglycolic acid were allowed to react for 6 h in a procedure similar to that for the preparation of Fl_{red}4a-CH_2SSCH_2COOH: yield, 275 mg (72%); R_f 0.11 (A); mp 182 °C; IR (KBr) 3290 (N(5)—H), 3265 (COOH), 1720 (C=OOR), 1705 (C(4)=O), 1660 (C(2)=O), 1140 sy, 1120 cm^{-1} as (OCOH); UV (CH_3CN) λ_{max} [ϵ (M^{-1} cm^{-1})] 352 (6500), 267 (sh), 222 nm (27 000); UV (6 N HCl) λ_{max} [ϵ (M^{-1} cm^{-1})] 381 (2500), 290 (sh), 262 (11 500), 213 nm (22 900); ^1H NMR ($CDCl_3$) δ 11.43 (s, COOH), 6.99 (m, 6-, 7-, 8-, 9-H), 5.17 (s, 5-H), 3.65 (s, 10-CH_3), 3.42 (s, CH_2COO), 3.35 (s, 3-CH_3), 3.29 (q, J = 14.0 Hz, a = 7.6 Hz, 4a-CH_2); MS (70 eV, 300 °C), m/e 380 (100%, M^+).

4a-(((Carbomethoxymethyl)dithio)methyl)-4a,5-dihydro-3,10-dimethylisoalloxazine (Fl'_{red}4a-CH_2SSCH_2COOMe). This was prepared by esterification of Fl'_{red}4a-CH_2SSCH_2COOH using a method simliar to that used for the preparation of Fl_{red}4a-CH_2SSCH_2COOMe: yield, 380 mg (96%); R_f 0.70 (A); mp 164 °C ($CHCl_3$/ether); IR (KBr) 3350 (N(5)—H), 1722 (C=OOR), 1710 (C(4)=O), 1660 (C(2)=O), 1320 sy, 1300 cm^{-1} as (OCOR); UV (CH_3CN) λ_{max} [ϵ (M^{-1} cm^{-1})] 358 (6300), 270 nm (12 600); ^1H NMR ($CDCl_3$) δ 6.96 (m, 6-, 7-, 8-, 9-H), 5.18 (s, 5-H), 3.59 (s, 10-CH_3), 3.55 (s, OCH_3), 3.32 (s, CH_2COO), 3.25 (s, 3-CH_3), 3.16 (q, J = 15.3 Hz, a = 4.2 Hz, 4a-CH_2); MS (70 eV, 350 °C) m/e 394 (16%, M^+), 243 (100, M^+ - $CH_2SSCH_2COOCH_3$).

Anal. Calcd for $C_{16}H_{18}O_4N_4S_2$ (M_r 394.5): C, 48.72; H, 4.60; N, 14.20; S, 16.25. Found: C, 48.71; H, 4.68; N, 14.29; S, 16.12.

6-(((Carboxymethyl)dithio)methyl)-3,10-dimethylisoalloxazine (Fl'_{ox}6-CH_2SSCH_2COOH). The remaining $CHCl_3$ solution from the aforementioned preparation of Fl'_{red}4a-CH_2SSCH_2COOH afforded the product after a workup similar to that used for the isolation of Fl_{ox}6-CH_2SSCH_2COOH: yield, 3 mg (1%); R_f 0.11 (B); ^1H NMR ($CDCl_3$) δ 7.79 (m, 7-, 8-, 9-H), 4.88 (s, 6-CH_2), 4.16 (s, 10-CH_3), 3.59 (s, 3-CH_3), 3.44 (s, CH_2COO).

6-(((Carbomethoxymethyl)dithio)methyl)-3,10-dimethylisoalloxazine (Fl'_{ox}6-CH_2SSCH_2COOMe). This was prepared from Fl'_{ox}6-CH_2SSCH_2COOH by a procedure similar to that used for the preparation of Fl_{ox}6-CH_2SSCH_2COOMe: yield, 2.8 mg (72%); R_f 0.47 (B); mp 151 °C ($CHCl_3$/ether); IR (KBr) 1730 (C=OOR), 1700 (C(4)=O), 1655 (C(2)=O), 1565 (C—C aromatic), 1290 sy, 1270 cm^{-1} as (COOR); UV (CH_3CN) λ_{max} [ϵ (M^{-1} cm^{-1})] 442 (11 200), 350 (9800), 268 (26 300), 225 nm (17 400); UV (6 N HCl) λ_{max} [ϵ (M^{-1} cm^{-1})] 379 (15 850), 266 (20 900), 217 nm (25 700); ^1H NMR ($CDCl_3$) δ 7.75 (m, 7-, 8-, 9-H), 4.65 (s, 6-CH_2), 4.15 (s, 10-CH_3), 3.78 (s, OCH_3), 3.60 (s, CH_2COO), 3.52 (s, 3-CH_3).

Bis(4a,5-dihydro-3-methyllumiflavin-4a-methyl) Disulfide (Fl_{red}4a-CH_2S-)$_2$. Fl_{ox}4a-CH_2SSCH_2COOH (40.8 mg, 0.10 mmol) and 2 mL of concentrated HCl were stirred together for 10 h at 30 °C in the dark. The solvent was removed and the residue dissolved in 2 mL of $CHCl_3$ before purification by column chromatography (silica/$CHCl_3$): yield, 27 mg (85%); R_f 0.29 (A); mp 195 °C dec ($CHCl_3$/ether); IR (KBr) 3330 (N(5)—H), 1715 (C(4)=O), 1670 cm^{-1} (C(2)=O); UV (CH_3CN) λ_{max} [ϵ (M^{-1} cm^{-1})] 360 (9500), 300 (sh), 270 (24 000), 225 nm (47 900); UV (6 N HCl) 401 (38 000), 300 (sh), 265 (3000), 234 nm (33 900); ^1H NMR ($CDCl_3$) δ 6.89 (s, 6-H), 6.48 (s, 9-H), 4.83 (s, 5-H), 3.60 (s, 10-CH_3), 3.27 (s, 3-CH_3), 2.99 (s, 4a-CH_2), 2.22 (s, 7-CH_3 and 8-CH_3); MS (70 eV, 350 °C), m/e 317 (0.5%, $M^+/2$ - 2).

Anal. Calcd for $C_{30}H_{34}N_8O_4S_2$ (M_r 637.8): C, 56.97; H, 5.37; N, 17.57; S, 10.05. Found: C, 56.11; H, 5.48; N, 17.30; S, 10.50. Formula weight as determined by ebullioscopy = 555 ± 110.

