Preparation and Some Properties of 6-Substituted Flavins as Active Site Probes for Flavin Enzymes[†]

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Received October 31, 1985; Revised Manuscript Received February 4, 1986

ABSTRACT: 6-Azidoflavins, 6-thiocyanatoflavins, and 6-mercaptoflavins at the lumiflavin, riboflavin, FMN, and FAD level were prepared from the corresponding 6-aminoflavins and some of their properties investigated. They are bound tightly by apoflavin enzymes which bind either riboflavin, FMN, or FAD. 6-Azidoflavins undergo facile photolysis. One major product was identified as 6-aminoflavin. A further product, which was formed also during acid decomposition of the azide, results from opening of the flavin benzene ring and is proposed to have a lumazine structure. 6-Thiocyanatoflavins are easily converted by dithiothreitol to 6-mercaptoflavins. The latter are stabilized against dimerization in the presence of reducing thiols. 6-Mercaptoflavins have a pK of 5.9, which corresponds to ionization of the 6-SH function. The neutral form is yellow, while the anion is green, due to a long-wavelength band ($\lambda_{max} \sim 600$ nm) extending beyond 700 nm. These properties suggest the use of these 6-substituted flavins for probing the active site of flavin enzymes. Because their reactive substituents are in close proximity to the flavin N(5)-position, these 6-substituted derivatives should also serve as useful probes of the environment around the flavin N(5), a position known to be involved in all flavin-mediated redox processes.

Our present understanding on the ways flavins interact with the protein at the active center of enzymes has resulted to a considerable extent from the use of flavin-derived active site probes (Ghisla et al., 1976; Walsh et al., 1978; Massey et al., 1979, 1984; Schopfer et al., 1981; Biemann et al., 1983; Kasai et al., 1983; Ghisla et al., 1984; Fitzpatrick et al., 1985; Krauth-Siegel et al., 1985). They might be differentiated into three broad classes with respect to their chemical properties: (i) flavin analogues that reflect active site properties by virtue of spectral effects induced upon binding; (ii) probes that react either with a protein functional group at the active site or with solvent-borne reactants (accessibility probes); (iii) photoaffinity labeling derivatives. Until the present, functions at positions 1, 2, 4, and 8 of the flavin molecule have been candidates for specific modifications, yielding information, respectively, about the interactions with the protein or solvent of positions in all three rings of the flavin structure. One part of the flavin, which

has been little explored in this context, is the portion of the benzene ring closest to the catalytically important N(5) position. Exceptions have been the use of isoflavin (6-methyl-8-norisoalloxazine) (Choong & Massey, 1981; Hastings et al., 1981) and of 6-hydroxyflavin coenzyme analogues, the latter having proven useful in the study of pK shifts induced upon binding, by virtue of their p$K \sim 7$ (Mayhew et al., 1974; Thorpe & Massey, 1983). We thus set out to attempt to introduce two types of functional groups into this part of the molecule. The azido function should serve as a photoaffinity label (Bayley & Knowles, 1977; Chowdhry & Westheimer, 1979; Lwowski, 1980), as has proven feasible in the case of 8-azidoflavins (Ghisla et al., 1984; Fitzpatrick et al., 1985). A mercapto function might serve as a probe for investigating the accessibility to this region of the flavin, by using its readily monitored reactivity with alkylating agents. In addition, it could also function as a chromophore sensitive to changes in the dipole moment of the active center, since it is expected to be comparable to 6-hydroxy- and 8-hydroxyflavins (Mayhew et al., 1974; Ghisla & Mayhew, 1976) and to 8-mercaptoflavins (Massey et al., 1979) and to have a pK in the physiological range. The synthesis and properties of some 6-substituted flavins have been described earlier in the context of the structural elucidation of 6-S-cysteinylriboflavin, the co-

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Scheme I: Structures of 6-Substituted Flavins at Lumiflavin, Riboflavin, FMN, and FAD Levels
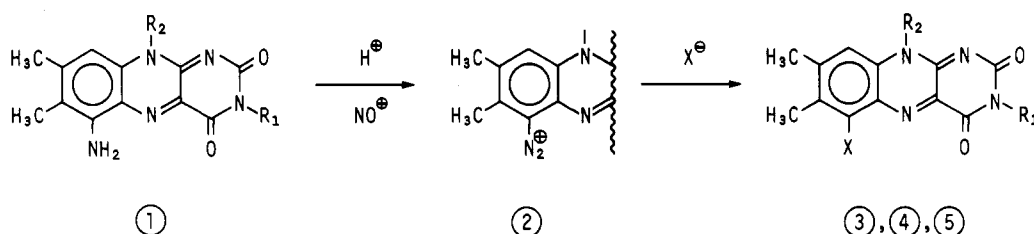
a: R_1, R_2=CH$_3$ (3-Methyl-6-X-lumiflavin)

b: R_1=H, R_2=D-Ribityl (6-X-riboflavin)

c: R_1=H, R_2=D-Ribityl-(5')-OPO$_3^=$ (6-X-FMN)

d: R_1=H, R_2=D-Ribityl-(5')-ADP (6-X-FAD)

3: X = -N$_3$

4: X = -SCN

5: X = -SH

factor of trimethylamine dehydrogenase (Ghisla et al., 1980). We thus attempted the synthesis of the flavin analogues mentioned above and here describe some of their properties. In work to be detailed later, it will be shown that 6-azidoflavins indeed can be excellent photoaffinity labels and that 6-thiocyanato- and 6-mercaptoflavins also are good analogues for probing the active sites of flavoproteins.

EXPERIMENTAL PROCEDURES

Materials. The conversion of riboflavin analogues into FAD derivatives was achieved with partially purified enzymes from *Brevibacterium ammoniagenes* (Spencer et al., 1976). FMN analogues were obtained from the FAD precursors by hydrolysis with *Naja naja* venom (Sigma Chemical Co., St. Louis, MO). The following proteins and their respective apoproteins were prepared as previously described: riboflavin-binding protein from egg white (Blankenhorn et al., 1975), flavodoxin from *Megasphera elsdenii* (Mayhew & Massey, 1969; Wassink & Mayhew, 1975), Old Yellow Enzyme from brewers bottom yeast (Abramovitz & Massey, 1976a,b), D-amino acid oxidase from hog kidney (Yagi et al., 1967; Massey & Curti, 1966), and lactate oxidase from *Mycobacterium smegmatis* (Choong et al., 1975; Sullivan et al., 1977). 3-Methyl-6-nitrolumiflavin and 6-nitroriboflavin were prepared by the method of Knappe (1979). 6-Amino-3-methyllumiflavin (**1a**) and 6-amino-riboflavin (**1b**) (see Scheme I) were obtained by reduction of the 6-nitro derivatives with alkaline dithionite (Ghisla et al., 1980). Riboflavin, FAD, and FMN were purchased from Sigma Chemical Co.

