Synthesis and Biological Evaluation of 7α,7α,7α,8α,8α,8α-Hexafluororiboflavin and 7α,7α,7α,8α,8α,8α-Hexafluoro-FMN

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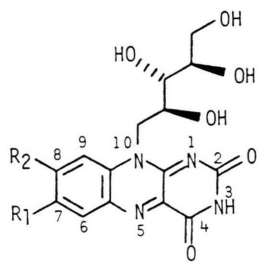
Riboflavin, 7α,7α,7α,8α,8α,8α-Hexafluoro-FMN, *Lactobacillus casei*, Apoflavodoxin, Oxidation-Reduction Potential

7α,7α,7α,8α,8α,8α-Hexafluororiboflavin (**4**) has been prepared and its oxidation-reduction potential determined polarographically to be +0.02 V with respect to the standard hydrogen electrode. Compound **4** does not by itself promote the growth of *Lactobacillus casei*. However, in the presence of low riboflavin (**1**) concentrations, the hexafluoro analog **4** has some growth enhancing activity. The FMN analog 7α,7α,7α,8α,8α,8α-hexafluororiboflavin 5′-phosphate (**10**) was also synthesized and found to bind tightly to apoflavodoxin from *Megasphaera elsdenii*. The dissociation constant (3.2×10^{-9} M) is about one order of magnitude larger than that of FMN (1.1×10^{-10} M). However, apoflavodoxin reconstituted with hexafluororiboflavin 5′-phosphate (**10**) has no coenzyme activity. Hexafluoro-FMN (**10**) was also unable to act as a coenzyme for luciferase from *Photobacterium fisheri*. Hexafluororiboflavin **4** did not inhibit the light riboflavin synthase from *Bacillus subtilis* to a significant extent ($K_i > 10^{-4}$ M).

The vital role which riboflavin (**1**) plays in cellular metabolism has led to the preparation of an array of structurally related molecules in the search for compounds which might function as useful riboflavin antagonists (antimetabolites) [1]. In living cells, riboflavin generally occurs as riboflavin 5′-phosphate (flavin mononucleotide, FMN) and flavin adenine dinucleotide (FAD) bound to specific proteins to form oxidative enzymes. While it has been demonstrated that substituents in positions 7 and 8 of the isoalloxazine nucleus have a relatively small influence on protein binding, such substituents can have a pronounced influence on the flavin's oxidation-reduction potential [2]. These positions therefore appear to be a logical location for the introduction of fluorine. Consideration of the similarity of the van der Waals radii of fluorine (1.35 Å) and hydrogen (1.20 Å), along with the fact that fluorine is the most electronegative element, suggests that such fluorinated flavins should be capable of binding to apoenzymes and also that they would have a higher oxidation-reduction potential than riboflavin itself. A variety of fluorinated analogs of riboflavin (**1**), including **2** [3], **3** [4], and **4** [5], have already been prepared. Compounds **2** and **3** are reported to be competitive antagonists of riboflavin (**1**) in *Lactobacillus casei* [6]. The present communication describes a modified synthesis and biological evaluation of 7α,7α,7α,8α,8α,8α-hexafluororiboflavin (**4**) as well as the preparation and apoflavodoxin binding properties of hexafluoro-FMN **10**.



1, $R_1 = R_2 = CH_3$
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4, $R_1 = R_2 = CF_3$

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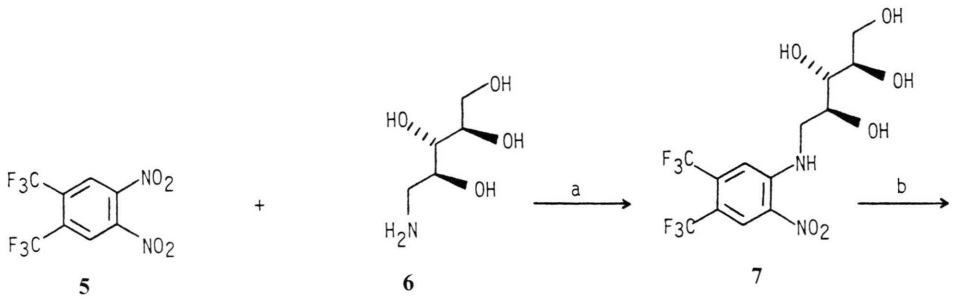
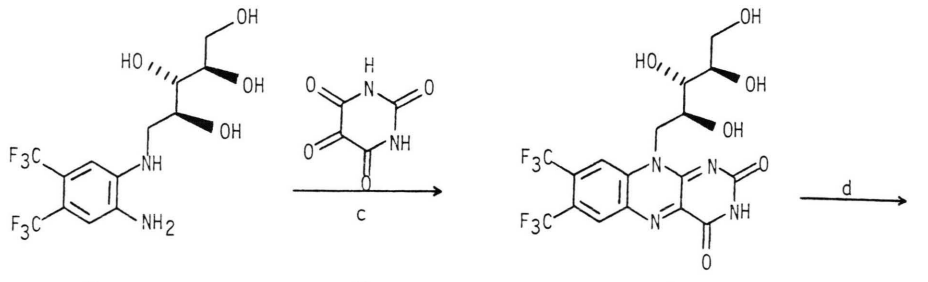
Chemistry