4a,5-Dihydro-3-methyl-4a,5-(2-thiapropano)lumiflavin (5,8,10,11-Tetramethyl-8*H*-benzo[*g*]thiazolo[3,4-e]pteridine-4,6-dione) (Fl_{red}4a,5-$(CH_2)_2S$). Fl_{ox} (1.35 g, 5.00 mmol) and 45.5 g (250 mmol) of dithiodiglycolic acid were dissolved in 150 mL of CH_3CN and 50 mL of water. The mixture was treated as for the preparation of Fl_{red}4a-CH_2SSCH_2COOH except that it was maintained at 50 °C for 34 h. The solvent was removed and the residue treated twice with a mixture of 80 mL of formic acid and 20 mL of acetic anhydride. The remaining solid was dissolved in $CHCl_3$ after the second treatment, which was then washed four times with 20-mL aliquots of water and once with 50 mL of saturated $NaHCO_3$ solution. The $CHCl_3$ was dried over anhydrous Na_2SO_4 and worked up as for Fl_{red}4a-CH_2SSCH_2COOEt: yield, 46 mg (3%); R_f 0.56 (A); mp 220 °C dec ($CHCl_3$/ether); IR (KBr) 1710 (C(4)=O), 1660 cm^{-1} (C(2)=O), UV (CH_3CN) λ_{max} [ϵ (M^{-1} cm^{-1})] 363 (5100), 307 (5000), 275 (12 900), 210 nm (29 500); ^1H NMR ($CDCl_3$) δ 6.87 (s, 6-H, 9-H), 5.28 (q, J = 9.38 Hz, a = 5.14 Hz, 5-CH_2), 3.68 (s, 10-CH_3), 3.39 (q, J = 11.2 Hz, a = 7.6 Hz, 4a-CH_2), 3.28 (s, 3-CH_3), 3.25 (s, 8-CH_3), 3.23 (s, 7-CH_3); MS (70 eV, 150 °C), m/e 332 (18%, M^+ + 2), 331 (45, M^+ + 1) 330 (100, M^+).

Anal. Calcd for $C_{16}H_{18}N_4O_2S$ (M_r 330.4): C, 58.16; H, 5.49; N, 16.96; S, 9.71. Found: C, 58.12; H, 5.52; N, 16.67; S, 9.83.

4a-(((Carbomethoxymethyl)sulfinyl)methyl)-4a,5-dihydro-3-methyl-

lumiflavin (Fl_{red}4a-CH_2SOCH_2COOMe). Procedure 1. Fl_{ox} (270 mg, 1.00 mmol) and 7.5 g (50 mmol) of thiodiglycolic acid were dissolved in 150 mL of CH_3CN and 100 mL of water. The solution was alternately irradiated at 50 °C for 2 h under anaerobic conditions and reoxidized with air in the dark until the green color disappeared. The workup was similar to that used for the preparation of Fl_{red}4a-CH_2SSCH_2COOH. The methyl ester was prepared in the usual manner. Diastereoisomer A: yield, 8 mg (4%); R_f 0.09 (A); mp 250 °C dec; IR (KBr) 3220 (N(5)—H), 1740 (C=OOR), 1730 (C(4)=O), 1670 (C(2)=O), 1320 sy, 1258 as (OCOR), 1040 cm^{-1} (S=O); UV (MeOH) λ_{max} [ϵ (M^{-1} cm^{-1})] 362 (6200), 300 (sh), 274 (12 300), 228 nm (17 000); ^1H NMR ($CDCl_3$) δ 6.90 (s, 6-H), 6.65 (s, 9-H), 4.97 (s, 5-H), 3.64 (s, 0-CH_3 and 10-CH_3), 3.62 (q, J = 13.6 Hz, a = 3 Hz, $SOCH_2$), 3.34 (s, 3-CH_3), 3.23 (q, J = 13.5, a = 22.8 Hz, 4a-CH_2), 2.23 (s, 7-CH_3 and 8-CH_3); MS (70 eV, 200 °C), m/e 406 (80%, M^+), 271 (100, M^+ - CH_2SOCH_2COOMe).

Anal. Calcd for $C_{18}H_{22}N_4O_5S$ (M_r 406.5): C, 53.19; H, 5.46; N, 13.78. Found: C, 52.12; H, 5.61; N, 13.12.

Diastereoisomer B: yield, 8 mg (4%); R_f 0.14 (A); mp 250 °C dec ($CHCl_3$/ether). IR (KBr) and UV (MeOH) were similar to that of A: UV (acetic acid) λ_{max} [ϵ (M^{-1} cm^{-1})] 364 (6200), 302 (sh), 275 (12 300), 248 nm (13 800); ^1H NMR ($CDCl_3$) δ 6.90 (s, 6-H), 6.70 (s, 9-H), 5.06 (s, 5-H), 3.70 (s, OCH_3), 3.66 (s, 10-CH_3), 3.61 (q, J = 14.4 Hz, a = 1,8 Hz, $SOCH_2$), 3.34 (s, 3-CH_3), 3.18 (q, J = 13.2 Hz, a = 18.8 Hz, 4a-CH_2), 2.24 (s, 8-CH_3), 2.23 (s, 7-CH_3); MS (70 eV, 225 °C), m/e 406 (16%, M^+), 271 (100, M^+ - CH_2SOCH_2COOMe).

Anal. Calcd for $C_{18}H_{22}N_4O_5S$ (M_r 406.5): C, 53.19; H, 5.46; N, 13.78. Found: C, 52.10; H, 5.94; N, 12.82.

Procedure 2. Fl_{red}4a-CH_2SCH_2COOMe (10 mg, 0.026 mmol) was dissolved in 2 mL of 30% H_2O_2 and 2 mL of acetone. The mixture was kept at 50 °C for 5 h and worked up in a procedure similar to that used for the preparation of Fl_{red}4a-CH_2SSCH_2COOEt.

4a-((*tert*-Butyldithio)methyl)-4a,5-dihydro-3-methyllumiflavin (Fl_{red}4a-CH_2SS-*t*-Bu). Fl_{red}4a-CH_2SSCH_2R (where R = Ph, COOH, COOMe, or COOEt) (100 mg, 0.24 mmol) was shaken with 3 mL of aqueous $NaHCO_3$ solution, 5 mL of CH_3CN, and 0.2 mL (2.23 mmol) of *tert*-butyl mercaptan for 60 s. The reaction mixture was added to 20 mL of water and then extracted three times with 20-mL aliquots of $CHCl_3$. The $CHCl_3$ was dried and removed to yield a residue which was purified by column chromatography (silica/$CHCl_3$): yield, 3.5 mg (4%); R_f 0.54 (A); mp 179 °C ($CHCl_3$/ether); IR (KBr) 3320 (N(5)—H), 2960 (C—$(CH_3)_3$), 1715 (C(4)=O), 1670 (C(2)=O), 1565 cm^{-1} (C—C aromatic); UV (CH_3CN) λ_{max} [ϵ (M^{-1} cm^{-1})] 358 (7760), 300 (sh), 274 (15 850), 218 nm (40 700); UV (6 N HCl) λ_{max} [ϵ (M^{-1} cm^{-1})] 401 (3240), 300 (sh), 270 (14 500), 230 (sh), 215 nm (29 500); ^1H NMR ($CDCl_3$) δ 6.82 (s, 6-H), 6.01 (s, 9-H), 4.87 (s, 5-H), 3.59 (s, 10-CH_3), 3.31 (s, 3-CH_3), 3.04 (s, 4a-CH_2), 2.18 (s, 7-CH_3), 2.18 (s, 8-CH_3), 1.17 (s, *t*-Bu); MS (70 eV, 175 °C), m/e 406 (33%, M^+), 271 (100, M^+ - CH_2SS-*t*-Bu).