3-Methyllumiflavin 6-(Diazonium perchlorate) (**2a**). One millimole of 6-amino-3-methyllumiflavin (**1a**) was dissolved in the minimal amount necessary of 70% HClO$_4$, chilled in ice, and then diluted with 2–3 g of ice. A solution of saturated NaNO$_2$ was then added dropwise until the color of the solution had turned golden orange. Solid sodium perchlorate was then added to saturate the solution, and the diazonium perchlorate was allowed to crystallize at ice temperature for approximately 20 min and filtered by suction. The crude orange crystals were washed with 1 mL of cold 1 M HClO$_4$, with a little cold methanol, dried under vacuum, and then stored dry at -20 °C. Under these conditions, the crude material ($\sim$150 mg) is stable for weeks. It was estimated to be >90% pure, since upon reduction with hydroxylamine it yielded 6-amino-3-methyllumiflavin, which was >90% pure as judged by high-performance liquid chromatography (HPLC). Since the riboflavin 6-diazonium salt (**2b**) could not be obtained as a crystalline product, it was converted in situ into its derivatives.

6-Azido-3-methyllumiflavin (**3a**). A total of 2.6 g ($\sim$9 mmol) of 6-amino-3-methyllumiflavin (**1a**) was dissolved in 10 mL of concentrated H$_2$SO$_4$, cooled in ice, added to 100 mL of ice water (50 mL of water, 50 g of ice), and then diazotized with 900 mg of NaNO$_2$. A total of 500 mg of urea was then added in order to destroy excess nitrite, followed by 5 g of solid sodium azide. The orange precipitate, which started forming within a few minutes, was filtered after 20 min, washed with cold water and methanol, and dried in vacuum in the absence of light, yield 1.75 g (60%). The compound was >95% pure by HPLC analysis. An additional 400 mg of material of lesser purity could be crystallized from the mother liquors upon standing: MS (EI), m/e (relative intensity) 311 (M$^+$, 4), 285 (M$^+$ + 2 - N$_2$, 71), 28 (N$_2$, 100); ^{1}H NMR (CF$_3$COOH) δ (ppm) 2.56 (s, 3 H, 7-CH$_3$), 2.79 (s, 3 H, 8-CH$_3$), 3.74 and 4.55 (2 $\times$ s, 2 $\times$ 3 H, 3-CH$_3$ and 10-CH$_3$), 7.85 (s, 1 H, 9-H); IR (KBr), strong band at 2120 cm^{-1} (-N$_3$), typical flavin bands (Müller et al., 1966) at 1200–1720 cm^{-1}.

6-Azidoriboflavin (**3b**). One millimole (400 mg) of 6-aminoriboflavin (**1b**) was diazotized as described earlier (Ghisla et al., 1980), treated with urea to destroy excess nitrite, and then reacted with 5 equiv ($\sim$300 mg) of sodium azide. After formation of a brown solution, and upon cessation of N$_2$ evolution, the same volume of water was added, and the compound was allowed to crystallize at ice temperature. The crude product obtained by filtration was recrystallized from acetic acid/water. The yield was 120 mg (30%) of orange-brown crystals, essentially pure as judged by HPLC and thin-layer chromatography analysis. Its UV–vis spectrum was essentially identical with that of the 6-azido-3-methyllumiflavin (**3a**), and the NMR spectrum was consistent with structure **3b**.

Acid-Catalyzed Decomposition of 6-Azidoflavins. 6-Azido-3-methyllumiflavin (**3a**) (400 mg) was added in small aliquots to ice-cold, concentrated H$_2$SO$_4$ over 20 min, care being taken to prevent heating. When the evolution of N$_2$ had ceased, the solution was diluted with 20 mL of ice/water and then allowed to stand overnight at 0–4 °C. A brown precipitate was then collected, which consisted of essentially pure 3,8-dimethyl-6-carboxy-7-(2-methyl-3-oxobut-2-enyl)lumazine (**8a**). The material can be recrystallized from glacial acetic acid/water: yield $\sim$300 mg (75%); ^{1}H NMR (Me$_2$SO-d_6) δ (ppm) 1.40 [s, 3 H, 7(3')-CH$_3$], 2.23 [d, J = 3.7 Hz, 3 H, 7(2')-CH$_3$], 3.25 and 3.90 (2 $\times$ s, 2 $\times$ 3 H, 3-CH$_3$ and 8-CH$_3$), 7.01 (d, J = 3.7 Hz, 1 H, 9-H); MS (CI/DI, isobutane), m/e (relative intensity) 319 (M$^+$ + 1, 3), 303 (M$^+$ - 15, 15), 287 (M$^+$ - 31, 100); IR (KBr), strong bands at 1270, 1490, 1580, 1630, and 1700 cm^{-1}. Anal. Calcd for C$_{14}$H$_{14}$N$_4$O$_5$: C, 52.83; H, 4.40; N, 17.61. Found: C, 52.93; H, 4.65; N, 17.60.

6-Azido-FAD (**3d**). One milliliter of a solution of 6-amino-FAD (**1c**) (A_{430} $\sim$ 17) was diluted at ice temperature with 1 mL of 1 N HCl; then, 2 μL of a saturated NaNO$_2$ solution was added, which caused the green solution to turn

golden yellow within 10 s. Then, 50 μL of saturated urea and, after ~10 s, 50 μL of saturated sodium azide were added; the latter induces N_2 evolution and formation of an orange-yellow solution. After ~30 s, the solution was treated with 100 μL of 2 M sodium acetate and subsequently with 200 μL of saturated trisodium phosphate to bring the pH to 4–4.5. The pH of the solution was then adjusted to 6–7 with solid bicarbonate and could be used without further manipulations for most enzyme reconstitution experiments. It could be freed of salts as described below for **4d**.