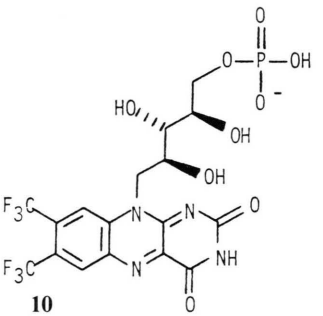
The reaction of 1,2-bis(trifluoromethyl)-4,5-dinitrobenzene (**5**) [7] with D-ribamine (**6**) [8] yielded



5 + **6** → (a) **7** → (b)



8 → (c) **9** → **4** → (d)



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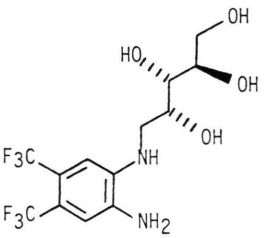

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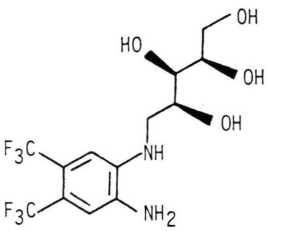

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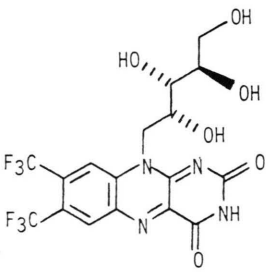

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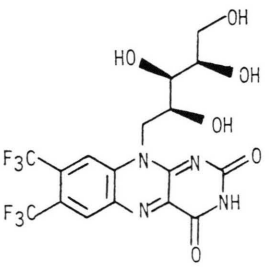

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Scheme I

[a] Isoamyl alcohol, 110 °C, 24 h.
[b] Zn, AcOH, 23 °C, 15 min.
[c] $B(OH)_3$, AcOH 23 °C, 72 h.
[d] H_2PO_3Cl, 23 °C, 19 h.

7. Reduction of the nitro group of compound **7** with zinc in acetic acid gave the intermediate diamine **8**, which without isolation was treated with boric acid and alloxan (**9**) in acetic acid to afford the hexafluororiboflavin **4**. Phosphorylation of **4** using monochlorophosphoric acid provided the target compound **10** [10]. The final product **10** migrated as a single spot in electrophoresis and in multiple thin layer chromatography systems (Table I). It also produced a single peak when subjected to HPLC (Table II).

The oxidation-reduction potential (E_0') of hexafluororiboflavin **4** at pH 7, 25 °C was determined by polarography to be +0.02 V with reference to the standard hydrogen electrode [11]. This value is significantly positive in comparison to those determined for riboflavin (E_0' − 0.21 V) [12] and a wide variety of riboflavin analogs [12–16].

The corresponding hexafluoro-D-araboflavin **13** and hexafluoro-D-xyloflavin **14** analogs were also prepared in a similar fashion from appropriate diamines **11** and **12**.

Table I. Thin-layer chromatography of hexafluororiboflavin 5′-phosphate.

I	*n*-butanol/ethanol/water (50:15:35, v/v)/cellulose
II	*n*-butanol/ethanol/water (50:15:35, v/v)/silicagel
III	*t*-butanol/water (60:40, v/v)/silicagel
IV	*n*-butanol/acetic acid/water (50:20:30, v/v)/cellulose
V	*n*-butanol/acetic acid/water (50:20:30, v/v)/silicagel

Retention values

	I	II	III	IV	V
FMN	0.27	0.11	0.54	0.35	0.27
F_6-FMN	0.51	0.14	0.68	0.39	0.45

Table II. Retention values of flavins studied by reverse phase HPLC[a].