4a,5-Dihydro-3-methyl-4a-(thiocyanatomethyl)lumiflavin (Fl_{red}4a-CH_2SCN). Fl_{red}4a-CH_2SSCH_2R (where R = Ph, COOH, COOMe, or COOEt) (100 mg, 0.245 mmol) was suspended in 10 mL of $CHCl_3$ and then vigorously shaken with 750 mg (15.3 mmol) of sodium cyanide in 3 mL of buffer (pH 9.4) for 2 min. The $CHCl_3$ phase was separated and dried, and the products were purified by column chromatography (silica/$CHCl_3$): yield, 15 mg (18%); R_f 0.37 (A); IR (KBr) 3325 (N(5)—H), 2158 (-SCN), 1718 (C(4)=O), 1670 (C(2)=O), 1560 cm^{-1} (C—C aromatic); UV (CH_3CN) λ_{max} [ϵ (M^{-1} cm^{-1})] 357 (7400), 300 (sh), 272 (15 800), 221 nm (33 900); UV (6 N HCl) λ_{max} [ϵ (M^{-1} cm^{-1})] 392 (4400), 301 nm (6800); MS (70 eV, 200 °C), m/e 343 (18%, M^+), 271 (100, M^+ - CH_2SCN). This procedure also yields 45 mg (58%) of (Fl_{red}4a-CH_2S-)$_2$.

Results

Irradiation of a mixture of Fl_{ox} and dithiodiglycolic acid (or its derivatives) in pure acetonitrile or acetonitrile–water mixtures gives Fl_{red}4a-CH_2SSCH_2R (R = Ph, COOH, COOEt) (eq 8).

$$^3Fl_{ox}^* + RCH_2SSCH_2COOH \rightarrow Fl_{red}4a\text{-}CH_2SSCH_2R + CO_2 \quad (8)$$

The UV/vis spectrum of Fl_{red}4a-CH_2SSCH_2COOH [λ_{max} 358 nm (ϵ 6500 M^{-1} cm^{-1}) and a less intense shoulder at 300 nm[7]] is characteristic of 4a-substituted 4a,5-dihydroflavins. The ^1H NMR exhibits a quartet at δ 2.50 ppm (J = 14.0, a = 7.4 Hz) attributable to the 4a-methylene group which is characteristic of RCH_2-substituents at the 4a-position of 4a,5-dihydrolumiflavins.[7] Considerable amounts (~30%) of unsubstituted dihydrolumiflavin (Fl_{red}) are also formed during the course of this reaction.

Table I. NMR Data for Thioflavins

compd	NMR data[a] C(4a)-CH_2S-	-SCH_2R
Fl_{red}4a-CH_2SSCH_2R	δ 3.19	δ 3.38
	a = 3.5	a = 0
	J = 12.2	J = ?
Fl_{red}4a-CH_2SCH_2R	δ 3.03	δ 3.08
	a = 15.3	a = 3.8
	J = 14.4	J = 14.9
Fl_{red}4a-CH_2SOCH_2R[b]	δ 3.23, 3.18	δ 3.62, 3.61
	a = 22.8, 18.8	a = 3.0, 1.8
	J = 13.5, 13.2	J = 13.6, 14.4
Fl_{red}4a,5-$(CH_2)_2S$	δ 3.39	δ 5.28
	a = 7.6	a = 5.1
	J = 11.2	J = 9.38

[a] R = COOMe; δ in ppm, a and J in Hz. [b] Data for both diastereomers are given.

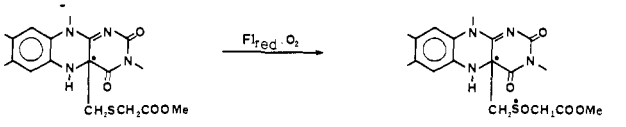
Irradiation of Fl_{red}4a-CH_2SSCH_2COOH, oxygen, and trace amounts of oxidized flavin (as a sensitizer) results in dealkylation to yield Fl_{ox} (eq 9). This reaction is analogous to the photo-

$$Fl_{red}4a\text{-}CH_2SSCH_2R + O_2 \xrightarrow{^3Fl_{ox}^*} Fl_{ox} + \text{oxidation products} \quad (9)$$

dealkylation of 4a-benzyl- and 4a-allyl-3-methyl-4a,5-dihydrolumiflavins reported earlier.[26] Thiodiglycolic acid and dithiodiglycolic acid derivatives react similarly with $^3Fl_{ox}^*$ (eq 10).

$$^3Fl_{ox}^* + S(CH_2COOH)_2 \rightarrow Fl_{red}4a\text{-}CH_2SCH_2COOH + CO_2 \quad (10)$$

Alternately reacting flavin or isoalloxazine photocatalytically with thiodiglycolic acid and then reoxidizing 1,5-dihydrolumiflavin by admission of oxygen in the dark give two additional products—the two diastereoisomeric sulfoxides of thiodiglycolic acid (Fl_{red}4a-CH_2S*OCH_2COOMe), A and B (eq 11). The C(4a) asymmetric



Fl_{red}4a-CH_2SCH_2COOMe Fl_{red}4a-CH_2SOCH_2COOMe (11)

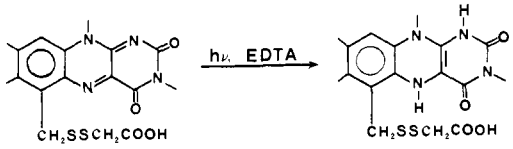
center causes splitting of the 4a-methylene protons in the ^1H NMR spectra of these compounds;[7] e.g., the 4a-α-protons of Fl_{red}4a-CH_2SSCH_2COOMe show an AB pattern with δ 3.19, a = 3.5 Hz, and J = 12.2 Hz (see Table I). A single sulfur bridge (as in Fl_{red}4a-CH_2SCH_2COOMe) results in even greater splitting of the methylene resonance and also in the splitting of the signal from the methylene adjacent to the carboxylate function. Furthermore, the introduction of a second asymmetric center (i.e., the sulfoxyl group) considerably increases the splitting of the 4a-methylene proton signal. For comparison we have included the ^1H NMR data for Fl_{red}4a,5-$(CH_2)_2S$ (vide infra, eq 17) in Table I.

Photocatalytic reaction of Fl_{ox} and Fl'_{ox} with thio- or dithiodiglycolic acid or its esters yields small quantities of C(6)-substituted products as identified by the absence of the H(6) signal and the presence of a singlet from the α-CH_2 group in the ^1H NMR spectrum and by the accompanying change in redox behavior. The ^1H NMR spectrum of Fl_{ox}6-CH_2SSCH_2COOMe shows a signal at δ 4.75 attributable to the C(6)-α-protons, a signal at δ 3.6 due to -SCH_2COO, and a signal due to the methyl ester ($COOCH_3$) at δ 3.8. The UV/vis spectrum of Fl_{ox}6-CH_2SSCH_2R (R = COOH, $COOCH_3$) (pH 7 buffer) shows only a slight shift in λ_{max} from 444 nm (in Fl_{ox}) to 445 nm whereas λ_{max} at 341 nm (in Fl_{ox}) is shifted to 364 nm upon C(6) alkylation.

In benzene solution, the longer wavelength λ_{max} for Fl_{ox}6-CH_2SSCH_2R shifts to 459 nm and a second λ_{max} appears at 487

(26) Blankenhorn, G.; Hemmerich, P. *Tetrahedron Lett.* **1979**, *35*, 1129–1134.