6-Thiocyanato-FAD (**4d**). Diazotization of 6-amino-FAD (**1c**) was carried out as described above, however in 0.1 M H_2SO_4. After addition of urea and 200 μL of saturated NH_4SCN solution, the solution was immediately treated with the amount of 2 M sodium acetate necessary to bring the pH to 4–4.5. At this point the formation of the 6-thiocyanate was followed by recording the absorption spectrum of an aliquot. The conversion was essentially complete after 20–30 min at this pH and ice temperature and is dependent on the concentration of NH_4SCN and pH. Thereafter, the pH of the solution was adjusted to 6–7, and the material could be used directly to reconstitute holoenzymes. The FAD derivative was freed of salt by chromatography on a column of Bio-Gel P-2 (100 × 2.5 cm) equilibrated and eluted with glass-distilled water. As with most flavins (Spencer et al., 1976), the 6-SCN-FAD migrates as a sharp band, well separated from a minor impurity at the FMN level.

6-Thiocyanatoriboflavin (**4b**). This was prepared analogously to the 6-thiocyanato-FAD; however, 1 N HCl or 1 M H_2SO_4 was used in the diazotization step.

6-Mercaptoflavins (**5b–d**). These compounds were prepared from the corresponding 6-thiocyanatoflavin by reaction at pH 7 with a small excess of dithiothreitol. Providing that a low concentration (≥10 μM) of ethylenediaminetetraacetic acid (EDTA) is present to prevent trace metal catalyzed oxidation of the thiols, the 6-mercaptoflavins are stable for several hours at 25 °C. The conversion is conveniently followed spectrophotometrically at 440 nm, where a large extinction increase is observed, or at 600 nm, where the mercaptoflavin anion has substantial absorption (Ghisla et al., 1980).

Instrumentation. Absorption spectra were recorded with a Shimadzu MPS 2000 or a Shimadzu UV-250 spectrophotometer at 25 °C, when not otherwise indicated. NMR spectra were obtained with a JEOL FX-90 instrument using tetramethylsilane as an internal standard. Infrared spectra in KBr pellets were measured with a Hitachi 270-30 instrument. Mass spectra were obtained with a JEOL DX-300 instrument. HPLC analysis and preparative separations were done with a Shimadzu LC-4A instrument using a Develosil (30 × 0.7 cm) RP_{18} column and the following elution conditions: for lumiflavin and riboflavin derivatives, 90% MeOH/0.01 M phosphate buffer, pH 6.0, at volume ratios 4/6 from 0 to 2 min, then a gradient to 10/0 from 2 to 14 min, and maintaining this to 20 min; for FMN and FAD derivatives, 90% MeOH/0.01 M phosphate, pH 6.0, 2/8 from 0 to 2 min, then a gradient to 5/5 from 2 to 14 min, and then to 9/1 from 14 to 18 min. For the lumazine derivatives the same gradient system was used but employing acetic acid, pH 2.5, instead of phosphate buffer.

RESULTS AND DISCUSSION

General Comments on Synthesis of 6-Substituted Flavin Coenzymes. In a preceding paper (Ghisla et al., 1980), the synthesis and diazotization of 6-aminoflavins have been described (Scheme I). The Sandmeyer reaction of the flavin diazonium cations (**2**) was also shown to be a suitable path
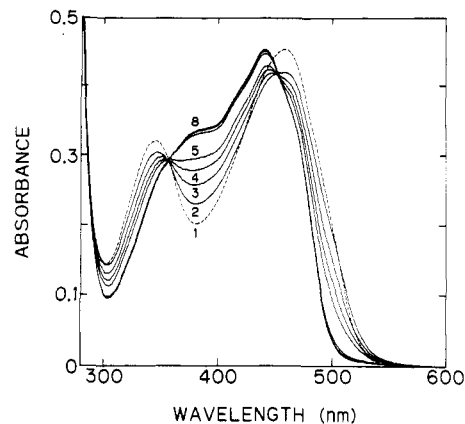

FIGURE 1: Formation of 6-thiocyanatoflavin (**4**) from flavin 6-diazonium cation (**2**). 6-Aminoriboflavin (**1b**) in dimethylformamide was diluted with 1 M sulfuric acid to yield an approximately 0.5 M solution. The diazonium cation **2b** was then generated as described in the text and the pH of the solution adjusted to pH 2.5 by addition of saturated sodium acetate solution (curve 1). To this species, 20 μL of a saturated solution of NH_4SCN was added, and the spectra of curves 2–8 were recorded after 2.5, 5, 7.5, 10, 20, 30, and 60 min, respectively. The final species consists of 90–95% 6-thiocyanatoriboflavin (**4b**) as judged by HPLC analysis. The formation of the FAD analogue **2d** proceeds analogously.

for the introduction of (pseudo)halogens into this position. However, the method involved the use of concentrated sulfuric acid and was reported to be prone to formation of side products, probably originating from decay of the diazonium salts. Strong acid was required because of the extremely low solubility of 6-aminoflavins and in particular of 6-aminoriboflavin (**1b**). The conditions of diazotization could be significantly improved in the present work for the preparation of 6-diazonium flavins (**2**) and their subsequent conversion products. Thus, 3-methyllumiflavin 6-(diazonium perchlorate) (**2a**) was obtained in crystalline form; this allowed the study of some of its properties and conversions. The preparation of 6-azidoflavins (**3**) and 6-thiocyanatoflavins (**4**) was miniaturized to the point where the conversion of nanomole to micromole quantities of flavins could be achieved quickly and essentially quantitatively. This was of particular importance for the synthesis of 6-substituted FAD and FMN derivatives **3c**, **3d**, **4c**, and **4d**. The attempted conversion of 6-azidoriboflavin (**3b**) and of 6-thiocyanatoriboflavin (**4b**) to the FMN **3c** and **4c** and FAD **3d** and **4d** derivatives with the system from *B. ammoniagenes*, which has been successful in the conversion of most riboflavin derivatives into their FAD counterparts (Spencer et al., 1976), unexpectedly failed, although conversion of 6-aminoriboflavin (**1b**) to 6-amino-FAD (**1d**) succeeded with excellent yields. The reason for the failure might reside in the steric requirements of these two substituents, which, due to hindrance with the ortho functions [7-CH_3 and N(5)] and their forming an angle at their proximal atom probably cannot be accomodated in the flavin plane. The preparation of the desired analogues was thus carried out via 6-amino-FAD (**1d**). Since under strongly acidic conditions the pyrophosphate bond of FAD will hydrolyze rapidly, a rapid and mild procedure was devised (cf. Experimental Procedures for details), which in the case of the preparation of 6-azido-FAD (**3d**) and 6-thiocyanato-FAD (**4d**) yielded the desired products in >90% purity as judged from their HPLC analysis. This is exemplified in Figure 1 for the preparation of 6-thiocyanatoflavins. That under the reaction conditions no modification of the FAD molecule occurs in the adenine moiety is evidenced by the tight binding to apoflavoenzymes such as apo-D-amino acid oxidase. This was also demonstrated directly by treating the FAD
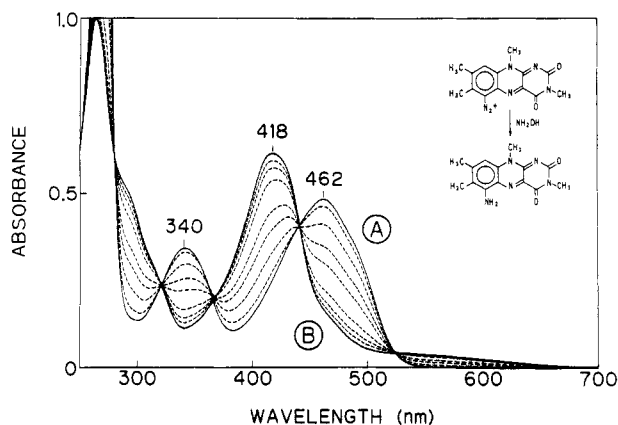
FIGURE 2: Absorption spectrum of 3-methyllumiflavin 6-diazonium cation (2a) and conversion to 6-amino-3-methyllumiflavin (1a). A total of 50 μL of a stock solution of 2a in acetonitrile containing 1% CH₃COOH was diluted into 950 μL of dilute acetic acid (pH 2.5) (curve A). A 50-μL aliquot of 1 M NH₂OH·HCl was then added, and the following curves were recorded after approximately 1, 4, 8, 12, 22, 32, and 42 min. The final spectrum (curve B) is identical with that of 6-amino-3-methyllumiflavin (1a) under the same conditions and was obtained after 80 min.