Compound	Retention time [min]
Riboflavin	45.6
FMN	26.1
Hexafluoroflavin	80.3
F_6-FMN	42.6

[a] Analytical HPLC from Hupe and Busch equipped with a Nucleosil 10 C_{18} column (Machery and Nagel), 250 × 4 mm; eluent 0.1 M ammonium formate pH 3.7 in 17% methanol; flow rate, 2.0 ml/min.

Biological Results and Discussion

The biological activity of hexafluororiboflavin **4** was tested in *Lactobacillus casei*, which is entirely dependent on an exogenous supply of riboflavin for growth [17]. The growth was monitored both by turbidemetry and by titration of the acids present in the medium. Examination of the data presented in Fig. 1 reveals that the hexafluororiboflavin **4** does not by itself promote the growth of the test organism over a wide range of concentrations. However, in the presence of a low concentration of riboflavin (**1**) which does not promote the maximum rate of growth, the hexafluororiboflavin **4** has some growth enhancing activity at relatively high concentrations. At very high concentrations the hexafluororiboflavin **4** is inhibitory. Similar results were obtained when the experiment was performed using an auxotrophic mutant of *Bacillus subtilis* which is riboflavin deficient.

An alternative experiment was also performed in which *L. casei* was exposed to an optimum concentration of the hexafluororiboflavin **4** of 3 mg/l and the concentration of riboflavin (**1**) varied (Fig. 2). The data show convincingly that hexafluororiboflavin **4** has a growth-promoting effect which is most pronounced at very low riboflavin (**1**) concentrations. Qualitatively the same results were obtained when the incubation time was varied from one to three days. In separate experiments in which the growth was monitored by determination of the acids produced rather than by turbidemetry, the production of acids was increased by hexafluororiboflavin **4**.

The binding of hexafluoro-FMN (**10**) to apoflavodoxin from *Megasphaera elsdenii* (courtesy of Professor S. Ghisla, Konstanz) was determined by fluorescence titration of the apoflavodoxin with hexafluoro-FMN (Fig. 3). The fluorescence of the analog is significantly quenched upon binding to the protein. The residual fluorescence of 4.8% in the titration curve is partially due to the presence of hexafluororiboflavin 4′-phosphate, which we were unable to remove by HPLC. Scatchard analysis of the data allowed the calculation of the dissociation constant (3.2×10^{-9} M), which is about one order of magnitude larger than that of FMN (1.1×10^{-10} M). However, apoflavodoxin reconstituted with hexafluororiboflavin 5′-phosphate (**10**) has no coenzyme activity [18]. Hexafluoro-FMN (**10**) was also unable

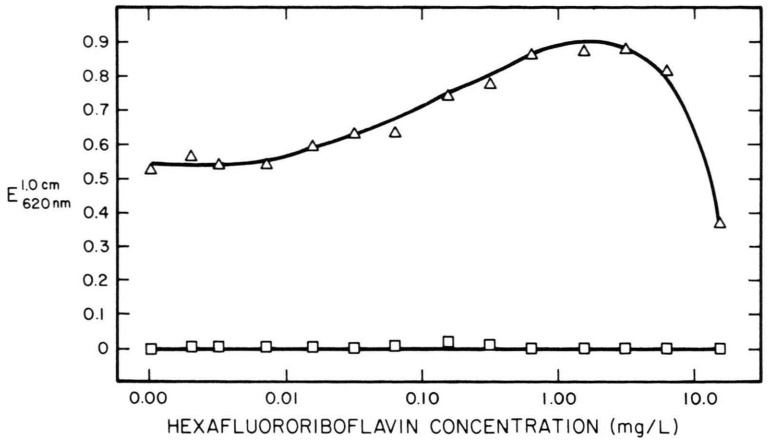

Fig. 1. Effect of varying concentrations of hexafluororiboflavin **4** on the growth of *Lactobacillus casei* in the absence of riboflavin (□) and in the presence of riboflavin at a concentration of 14 µg/l (△). The incubation time was 24 h.