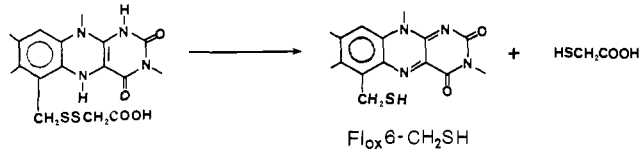
nm. In pH 9 buffer the extinction coefficient at λ_{max} 394 nm (ϵ 13 500) exceeds that of 445 nm (ϵ 10000). Similar observations were reported by Hemmerich et al.[27] for flavins substituted in the 6- or 9-positions with electron-withdrawing groups. Anaerobic photoreduction of $Fl_{ox}6\text{-}CH_2SSCH_2COOH$ with EDTA results in a spectral change which is characteristic of reactions of the type: $Fl_{ox} \rightarrow Fl_{red}$. The striking similarity between these changes and those observed for reaction of unsubstituted flavins with EDTA[4] suggests a simple 1,5-reduction as shown in eq 12. In the absence



(12)

$Fl_{ox}6\text{-}CH_2SSCH_2COOH$ $Fl_{red}6\text{-}CH_2SSCH_2COOH$

of O_2 and light, the latter compound undergoes a subsequent slow reaction to give a product with a UV spectrum very similar to that of the original oxidized 6-substituted lumiflavin. The absence of reducible substrates (other than the disulfide moiety of the 6-side chain) strongly suggests that the reaction is an intramolecular redox reaction resulting in the formation of 6-mercaptomethyl-3-methyllumiflavin ($Fl_{ox}6\text{-}CH_2SH$) (eq 13). Additional evidence



$Fl_{ox}6\text{-}CH_2SH$

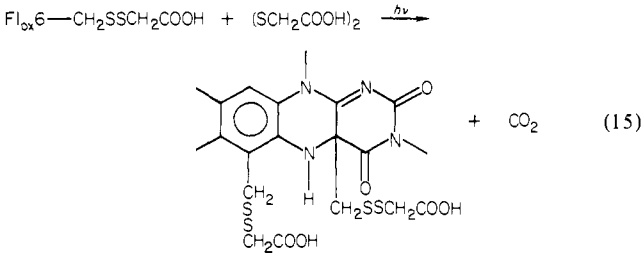
(13)

for the formation of $Fl_{ox}6\text{-}CH_2SH$ was obtained from the inability of the reaction products to undergo another subsequent dark reaction after treatment with light and excess EDTA. Further irradiation of $Fl_{ox}6\text{-}CH_2SH$ with EDTA forms 6-mercaptomethyl-3-methyl-1,5-dihydrolumiflavin ($Fl_{red}6\text{-}CH_2SH$), which cannot be reoxidized in absence of oxygen or other oxidants (eq 14). Admission of air to the reaction solution reoxidized

$$Fl_{ox}6\text{-}CH_2SH \xrightarrow{\text{EDTA, } h\nu} Fl_{red}6\text{-}CH_2SH \qquad (14)$$

$Fl_{red}6\text{-}CH_2SH$ to $Fl_{ox}6\text{-}CH_2SH$ as demonstrated by the regeneration of the $Fl_{ox}6\text{-}CH_2SH$ UV spectrum.

Irradiation of $Fl_{ox}6\text{-}CH_2SSCH_2COOH$ with dithiodiglycolic acid gives the 4a-adduct (eq 15). This product was characterized

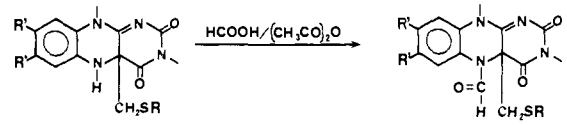


(15)

$Fl_{red}4a\text{-}CH_2SSCH_2COOH\text{-}6\text{-}CH_2SSCH_2COOH$

by UV/vis spectroscopy; its spectrum closely resembled that of the photoadducts obtained by reaction of hetero-substituted carboxylic acids with $^3Fl_{ox}$*. C(6)-Alkylation by a (methyldithio)alkyl moiety results in effective fluorescence quenching of the oxidized flavin. The fluorescence maximum is shifted from 522 nm in the unsubstituted lumiflavin to 508 nm in the C(6)-alkylated derivative. Oxidation with hydrogen peroxide in glacial acetic acid restores approximately 60% of the fluorescence relative to unsubstituted lumiflavin.

Treatment of $Fl_{red}4a\text{-}CH_2SSCH_2R$ (R = Ph, COOH, COOMe, COOEt), $Fl_{red}4a\text{-}CH_2SCH_2COOH$, and $Fl'_{red}4a\text{-}CH_2SSCH_2R$ (R = COOH, COOMe) with acetic anhydride and formic acid results in formylation at the N(5)-position (eq 16). The for-



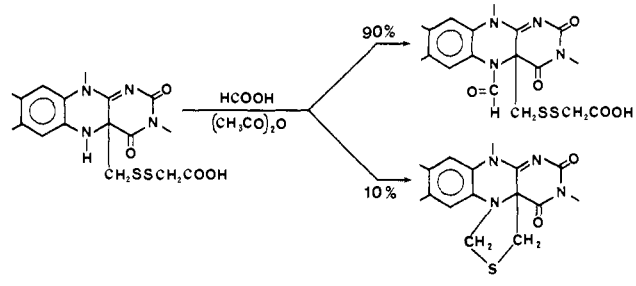
(16)

R'= H, Me
R = SCH_2Ph, SCH_2COOH, SCH_2COOMe, SCH_2COOEt, CH_2COOH, $SCH_2\text{-}4aFl_{red}$

$Fl_{red}4a\text{-}CH_2SR\text{-}5\text{-}CHO$

mylated products have UV/vis spectra with λ_{max} ~320 nm (ϵ 9100 M^{-1} cm^{-1}) and a shoulder at ~260 nm, characteristic of such compounds.[28]

When the flavin photoalkylation reaction solution was treated with formic acid/acetic anhydride, another side product was isolated, **5,8,10,11-tetramethyl-8*H*-benzo[*g*]thiazolo[3,4-*e*]pteridine-4,6-dione,** [$Fl_{red}4a,5\text{-}(CH_2)_2S$] (eq 17). This was charac-



$Fl_{red}4a,5\text{-}(CH_2)_2S$

(17)

terized by its ^1H NMR spectrum; AB quartets are observed from both the N(5)-methylene and the C(4a)-methylene group. The UV/vis spectrum of the adduct strongly resembles that of 4a-substituted, 4a,5-dihydrolumiflavins, as expected. However, molecular oxygen and light (in the presence of lumiflavin as sensitizer) fail to reoxidize $Fl_{red}4a,5\text{-}(CH_2)_2S$ as usually occurs with other dihydroflavin-4a-adducts.[26] Since the product could not be obtained from the reaction of pure $Fl_{red}4a\text{-}CH_2SSCH_2COOH$ with formic acid/acetic anhydride, ring closure must occur in the presence of Fl_{red} or $Fl_{red}5$-alkyl adducts.

The 5-formyl-4a-(((methyl)dithio)alkyl)-4a,5-dihydrolumiflavins hydrolyze preferentially at the N(5)-formyl group in acidic solution to give 4a-(((methyl)dithio)alkyl)-4a,5-dihydrolumiflavins whereas under basic conditions the disulfide bridge is hydrolyzed preferentially to give the 5-formyl-1,5-dihydrolumiflavins followed by the slower, base-catalyzed hydrolysis of the formyl group (see eq 35).