FIGURE 3: Spectra of 6-azidoriboflavin (3b) and pH dependence. Aliquots (50 μL) of 6-azidoriboflavin in dimethylformamide were diluted with 1 mL each of 0.1 M phosphate, pH 6.0 (—), 50 mM NaOH (– –), or 20% HClO₄ (–·–). Extinction coefficients were determined by titration of 6-azidoriboflavin at pH 7.0 with a standardized solution of riboflavin-binding protein (see text for details).

derivatives 3d and 4d with *Naja naja* venom and HPLC analysis of the products. In both cases, as well as when ATP and *N. naja* venom were used, the product was indistinguishable from authentic adenosine (HPLC: 10% MeOH/0.01 M phosphate buffer, pH 6.0). The synthetic path is outlined in Scheme I.

Some Properties and Reactions of Flavin 6-Diazonium Cations. Since the flavin 6-diazonium cations (2) are the key intermediates for the synthesis of further 6-substituted flavins and also in view of their potential use as active site probes, this class of compounds was investigated in some detail. The lumiflavin 6-(diazonium perchlorate) (2a) is stable in acetonitrile containing a trace of acid; however, in the presence of nucleophilic solvents and of base, the chromophore is destroyed irreversibly. In methanol containing traces of ammonia, or in basic or neutral aqueous solution, a chromophore with a λmax at 365 nm is formed immediately, which is not stable and decays further. An indication to its possible structure is suggested by the loss of the typical strong absorption band at λ 260–265 nm, suggesting that the aromatic system of the benzene ring has been suppressed. The absorption spectrum (Figure 2) of the 6-diazonium flavin cation (2a) retains the two maxima in the visible–near-UV region of normal flavin, indicating only a minor perturbation of the chromophore; this contrasts with the spectra of other 6-substituted flavins to be detailed below. 8-Diazonium flavins were found to yield 8-azidoflavins in good yield upon reaction with hydroxylamine (Fitzpatrick et al., 1985). In the present case, a completely different course of reaction was observed (Figure 2), in which 6-aminoflavin (1) was obtained essentially quantitatively. The latter was identified by its typical absorption spectrum (Ghisla et al., 1980) and by HPLC comparison with authentic material. This further confirms the structure of the flavin 6-diazonium structure (2). The different reactivity of 6-substituted flavins as compared with the 8-substituted analogues was observed also in attempts to convert the 6-diazonium cation 2 to 6-hydroxyflavin by a method published earlier for 8-hydroxyflavin (Ghisla & Mayhew., 1980), consisting of the acetolysis of 2 in glacial acetic acid followed by hydrolysis. In the present case, two main, highly fluorescent products having a flavin-like spectrum (λmax at 435, 365, and 265 nm) were obtained, which were, however, not the expected 6-OR derivatives and were not identified further.
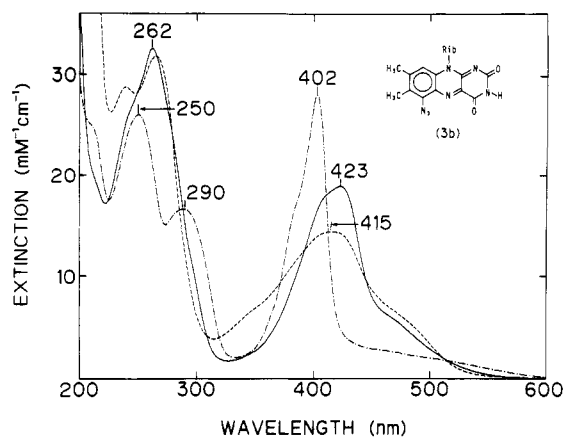
Properties and Reactions of 6-Azidoflavins. 6-Azidoriboflavin (3b) and 6-azido-3-methyllumiflavin (3a) are stable when stored as a solid in the dark. Their structure is confirmed by the ¹H NMR spectra, in which all the resonances of the parent compound are present (cf. Experimental Procedures), and by the IR-spectrum, which shows the characteristic strong –N₃ band at 2120 cm⁻¹. They have absorption spectra (Figure 3) lacking the typical two-banded shape of the oxidized isoalloxazine chromophore. These spectra are, on the other hand, similar to those of other 6-substituted flavins having electron-donating functions (Mayhew et al., 1974; Schöllnhammer & Hemmerich, 1974; Ghisla et al., 1980). The outstanding feature of 6-azidoflavins is their sensitivity to light as demonstrated in Figure 