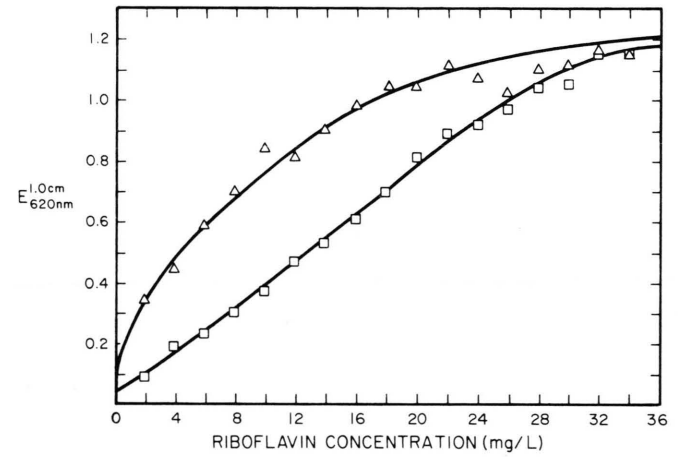

Fig. 2. Effect of varying concentrations of riboflavin on the growth of *Lactobacillus casei* in the absence (□) and in the presence (△) of hexafluororiboflavin **4** at a concentration of 3 mg/l.

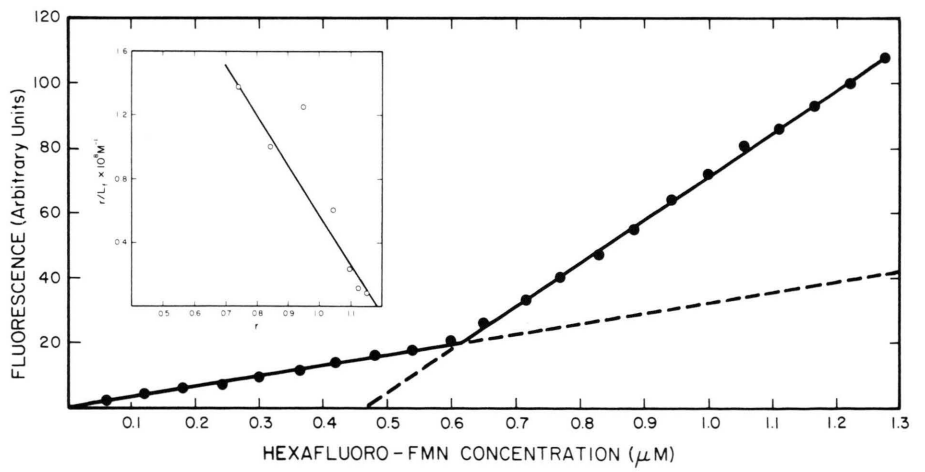

Fig. 3. Binding of hexafluororiboflavin 5′-phosphate (**10**) by apoflavodoxin from *M. elsdenii*. A solution containing 5.7×10^{-7} M apoflavodoxin, 10 mM sodium acetate pH 6.0 and 0.2 M sodium chloride was titrated with 2.45×10^{-5} M hexafluoro-FMN (**10**) at 23 °C. Fluorescence was monitored as described in the Experimental Section. After each addition of flavin, the system was allowed to reach equilibrium before reading. Inset: Scatchard plot of the binding of hexafluoro-FMN (**10**) to apoflavodoxin; r = the fraction of binding sites that are occupied, L_f = the concentration of free ligand.

to act as a coenzyme for luciferase from *Photobacterium fisheri* [19]. Hexafluororiboflavin **4**, as well as analogs **13** and **14**, did not inhibit the light riboflavin synthase from *Bacillus subtilis* [20] to a significant extent ($K_i > 10^{-4}$ M). The dihydro forms of these substances also did not inhibit the enzyme.

Several other riboflavin analogs, which are inactive when administered alone, have been observed to stimulate the growth of *L. casei* when provided at lower analog:riboflavin ratios and to inhibit growth at higher ratios. These include lumiflavin [21], L-lyxoflavin [22], D-galactoflavin [22], 7-ethyl-8-chloro-10-(1′-D-ribityl)isoalloxazine [23], and roseoflavin [24].