$Fl_{red}4a\text{-}CH_2SSCH_2R$ (R = Ph, COOH, COOMe, COOEt) also undergoes acid-catalyzed rearrangement to give the "symmetrical" compound, bis(4a,5-dihydro-3-methyllumiflavin-4a-methyl) disulfide (eq 18). Under basic conditions the 4a-methyl-4a,5-di-

$$Fl_{red}4a\text{-}CH_2SSCH_2R \xrightarrow{H^+} (Fl_{red}4a\text{-}CH_2S\text{-})_2 + (RCH_2S\text{-})_2 \qquad (18)$$

hydrodithioglycolic acid derivatives of lumiflavin and isoalloxazine decompose to give Fl_{red} and Fl_{ox} or Fl'_{red} and Fl'_{ox}, respectively. Reaction with aqueous base in the presence or absence of O_2 is characterized by a pronounced lag phase in the appearance of Fl_{ox} or disappearance of 4a-adduct.

Treatment of $Fl_{red}4a\text{-}CH_2SSCH_2R$ (R = Ph, COOH, COOMe, COOEt) with mercaptans (such as *tert*-butyl mercaptan) results in mercaptide exchange. Addition of cyanide ions to the 4a,5-dihydroflavin- or 4a,5-dihydroisoalloxazine-4a-methyl disulfide

(27) Schollnhammer, G.; Hemmerich, P. *Eur. J. Biochem.* **1974**, *44*, 561–577.

(28) Ghisla, S.; Hartmann, U.; Hemmerich, P.; Mueller, F. *Liebigs Ann. Chem.* **1973**, *1973*, 1388–1415.

adducts gives the unsubstituted dihydroflavins and the thiocyanate adduct, $Fl_{red}4a\text{-}CH_2SCN$, as was characterized from its IR, UV/vis, and mass spectra. In addition to the expected 4a-adducts, the mass spectral studies indicate that the 4a side chain is extended by the insertion of one (or more) CH_2S groups (eq 37–40).

Discussion

Photoalkylation of Lumiflavin. The earlier report[1] that photoalkylation of 3-benzyllumiflavin in the presence of (benzyldithio)diglycolic acid, dithiodiglycolic acid or its monomethyl or -ethyl ester results in S–S bond rupture, with the formation of a flavin–4a-sulfur adduct (eq 7), could not be verified with 3-methyllumiflavin, even when carried out under identical reaction conditions. In our hands, the reaction leads only to decarboxylation of the dithioglycolic acid derivative and formation of the 4a-R-SCH_2-4a,5-dihydroflavin adduct (eq 8). Our results corroborate the previous observations of photodecarboxylation reactions reported by Walker et al.[7] We have also found that the same reaction occurs with thioglycolic acid derivatives and that scission of the methylene–sulfur bond does not occur (eq 19) as claimed

$$^3Fl_{ox}{}^* + RCH_2SCH_2COOH \rightarrow Fl_{red}5\text{-}CH_2R + [S{=}CH_2] + CO_2 \quad (19)$$

previously.[2] We find that reaction of $^3Fl_{ox}{}^*$ with thioglycolic acid gives only $Fl_{red}4a\text{-}CH_2SCH_2COOH$ (eq 10).

Reaction of both thio- and dithioglycolic acid with $^3Fl_{ox}{}^*$ follows the general mechanism of photodecarboxylation of α-hetero carboxylic acids. In addition to the well-known reductive C(4a)- and N(5)-alkylation of flavins by α-hetero carboxylic acids, a minor reaction also leads to alkylation at C(6). Attempts to rearrange the 4a- or 5- adducts to the C(6)-substituted lumiflavin with strong acid or base failed, and so we postulate that C(6)-substitution occurs during photoreduction.

Whereas lumiflavin loses its redox reactivity when substituted in the 4a-position, substitution at C(6) does not significantly influence the redox capability for reversible electron transfer. Thus, under the aerobic reaction conditions employed, we isolated only the oxidized form of the C(6) adduct.

Isoalloxazine shows the same general product distribution as does lumiflavin except that 8-methylisoalloxazine is also formed. This unusual alkylation reaction probably proceeds by the formation of 8-(((carboxymethyl)dithio)methyl)-1,5-dihydro-3-methyllumiflavin followed by scission of the CH_2–S bond. In Scheme I we have summarized the products observed for reaction of Fl'_{ox} and Fl_{ox} with thio- and dithioglycolic acid derivatives.

As shown herein, there are several reactive positions of the flavin nucleus which can undergo substitution, not all of which, however, lead to stable products. The substitution reactions and the observed formation of reduced flavin can be explained by radical mechanism proposed by Bruice et al.[29,44] (eq 20–26) on the basis

$$RSCH_2COOH + {}^3Fl^* \rightarrow RS{-}\dot{C}H_2COO^- + Fl{\cdot}H \quad (20)$$

$$2Fl{\cdot} \rightarrow Fl_{ox} + Fl_{red} \quad (21)$$

$$R{-}S\dot{C}H_2COO^- \rightarrow R{-}S\dot{C}H_2 + CO_2 \quad (22)$$

$$R{-}S\dot{C}H_2 + Fl{\cdot}H \rightarrow HFl_{red}{-}CH_2SR \quad (23)$$

$$R{-}S\dot{C}H_2 + Fl{\cdot} \rightarrow R{-}S^+{=}CH_2 + Fl_{red}{}^- \quad (24)$$

$$R{-}S\dot{C}H_2 + Fl_{ox} \rightarrow R{-}S^+{=}CH_2 + Fl{\cdot} \quad (25)$$

$$R{-}S^+{=}CH_2 \rightarrow R^+ + CH_2S \quad (26)$$

of spin trapping of the $RXCH_2{\cdot}$ moieties and other experimental observations.

The decarboxylation of the carboxylate radical (eq 22) is fast and possibly concerted as is in the oxygen–oxygen bond and the carbon–carbon bond cleavage in the thermal hydrolysis of *tert*-butylphenyl peroxyacetate[30] (eq 27). $^3Fl^*$ shows a phosphorescence maximum at 610 nm[31] corresponding to an energy of 1.83 eV. Thus, the flavin triplet must have an energy of 177 kJ mol^{-1} above the ground state. If this energy is used in a reaction with a substrate, it corresponds to an increase of the redox potential for the couple

$$PhCH_2COO\text{-}t\text{-}Bu \rightarrow (PhCH_2{\cdot\cdot}\,C(=O){\cdot\cdot}\,O{\cdot\cdot}\,O\text{-}t\text{-}Bu) \rightarrow PhCH_2{\cdot} + CO_2 + t\text{-}Bu{\cdot} \quad (27)$$