4. Thus, irradiation with the light of a desk lamp causes marked changes in the visible spectrum, concomitant with disappearance of the starting material as analyzed by HPLC (Figure 4, A, inset, and 4B). The light sensitivity is, however, at least 1 order of magnitude smaller than that of 8-azidoflavins (Ghisla et al., 1984; Fitzpatrick et al., 1985). A multitude of products appears during irradiation. Although several types of reaction can be expected upon photolysis of aromatic azides (Abramovitch & Kyba, 1971; Reiser & Wagner, 1971), this finding is in partial contrast to what was observed with 8-azidoflavins where several photoproducts could be identified readily (Ghisla et al., 1984; Fitzpatrick et al., 1985). The ratio of the products also changes upon further irradiation, indicating secondary photoreactions. A major product was identified as 6-aminoflavin (1a) by comparison of its properties with an authentic sample. It might be formed by intermolecular reduction. The products other than 6-aminoflavin formed upon irradiation (cf. inset of Figure 4A) have a much shorter retention time on HPLC than any 6-substituted riboflavin or lumiflavin. This indicates that a major modification or breakdown of the isoalloxazine nucleus occurs. One of these products has a retention time and fluorescence characteristics (excitation maximum 410 nm, emission maximum 480 nm) matching those of the main protic solvent decay product of the lumazine derivative 8 to be described below. A major compound to be expected from photoreaction of azides (Abramovitch & Kyba, 1971; Reiser & Wagner, 1971) is the product of insertion of the nitrene into water, i.e., the hydroxylamine derivative. Such a compound was identified as a product from the photolysis of 8-azidoflavins (Fitzpatrick et al., 1985). The synthesis of 6-(hydroxylamino)flavin was attempted by starting from 6-chloroflavin
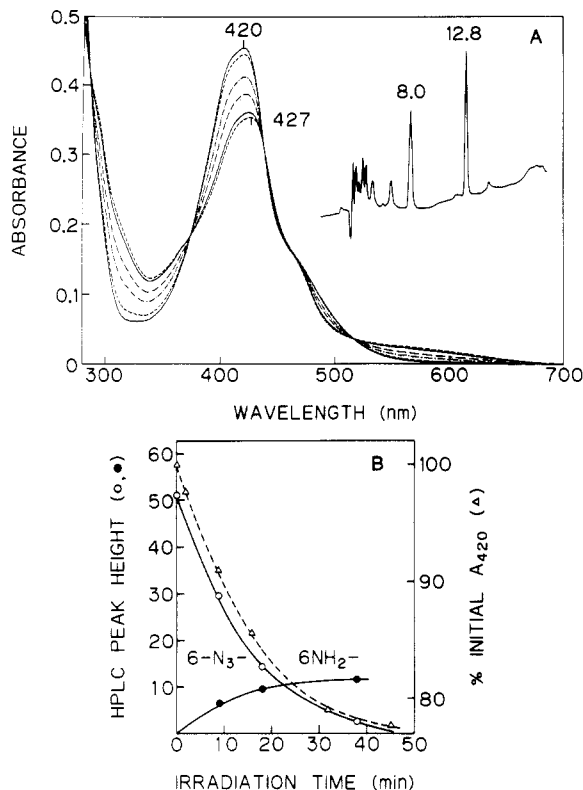
FIGURE 4: (A) Spectral course of photochemical reaction of 6-azi-do-3-methyllumiflavin (3a). 6-Azido-3-methyllumiflavin, 2.38×10^{-4} M in 0.1 M phosphate buffer, pH 7.0, was irradiated with a 15-W daylight fluorescent desk lamp (approximately 25 cm from the sample). (—) Starting spectrum. (---), (—·—), (—–), (—), and (---) were obtained after 1.5, 9, 17, 31, and 44 min, respectively. The inset shows the HPLC analysis of a sample obtained after 18-min irradiation. The elution gradient was 0.01 M phosphate buffer, pH 6.0/90% methanol as described under Experimental Procedures. 6-Azido-3-methyllumiflavin (3a) elutes at 12.8 min and 6-amino-3-methyl-lumiflavin (1a) after 8.0 min. (B) Concentration dependence of the components of the experiment of (A) on the time of irradiation. (△) Absorbance changes at 420 nm; (○) and (●) relative peak height of the signals corresponding to 6-azido- and 6-amino-3-methyllumiflavin, respectively, obtained from HPLC elution profiles such as that shown in the inset of (A).

(Ghisla et al., 1980). The major product obtained after incubation of this compound with excess hydroxylamine in dry dimethylformamide in the presence of K_2CO_3 was, however, 6-aminoflavin, which might have been formed from a primary 6-(hydroxylamino)flavin and its subsequent reduction by excess hydroxylamine. The elucidation of the structure of degradation product(s) is of importance in view of the projected use of 6-azidoflavins as photoaffinity labels. In view of the complexity of the mixture obtained, this promised to be a very difficult task. In order to obtain at least partial insight into the reaction pathways of photochemically generated flavin-6-nitrenes, we resorted to chemical generation and attempted the structural elucidation of at least some of the products, as detailed in the next section.