$$^3Fl^* + e^- \rightleftharpoons Fl{\cdot} \quad (28)$$

of 1830 mV. Thus, $^3Fl^*$ is an extremely strong 1-e$^-$ oxidizing agent compared to the flavin in the ground state (eq 20). Once formed, the flavin radical may then disproportionate with a rate constant of 2×10^8[32] to form both ground-state oxidized and reduced flavin (eq 21). In competition with its disproportionation, flavin radical couples to R–S–$CH_2{\cdot}$ (eq 23), with the site of coupling to flavin radical being determined by the spin density of its various positions[9,33] and by other factors.[34] Thus, the positions of radical coupling (4a, 5, 6, and 8) follow from the distribution of high spin density of the flavin radical. In this study the formation of the expected flavin 6-adducts has been established for the first time. Also formation of the predicted 8-adducts could be shown with isoalloxazines bearing no alkyl groups in the 7- and 8-positions. Thus, eq 20–26 explains the considerable amount of dihydroflavin found after each preparative photoalkylation. Other photoalkylation products found were 1,5-dihydrolumiflavin and 5-alkylated 1,5-dihydrolumiflavin; the former was trapped as 5-formyl-1,5-dihydro-3-methyllumiflavin and the latter has been isolated elsewhere[7] from reaction of α-activated carboxylic acids with $^3Fl_{ox}{}^*$. Although 5-(((carboxymethyl)dithio)methyl)-1,5-dihydro-3-methyllumiflavin may be formed in reactions of the type investigated herein, it is probably highly unstable due to the presence of two redox centers coupled to each other (the oxidizing disulfide moiety and the reducing flavin moiety). Evidence for the instability of potential N(5)-adducts is suggested by the rates of hydrolysis of N(5)-alkylated derivatives.[35,36]

The anaerobic oxidation observed after photoreduction of $Fl_{ox}6\text{-}CH_2SSCH_2COOH/Me$ with EDTA is presumably due to the slow reoxidation of reduced flavin by the disulfide bond in the C(6) side chain (eq 13). This is plausible in that the conjugated flavin nucleus should increase the redox potential of the disulfide. The same behavior has been observed for "high potential" disulfides such as 5,5′-dithiobis(2-nitrobenzoic acid) [(Ph-4-NO_2,2-COOH,1-S–)$_2$, Ellman's reagent][37] (eq 29). The

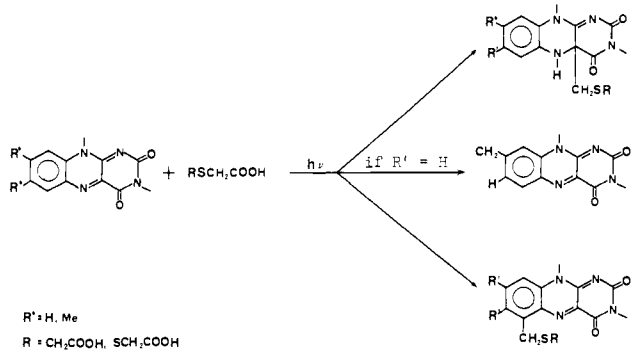
$$Fl_{red} + (\text{Ph-4-}NO_2\text{,2-COOH,1-S-})_2 \rightarrow Fl_{ox} + 2\ \text{Ph-4-}NO_2\text{,2-COOH,1-SH} \quad (29)$$

redox potential of Ellman's reagent is approximately 800 mV higher ($E^{\circ\prime}$ = 600 mV) than that for lumiflavin ($E^{\circ\prime}$ = −210 mV) and thus will readily oxidize Fl_{red}. After oxidation of the flavin nucleus by the side chain, photoreduction with EDTA can again be performed (eq 14). Further reoxdiation in a subsequent dark reaction was not observed.

Formation of the sulfoxide side products by photoalkylation is linked to the ability of the flavin/dihydroflavin systems to oxygenate suitable compounds.[38–42] A mechanism for this reaction

(29) Novak, M.; Miller, A.; Bruice, T. C.; Tollin, G. *J. Am. Chem. Soc.* **1979**, *102*, 1465–1467.
(30) Bartlett, P. D.; Ruechardt, C. *J. Am. Chem. Soc.* **1960**, *82*, 1756–1762.
(31) Sun, M.; Moore, T. A.; Song, P. S. *J. Am. Chem. Soc.* **1972**, *94*, 1730–1740.
(32) Faraggi, M.; Hemmerich, P.; Pecht, I. *FEBS Lett.* **1975**, *51*, 47–51.
(33) Ehrenberg, A.; Mueller, F.; Hemmerich, P. *Eur. J. Biochem.* **1967**, *2*, 286–293.
(34) Tedder, J. M. *Angew. Chem., Int. Ed. Engl.* **1982**, *21*, 401–410.
(35) Kemal, C.; Bruice, T. C. *J. Am. Chem. Soc.* **1976**, *98*, 3955–3964.
(36) Eberlein, G. A.; Bruice, T. C. *J. Am. Chem. Soc.* **1983**, *105*, 6679–6684.
(37) Eberlein, G. A. *Ph.*D. Dissertation, University of Konstanz, Konstanz, Germany, 1980.
(38) Hayaishi, O. In "Molecular Mechaisms of Oxygen Activation"; Hayaishi, O., Ed.; Academic Press: New York, 1974; pp 1–28.
(39) Flashner, M. S.; Massey, V. In ref 38, pp 245–283.

Scheme I



probably involves the flavin 4a-hydroperoxide (Fl_{red}4a-OOH) since this reactive species is known to oxidize several compounds including amines, activated aromatic rings, and cyclic sulfides[42-44] (eq 30 and 31). The sulfoxide product is formed by air oxidation

$$Fl_{red} + O_2 \rightarrow [Fl_{red}4a\text{-}OOH] \quad (30)$$

$$[Fl_{red}4a\text{-}OOH] + Fl_{red}4a\text{-}CH_2SCH_2R \rightarrow Fl_{ox} + Fl_{red}4a\text{-}CH_2S^*OCH_2R + H_2O \quad (31)$$

$$R = COOH, COOMe$$

when unsubstituted dihydroflavin is present in the reaction solution. Oxidation of the sulfide bond by hydrogen peroxide formed during reaction of reduced flavin and molecular oxygen does not occur since hydrogen peroxide (15%) does not react appreciably with Fl_{red}4a-CH_2SCH_2COOMe. This is in accord with the much higher reactivity of N(5)-alkylated flavin 4a-hydroperoxides than hydrogen peroxide for the oxidation of iodide or suitable organic substrates.[44,45]

Hydrolysis of Substituted Lumiflavins. Reaction of Fl_{red}4a-CH_2SSCH_2COOMe with hydroxide ion follows the usual mechanism of disulfide hydrolysis.[46-49] Attack of HO^- on either of the sulfur atoms results in S–S-bond cleavage with formation of a mercaptan and (unstable) sulfenic acid (eq 32 and 33). The

$$Fl_{red}4a\text{-}CH_2SSCH_2R + HO^- \rightarrow [Fl_{red}4a\text{-}CH_2S^-] + [HOSCH_2R] \quad (32)$$

$$Fl_{red}4a\text{-}CH_2SSCH_2R + HO^- \rightarrow [Fl_{red}4a\text{-}CH_2SOH] + RCH_2S^- \quad (33)$$

products of eq 32 or 33 could not be isolated due to their instability and disproportionation. Under anaerobic conditions, approximately 75% of Fl_{red}4a-CH_2SSCH_2R is converted to oxidized flavin, possibly through an intermediate flavin species (eq 32, 33). Since Fl_{red} is the only other product found from the reaction of Fl_{red}4a-CH_2SSCH_2R (R = Ph, COOH, COOMe, COOEt) with mercaptan (under anaerobic conditions), there must be an oxidant generated during the hydrolysis reaction. The flavinoid product of eq 33 may form oxidized flavin, water, and thioformaldehyde, as shown in eq 34. Similarly, the flavinoid product of eq 32 may

$$Fl_{red}4a\text{-}CH_2SOH \rightarrow Fl_{ox} + [CH_2{=}S] + H_2O \quad (34)$$

give 1,5-dihydroflavin and thioformaldehyde (eq 35). Fl_{red}4a-

$$Fl_{red}4a\text{-}CH_2S^- \rightarrow Fl_{red}^- + [CH_2{=}S] \quad (35)$$

CH_2SSCH_2R (where R = COOH) reacts $\sim 10^3$ times slower with HO^- than Fl_{red}4a-CH_2SSCH_2R (where R = Ph, COOMe, COOEt); this is presumably due to the shielding effect of the carboxylate group.