Reaction Course of Chemically Generated 3-Methyllumi-flavin-6-nitrene (6). The transient nitrene 6 derived from 6-azido-3-methyllumiflavin (3a) was generated with concentrated sulfuric acid at 0 °C (Abramovitch & Kyba, 1971). The crystalline product obtained in good yield (75%) was homogeneous by HPLC when analyzed in a weakly acidic system (pH 2.5). It was found, however, to decay in practically every protic solvent (HPLC analysis), in particular in the presence of trace amounts of base or at high pH. This behavior is a common feature of 8-substituted lumazines and has been shown to involve addition of solvent to the lumazine nucleus
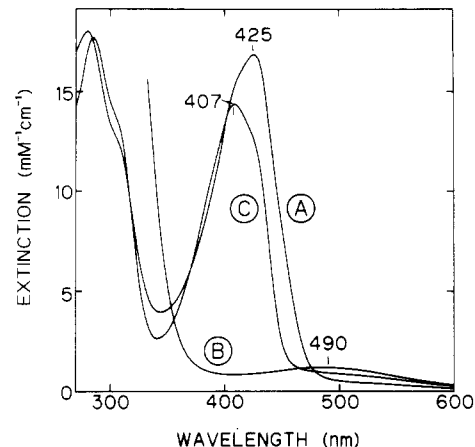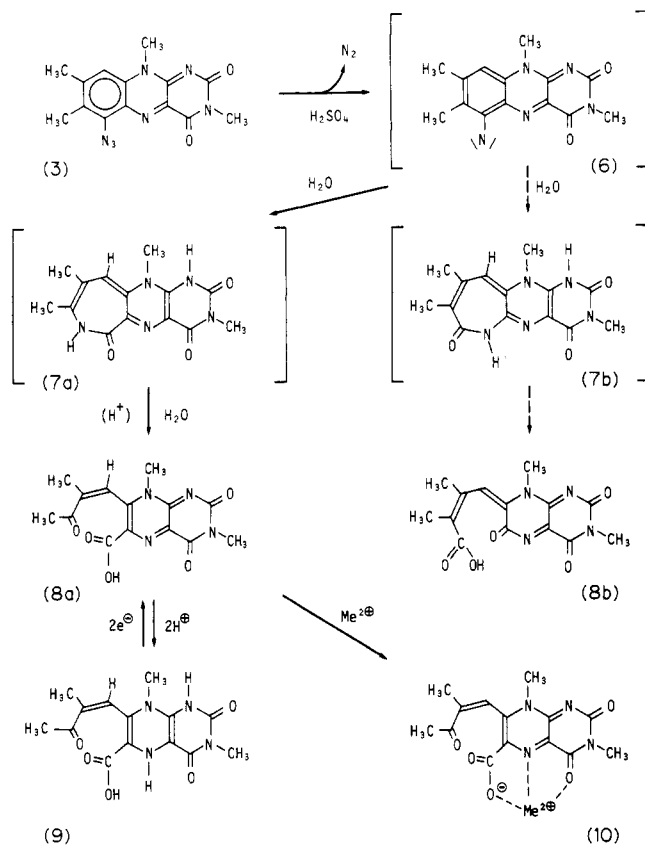

FIGURE 5: Spectral properties and redox reaction of 3,8-dimethyl-6-carboxy-7-(2-methyl-3-oxobut-2-enyl)lumazine (8a). The product from acid degradation of 6-azido-3-methyllumiflavin (3a) was dissolved in dimethyl sulfoxide containing 0.3% acetic acid and then diluted 20-fold in 0.1 M acetate buffer, pH 5.0 (curve A). To this a grain of solid dithionite was added, and spectrum B was recorded after careful mixing. Admission of air generated the spectrum of curve C. Repetition of the dithionite/air cycles generates the species of curves B and C.

(Pfleiderer et al., 1971; Ram et al., 1982). Its spectrum (Figure 5) clearly indicates that the isoalloxazine chromophore has been drastically modified; the absence of the typical strong UV absorption, which is present in all flavins having an intact xylene subnucleus (Visser & Müller, 1979), suggests that the latter has been modified. The IR spectrum reveals new bands attributable to carbonyl vibrations, which are not present in the parent compound. The ^1H NMR spectrum shows the presence of the same number of hydrogens as in the 6-azidoflavin (3a) and no additional couplings; the chemical shifts indicate a difference in environment of the methyl groups previously at C(7) and C(8) in 3a. The elemental analysis agrees very well with the formula $C_{14}H_{14}N_4O_5$, consistent with either of the two structures 8a or 8b (Scheme II). This interpretation is substantiated by several lines of evidence. As required, the compound forms a crystalline derivative with NH_2OH, the spectrum of which is only marginally different from that of the parent compound (λ_{max} at 430, 285, and 263 nm, in methanol, not shown). The elemental analysis of this hydroxylamine derivative is consistent with the formula C_{14}-$H_{15}N_5O_5$, which would be derived from addition at the oxo function of 8a. This argues against structure 8b in which the carbonyl function would be part of an amide and in which formation of such a derivative would not be facile and could be expected to have a major effect on the chromophore. The absorption spectrum of the compound is comparable in its λ_{max} and extinction coefficients (Figure 5) to those of 8-substituted lumazines. These have λ_{max} around 400 nm and similar extinction coefficients (Pfleiderer et al., 1966) and should be different from those of 8-substituted 6-oxolumazines (Pfleiderer, 1957). As required for a lumazine system, the molecule undergoes reduction by dithionite, presumably to the reduced form 9 (Figure 5). 5,8-Dihydrolumazines are reoxidized readily by oxygen, as observed (Mengel et al., 1977; Harzer & Ghisla, 1979). Of significance also is the long-wavelength band of the reduced species. Such a band is not present in normal reduced 8-substituted 7,8-dihydrolumazines (Pfleiderer & Mengel, 1971; Lund, 1975; Harzer & Ghisla, 1979) nor in reduced 6-oxolumazines (Pfleiderer, 1957); it was, however, observed in reduced lumazines carrying a —C=O function at position 6, the band being ascribed to exocyclic conjugation (Mengel et al., 1977). The long-wavelength band thus is

Scheme II: Proposed Reaction Course of Flavin-6-nitrene Generated in Sulfuric Acid and Structures of Intermediates and Products

FIGURE 6: Spectral course of Cu^{2+} complexation of 3,8-dimethyl-6-carboxy-7-(2-methyl-3-oxobut-2-enyl)lumazine (8a). The compound, 100 μL of a stock solution in dimethyl sulfoxide containing 0.3% acetic acid, was diluted into 1.0 mL of 0.1 M acetate buffer, pH 5.0 (curve A). A total of 20 μL of a 0.5 M solution of $Cu(NO_3)_2$ in the same buffer was then added to generate the spectrum of curve B. Addition of a small amount of solid dithionite led to formation of the spectrum of curve C. Upon being mixed with air, a spectrum identical with that of curve C of Figure 5 was obtained.

consistent also with structure 8a (Scheme II). In addition, a differentiation of structures 8a and 8b was attempted by complexation with transition metals. It has been shown (Schöllnhammer & Hemmerich, 1974) that 6-hydroxyflavins form chelate complexes with Cu^{2+}, and this feature was utilized for obtaining information on the nature of the 6-substitution in these compounds. Addition of Cu^{2+} to a freshly prepared solution of the compound indeed caused a major spectral change (Figure 6), which is ascribed to formation of the complex 10. Reduction of the system leads to a species having a pronounced band maximal at 520 nm, which can be reasonably ascribed to the reduced species 10. The same type of spectral effect was observed upon addition of Ni^{2+} (not shown).

Finally, a peculiar property of the rearrangement product should be mentioned briefly. As shown in Figure 5, dithionite reduction and reoxidation of the primary product (having a λ_{max} at 425 nm) generates a new species with a maximum at 407 nm and an intense blue fluorescence, both typical of 8-substituted lumazines. Subsequent reduction/oxidation cycles do not restore the original 425 nm absorbing species. The new product(s) do(es) not complex with metals, and one of its (their) major components has on HPLC a much longer retention time, as compared to the starting material, indicating the loss of a charge. These observations could reflect isomerizations of the side chain at position 7 of 8a; they would also be consistent with decarboxylation at position 6 of lumazine. The reaction course of the 6-azidoflavin (3) in concentrated sulfuric acid might thus be envisaged to parallel at least some of the reactions occurring during photolysis and to proceed as shown in Scheme II. Similar sequences of events are amply documented in the literature (Abramovitch & Kyba, 1971; Reiser & Wagner, 1971). The primary products(s) of ring expansion 7 is (are) likely not to be stable under strongly acidic
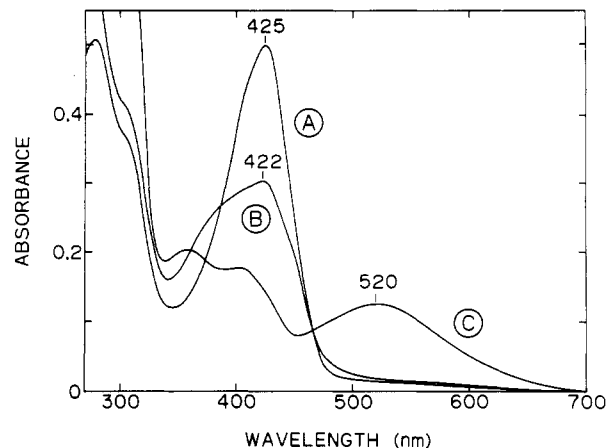
conditions. This is the probable reason for which it could not be isolated as such but only in the form of its hydrolysis product 8a. It is likely, however, that cyclic amides such as 7a and 7b are among the products observed upon photolysis (cf. Figure 4A).

Binding of 6-Azidoflavins to Apoproteins. 6-Azidoriboflavin binds tightly to the apoprotein of hen egg white riboflavin-binding protein, with a K_d value < 10^{-8} M at pH 7.0, 20 °C (results not shown). By standardization of the apoprotein with pure riboflavin, it is thus possible to determine the extinction coefficient of both the free and bound 6-azidoriboflavin. The free flavin has a λ_{max} at 420 nm ($\epsilon = 19\,000$ M^{-1} cm^{-1}). On binding to the riboflavin-binding protein, the λ_{max} shifts to 432 nm ($\epsilon = 15\,100$ M^{-1} cm^{-1}). 6-N_3-FMN was prepared from 6-N_3-FAD by addition of a trace of *N. naja* venom. If one assumes that 6-N_3-FMN has approximately the same spectrum as 6-azidoriboflavin (an assumption based on observations with many different substituted flavins), the extinction coefficient of 6-N_3-FAD at its λ_{max}, 428 nm, can be estimated as 17 500 M^{-1} cm^{-1}. Thus, as with normal flavin, the FAD level has a slightly longer wavelength maximum and an approximately 10% lower extinction than the FMN-level flavin. 6-Azido-FMN has been found to bind tightly to apoflavodoxin and to the apoprotein of Old Yellow Enzyme. Bound to apoflavodoxin, the λ_{max} is 428 nm, $\epsilon = 16\,800$ M^{-1} cm^{-1}, while the Old Yellow Enzyme form has $\lambda_{max} = 423$ nm, $\epsilon = 15\,700$ M^{-1} cm^{-1}. Bound to the apoenzyme of lactate oxidase, the 6-N_3-FMN has a λ_{max} at 428 nm; in this case, the extinction coefficient could not be determined because of instability of the apoprotein. 6-Azido-FAD also binds to the apoprotein of D-amino acid oxidase, the λ_{max} shifting from 428 to 422 nm, with essentially no change in extinction coefficient. Benzoate also binds strongly to the artificial enzyme, as it does with native enzyme (Yagi & Ozawa, 1962; Quay & Massey, 1977), to give a resolved spectrum with a double maximum, 432 and 488 nm, $\epsilon = \sim 16\,000$ M^{-1} cm^{-1}.

The properties of these artificial flavoproteins, including their reactions with substrates and other ligands, and the effect of light irradiation will be detailed in a separate paper (V. Massey, S. Ghisla, and K. Yagi, unpublished results).

Properties of 6-Thiocyanatoflavins and 6-Mercaptoflavins. Previous studies had shown the conversion of 6-thiocyanato-
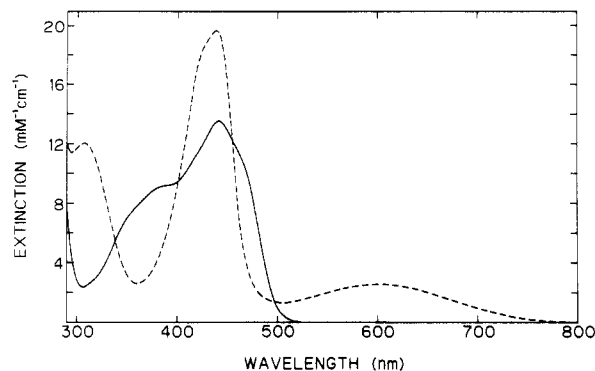
FIGURE 7: Absorption spectra of 6-thiocyanatoriboflavin (**4b**) and 6-mercaptoriboflavin (**5b**). 6-Thiocyanatoriboflavin (**4b**) in 0.05 M potassium phosphate buffer, pH 7.0, containing 100 μM EDTA (—) was reacted with 0.4 mM dithiothreitol to produce 6-mercaptoflavin (**5b**) (--). Extinction coefficients were determined by titration with riboflavin-binding protein, as shown in Figure 8.
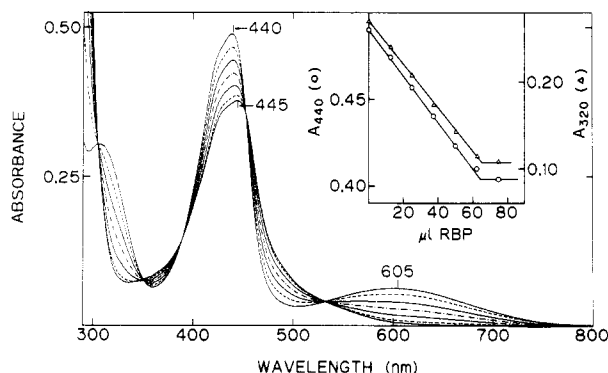