Cyanide ion reacts similarly to hydroxide ion with Fl_{red}4a-CH_2SSCH_2COOH to give thiocyanate and mercaptide. Attack of cyanide on the sulfur closest to the flavin moiety (eq 36) results

$$Fl_{red}4a\text{-}CH_2SSCH_2R + CN^- \rightarrow Fl_{red}4a\text{-}CH_2SCN + RCH_2S^- \quad (36)$$

in a reasonably stable dihydroflavin 4a-methyl thiocyanide. On the other hand, cyanide attack at the other sulfur atom (eq 37)

$$Fl_{ref}4a\text{-}CH_2SSCH_2R + CN^- \rightarrow Fl_{red}4a\text{-}CH_2S^- + RCH_2SCN \quad (37)$$

results in formation of an alkyl thiocyanide and Fl_{red}4a-CH_2S^- which undergoes decomposition (eq 35). Reaction of Fl_{red}4a-CH_2S^- with thioformaldehyde affords a chain-lengthened species (eq 38–41) which can react with Fl_{red}4a-CH_2SSCH_2R (R = Ph,

$$Fl_{red}4a\text{-}CH_2S^- + n[CH_2{=}S] \rightarrow Fl_{red}4a\text{-}CH_2S(CH_2S)_n^- \quad (38)$$

$$Fl_{red}4a\text{-}(CH_2S)_nCH_2S^- + Fl_{red}4a\text{-}CH_2SSCH_2R \rightarrow Fl_{red}4a\text{-}(CH_2S)_nCH_2SSCH_2R \quad (39)$$

$$\rightarrow Fl_{red}4a\text{-}(CH_2S)_nCH_2SSCH_2\text{-}4aFl_{red} \quad (40)$$

$$\rightarrow Fl_{red}4a\text{-}(CH_2S)_nCH_2SNu \quad (41)$$

$$n = 0, 1, 2, \ldots$$

$$Nu = CN^-, RS^-$$

COOH, COOMe, COOEt) to give several different 4a,5-dihydro-3-methyl-4a-methyl thiocyanides (and disulfides) with extended length of the dihydroflavin-4a side chain.

If excess mercaptide is present in the reaction solution, the following rearrangements occur (eq 42 and 43).

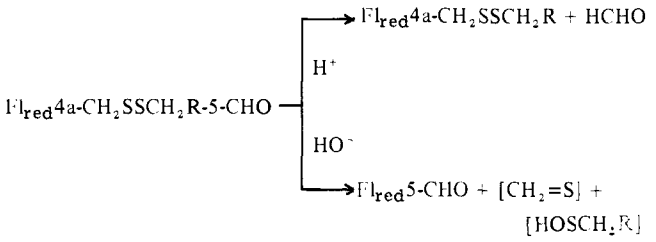
$$Fl_{red}4a\text{-}CH_2SSCH_2R + R'S^- \rightleftharpoons Fl_{red}4a\text{-}CH_2SSR' + RCH_2S^- \quad (42)$$

$$Fl_{red}4a\text{-}CH_2SSCH_2R + R'S^- \rightleftharpoons Fl_{red}4a\text{-}CH_2S^- + RCH_2SSR' \quad (43)$$

The reaction products of eq 42 and 43 are also in equilibrium with the products of eq 38–41, which results in the formation of other possible disulfides and mercaptides. This was demonstrated by quenching the reaction with acid before all of the Fl_{red}4a-CH_2S^- decomposed (eq 35). When the reaction was allowed to reach completion the only products isolated were Fl_{red} and Fl_{ox} under anaerobic and aerobic conditions, respectively.

Formylation at the N(5)-position of Fl_{red}4a-CH_2SSCH_2R (R = Ph, COOH, COOMe, COOEt) (eq 16) did not result in appreciable stabilization of the proposed intermediate, 5-formyl-4a,5-dihydro-4a-methylmercaptide-3-methyllumiflavin. When HO^-, CN^-, or RS^- were reacted with 1,5-dihydro-5-formyl-4a-methyldithioalkyl-3-methyllumiflavin, the only product isolated was 5-formyl-1,5-dihydro-3-methyllumiflavin (eq 44). The autocatalytic reaction profiles for reaction of Fl_{red}4a-CH_2SSCH_2R (R = Ph, COOH, COOMe, COOEt) with HO^- and RS^- were comparable to those for reaction of the 5-formylated analogues with the same nucleophiles. On the other hand, acid-catalyzed hydrolysis resulted in loss of the formyl group (eq 44). Formylation of Fl_{red}4a-CH_2SSCH_2COOH with formic acid/acetic anhydride[7] (eq 17) afforded an additional product which was devoid of redox activity. This compound does not undergo photodealkylation in the presence of oxygen, unlike 4a-alkylated 4a,5-dihydroflavins.[28] The product Fl_{red}4a,5-$(CH_2)_2S$ demonstrates these unusual properties because the additional ring gives it considerable stability. For example, reoxidation of Fl_{red}4a,5-

(40) Massey, V.; Hemmerich, P. *Enzymes, 3rd Ed.* 1970–1976 **1975**, *12*, 191–252.
(41) Ballou, D. P. In "Flavins and Flavoproteins"; Massey, V.; Williams, C. H., Eds.; Elsevier: Amsterdam, 1982; pp 301–310.
(42) Ball, S.; Bruice, T. C. *J. Am. Chem. Soc.* **1980**, *102*, 6498–6503.
(43) Bruice, T. C. In "Biomimetic Chemistry"; Dolphin, D., McKenna, C., Murakami, Y., Tabushi, I. Eds.; American Chemical Society: Washington, DC, 1980; Adv. Chem. Ser. No. 191, pp 89–119.
(44) Bruice, T. C. *Acc. Chem. Res.* **1980**, *13*, 256–261.
(45) Kemal, C.; Chan, T. W.; Bruice, T. C. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 405–409.
(46) Birch, S. F.; Cullum, T. V.; Dean, R. A. *J. Inst. Pet.* **1972**, *39*, 206. (*Chem. Abstr. 48*, 4428e).
(47) Foss, O. In "Organic Sulfur Compounds"; Pergamon Press: Elmsford, NY, 1961; Vol. 1, p 83.
(48) Kice, J. L.; Ekman, G. E. *J. Org. Chem.* **1975**, *40*, 711–716.
(49) Parker, A. J.; Kharasch, N. *Chem. Rev.* **1959**, *59*, 583–628.