FIGURE 8: Titration of 6-mercaptoriboflavin (**5b**) with riboflavin-binding protein. 6-Mercaptoriboflavin (**5b**) (volume = 1 mL) prepared as described in Figure 7 (curve with highest absorbance at 440 nm) was titrated with 12.5-μL aliquots of 3.81 × 10⁻⁴ M aporiboflavin-binding protein (successive curves). The absorbance values at 440 and 320 nm, corrected for dilution, are shown in the inset. The end point of 65 μL of riboflavin-binding protein permits the extinction coefficient of the starting 6-mercaptoriboflavin (**5b**) to be calculated: ϵ_{440} = 19 600 M⁻¹ cm⁻¹.

flavins to 6-mercaptoflavins by reductive elimination of cyanide, carried out with $Na_2S_2O_4$ or photochemically (Ghisla et al., 1980). The ready dimerization of the 6-mercaptoflavin by oxidation (Ghisla et al., 1980), however, did not seem to make it an attractive candidate as an active site probe for flavoproteins. Such problems, however, are easily overcome by treatment of 6-thiocyanatoflavins with dithiothreitol, which brings about their rapid conversion to the corresponding 6-mercaptoflavin (second-order rate constant: ~1 × 10³ M⁻¹ min⁻¹ for 6-thiocyanatoriboflavin at pH 7.0, 20 °C) and at the same time stabilizes the 6-mercaptoflavin against oxidative dimerization (cf. Figure 7). Thus, the conversion can be carried out with nearly stoichiometric concentrations of dithiothreitol, and provided that EDTA (>10 μM) is present to prevent trace metal catalyzed oxidation of thiols, the resulting 6-mercaptoflavins are stable for hours, permitting their incorporation into apoproteins. Under the conditions employed, this conversion does not appear to result from reduction of the flavin and elimination of cyanide, as found earlier for the low-pH photochemical reaction (Ghisla et al., 1980). Details of the possible mechanism will be published later (V. Massey, S. Ghisla, and K. Yagi, unpublished results).

The extinction coefficients were determined by titration of the 6-mercaptoriboflavin with riboflavin-binding protein, as shown in Figure 8. Riboflavin-binding protein is known to bind to neutral flavins with great avidity but weakly, or not
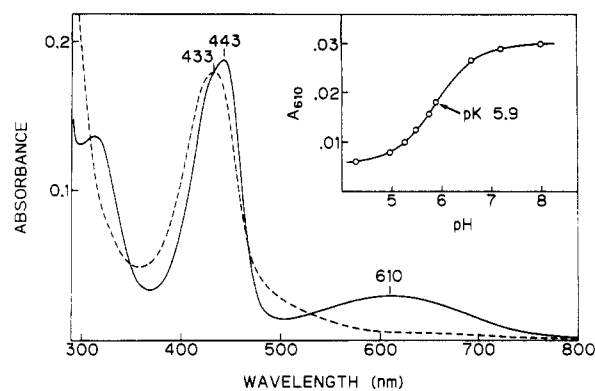

FIGURE 9: Spectra of 6-mercapto-FAD (**5d**) in its neutral (protonated) and anionic states. 6-Thiocyanato-FAD (**4d**) (~10 μM) in 0.02 M pyrophosphate buffer, pH 8.0, containing 1 mM EDTA was converted to 6-mercaptoflavin by addition of 5 mM dithiothreitol (—). The pH was then lowered stepwise by the addition of small amounts of finely powdered citric acid and the spectrum recorded after each addition. Isosbestic points at 530, 468, 434, and 350 nm were maintained to the lowest pH used, pH 4.3 (--). The inset shows the absorbance at 610 nm as a function of pH, yielding a pK of 5.9.

at all, to negatively charged flavins (Blankenhorn et al., 1975; Becvar & Palmer, 1982). The pronounced spectral shifts seen with riboflavin-binding protein are similar to those found with 6-hydroxy- and 8-hydroxyriboflavin (Massey, 1980) and with 8-mercaptoriboflavin (Massey et al., 1979) and are again consistent with preferential binding of the neutral flavin, even though the titration was carried out at a pH where the free flavin was in its anionic form (cf. Figure 9).

The pK of the thiol function of 6-mercapto-FAD was determined by recording the spectrum in the presence of excess dithiothreitol and by changing the pH by the addition of powdered citric acid. Figure 9 shows the spectra in the anionic and neutral forms, and the inset of A_{610} vs. pH shows a pK of 5.9, slightly lower than the value of 6.4 given by Steenkamp et al. (1978) for 6-mercapto-FMN. This is consistent with the previously determined pK values of hydroxy- and mercaptoflavins substituted at the 6- and 8-positions. The pK of 6-hydroxyriboflavin is 7.1 (Mayhew et al., 1974), while that of 8-hydroxyriboflavin is 4.8 (Ghisla & Mayhew, 1976), a difference of 2.3 pH units. 8-Mercaptoriboflavin has a pK of 3.8 (Massey et al., 1979), 2.1 pH units below that of 6-mercapto-FAD. This property of 6-mercaptoflavins, and the pronounced spectral differences between the neutral and anionic forms, should make them useful probes of the protein environment of the bound flavin, as has proved possible with the other 6- and 8-substituted hydroxy- and mercaptoflavins. Furthermore, the chemical reactivity of the mercapto function toward peroxides and alkylating agents should serve to probe solvent accessibility to this portion of the flavin in various flavoproteins, as has proved possible with flavins carrying sulfur substituents at the 2-, 4- and 8-positions (Schopfer et al., 1981; Biemann et al., 1983; Massey et al., 1984).

ACKNOWLEDGMENTS

Registry No. **1a**, 72184-57-7; **1b**, 73652-55-8; **1d**, 101760-86-5; **2a**, 101760-81-0; **3a**, 101760-82-1; **3b**, 101760-83-2; **3c**, 101760-92-3; **3d**, 101760-85-4; **4b**, 73647-56-0; **4d**, 101760-87-6; **5b**, 101760-88-7; **5c**, 101760-89-8; **5d**, 101760-90-1; **6**, 101760-91-2; **8a**, 101760-84-3; Cu, 7440-50-8; old yellow enzyme, 9001-68-7; lactate oxidase,

9028-72-2; D-amino acid oxidase, 9000-88-8.

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