R = Ph, COOMe, COOEt

(44)

$(CH_2)_2S$ with $NaNO_2$[2] and acetic acid to form Fl_{ox} was significantly slower than with either N(5)- or C(4a)-alkylated 1,5- or 4a,5-dihydroflavins.

The disulfides studied herein also undergo acid- and cyanide-catalyzed disulfide exchange to give (Fl_{red}4a-CH_2S-)$_2$.[50,51] The extinction coefficient for this compound at 360 nm is 9550 M^{-1} cm^{-1}, or approximately 1.5 times that for the monomeric compound. The fixed arrangement of the two flavin moieties accounts for this reduced absorption when compared to normal free flavins. This effect was also found by Knappe and Rothfelder[52] for the dimeric forms of dideazaflavins.

Conclusions

Photoexcited 3-methyllumiflavin does not mimic the enzymatic reaction of flavoenzymes with disulfides. Photoalkylation of Fl_{ox} with thioglycolic acid derivatives provides covalent flavin adducts at the 4a-, 5-, 6-, and 8-positions. We have also found that the decomposition of flavin–4a-adducts, to give either Fl_{red} or Fl_{ox}, depends on the electronegativity of the 4a-side chain as has been suggested for the methanol dehydrogenation/formaldehyde reduction by flavins.[53]

Acknowledgment. This work was initiated under the guidance of the late Professor Peter Hemmerich and continued with the hospitality of Professor Wolfgang Pfleiderer. To these individuals we extend our gratitude. This work was also supported by a grant of the Deutsche Forschungsgemeinschaft and of the Studienstiftung des Deutschen Volkes and by the Natural Sciences and Engineering Research Council of Canada.

Registry No. $PhCH_2SSCH_2Ph$, 150-60-7; $HSCH_2CO_2H$, 68-11-1; Fl_{ox}, 18636-32-3; Fl_{red}4a-CH_2SSCH_2Ph, 89322-24-7; Fl_{red}4a-$CH_2SSCH_2CO_2H$, 89322-25-8; Fl_{red}4a-$CH_2SSCH_2C(O)OMe$, 89322-26-9; Fl_{red}4a-$CH_2SSCH_2C(O)OEt$, 89322-27-0; Fl_{red}4a-$CH_2SSCH_2C(O)OMe$-5-CHO, 89322-28-1; Fl_{red}4a-$CH_2SSCH_2CO_2H$-5-CHO, 89322-29-2; Fl_{red}4a-$CH_2SCH_2CO_2H$, 89322-30-5; Fl_{ox}6-$CH_2SSCH_2CO_2H$, 89322-31-6; Fl_{ox}6-$CH_2SSCH_2C(O)OMe$, 89322-32-7; Fl'_{red}4a-$CH_2SSCH_2CO_2H$, 89322-33-8; Fl'_{ox}, 4074-59-3; Fl'_{red}4a-$CH_2SSCH_2C(O)OMe$, 89322-34-9; Fl'_{ox}6-$CH_2SSCH_2CO_2H$, 89322-35-0; Fl'_{ox}6-$CH_2SSCH_2C(O)OMe$, 89322-36-1; (Fl_{red}4a-$CH_2S)_2$, 89322-37-2; Fl_{red}4a,5-$(CH_2)_2S$, 89322-38-3; Fl_{red}4a-$CH_2S(O)CH_2C(O)OMe$ (isomer 1), 89322-39-4; Fl_{red}4a-$CH_2S(O)CH_2C(O)OMe$ (isomer 2), 89322-40-7; Fl_{red}4a-CH_2SSBu-*t*, 89322-41-8; Fl_{red}4a-CH_2SCN, 89322-42-9; *N*-bromophthalimide, 2439-85-2; *N*-(benzylthio)phthalimide, 14204-26-3; (benzyldithio)glycolic acid, 83167-33-3; dithiodiglycolic acid, 505-73-7; dithiodiglycolic acid monoethyl ester, 1665-63-0; thiodiglycolic acid, 123-93-3; *tert*-butyl mercaptan, 75-66-1.

(50) Pryor, A. W. In "Mechanisms of Sulfur Reactions"; McGraw-Hill: New York, 1962; p 56.
(51) Smiles, S.; McCleland, E. E. *J. Chem. Soc.* **1922**, *121*, 86–90.
(52) Knappe, W.-R.; Rothfelder, F. *J. Chem. Res. Miniprint* **1978**, 1325–1346.
(53) Bruice, T. C. In "Progress in Bioorganic Chemistry"; Kaiser, E. T., Ed.; Wiley: New York, 1976; Vol. 4.

Effect of Hydrostatic Pressure on the Transfer of a Fluorescent Phosphatidylcholine between Apolipoprotein–Phospholipid Recombinants

William W. Mantulin,* Antonio M. Gotto, Jr., and Henry J. Pownall

Contribution from the Department of Medicine, Baylor College of Medicine and the Methodist Hospital, Houston, Texas 77030. *Received October 17, 1983*

Abstract: The effect of high hydrostatic pressure on the mechanism of transfer for lipophiles between membranes was investigated. The transfer of fluorescent phosphatidylcholine (1-myristoyl-2-[9′-(3′-pyrenyl)nonanoyl]phosphatidylcholine) between recombinant complexes of apolipoprotein A-I and 1-palimitoyl-2-oleoylphosphatidylcholine occurred via the aqueous compartment. High hydrostatic pressure retarded the rate of transfer, resulting in a positive activation volume for the reaction. The calculated free energy of activation was approximately 23 kcal/mol. The positive activation volume presumably corresponds to the work necessary to overcome pressure-induced compression of the membrane (protein–lipid complex) before transfer can occur.

The spontaneous passive transfer of phospholipids between lipid compartments (e.g., membranes or lipoproteins) is an important mechanism in metabolism.[1] Kinetic transfer studies of phospholipids,[2,3] in particular, and lipophiles,[4–6] in general, support the mechanism in which dissociation from the lipid surface is the rate-limiting step and the spontaneous transfer of monomeric phospholipid occurs via the aqueous phase. Experimental data suggests that the rate of transfer is a function of the lipid's aqueous solubility.[2] In this communication we report that high hydrostatic pressure (up to 1500 bar) slows the rate of transfer for a pyr-

(1) Mantulin, W. W.; Pownall, H. J. In "The Excited States of Biopolymers"; Steiner, R. F., Ed.; Plenum Press: New York, 1983; pp 163–202.
(2) Massey, J. B.; Gotto, A. M., Jr.; Pownall, H. J. *Biochemistry* **1982**, *21*, 3630–3636.
(3) Roseman, M. A.; Thompson, T. E. *Biochemistry* **1980**, *19*, 439–444.
(4) Pownall, H. J.; Hickson, D. L.; Smith, L. C. *J. Am. Chem. Soc.* **1983**, *105*, 2440–2445.
(5) Doody, M. C.; Pownall, H. J.; Kao, Y. J.; Smith, L. C. *Biochemistry* **1980**, *19*, 108–116.
(6) Pownall, H. J.; Hickson, D. L.; Gotto, A. M., Jr.; Massey, J. B. *Biochim. Biophys. Acta* **1982**, *712*, 169–176.

0002-7863/84/1506-3317$01.50/0