Experimental Section

Melting points were determined using a Thomas Hoover Unimelt apparatus and are uncorrected. Visible and UV absorbance were measured with a PM 6 photometer (Zeiss). IR spectra were recorded on a Beckman IR-33 spectrophotometer. NMR spectra were recorded on Varian EM-360 60 MHz or FT-80 80 MHz instruments. Chemical shifts are reported in parts per million relative to Me_4Si as internal standard. Mass spectra were recorded on a CEC 21-220 or on a Dupont model 21-492B spectrometer using an ion-source temperature of 230–270 °C, an ionization potential of 70 eV, and an ionizing current of 100 μA. Elemental analyses were performed by the Purdue Microanalytical Laboratory. 6,7-Dimethyl-8-ribityllumazine was synthesized [25]. Light riboflavin synthase from *Bacillus subtilis* (specific activity 113 U/mg) was prepared as described [20]. Thin layer chromatography was performed with precoated silica gel plates (Cellulose F_{254}, Merck). Solvent systems are described in Table I. Electrophoresis was performed on Cellulose acetate strips (Machery and Nagel, Duren) in 0.1 M borate buffer pH 8.2 at 6 V/cm.

4,5-Bistrifluoromethyl-2-nitro-N-(1′-D-ribityl)aniline (**7**)

D-Ribamine [8] (**6**, 0.15 g, 1.0 mmol) and 1,2-bis(trifluoromethyl)4,5-dinitrobenzene [7] (**5**, 0.30 g, 1.0 mmol) were added to isoamyl alcohol (7.5 ml). The stirred solution was heated at 110 °C for 24 h. The reaction mixture was concentrated by evaporation of the solvent, yielding an orange-yellow solid (0.29 g, 71%): m.p. 107–109 °C (lit. [5] m.p. 118–120 °C); IR (KBr) 3500-3100, 1637, 1573, 1315, 1130 cm^{-1}; NMR (acetone-d_6) δ 9.11 (br s, 1 H), 8.69 (s, 1 H), 7.81 (s, 1 H), 3.81 (m, 7 H), 4.18 (br s, 4 H); mass spectrum, *m/e* (relative intensity) 409 ($M^+ + 1$, 9), 389 (11), 287 (23), 270 (10), 258 (100), 230 (12), 214 (42). Anal. ($C_{13}H_{14}F_6N_2O_6$) C, H, N, F.

7α,7α,7α,8α,8α,8α-Hexafluoro-D-riboflavin (**4**) [5]

4,5-Bistrifluoromethyl-2-nitro-N-(1′-D-ribityl)-aniline (**7**, 122.4 mg, 0.3 mmol) and activated zinc dust (0.30 g) were added to glacial acetic acid (2 ml). The solution was stirred for 15 min under a nitrogen atmosphere and then filtered through Celite. The solid material was washed with acetic acid (2 ml). To the combined organic solutions was added a solution of alloxan monohydrate (48 mg, 0.3 mmol) and boric acid (100 mg, 1.6 mmol) in acetic acid (4 ml) which had been warmed to 60 °C. The resulting solution was stirred for 30 s and then allowed to stand in the dark for 3 days. The work-up procedure was also performed in the dark. The solvent was evaporated and the residue was washed with methanol (10 × 10 ml). Water was added and the suspension was filtered. The solvent was evaporated from the filtrate and the residue was recrystallized from chloroform:methanol (10:1) to afford a yellow solid (80 mg, 50%): m.p. 225 °C (dec) [lit. [5] m.p. 223.5–225 °C (dec)]; UV ε_{427} = 10200 M^{-1} cm^{-1}; IR (KBr) 3600–3100, 1710, 1696, 1140 cm^{-1}; mass spectrum, *m/e* (relative intensity) 484 (M^+, 0.3), 367 (7), 351 (16), 350 (100), 331 (15), 328 (9), 280 (7), 279 (6), 260 (5), 248 (12). Anal. ($C_{17}H_{14}F_6N_4O_6\,3H_2O$) C, H, N, F.

7α,7α,7α,8α,8α,8α-Hexafluororiboflavin 5′-phosphate (**10**)

Hexafluororiboflavin, (**4**, 7.0 mg, 14.5 μmol) was dissolved in 0.5 ml (6.7 mmol) of freshly prepared monochlorophosphoric acid [10]. After standing at room temperature for 19 h under protection from light and moisture, the reaction was terminated by the addition of 5 ml of water. The pH of the solution was adjusted to 3 by the slow addition of 25% NH_4OH. This solution was directly applied to a preparative HPLC column (Liquid Chromatograph 830 from Dupont, Lichrosorb RP 18 column, 16 × 250 mm; eluent, 0.1 M ammonium formate pH 3.7 in 20% methanol). Fractions were collected

and methanol was removed by evaporation under reduced pressure. The remaining solution was lyophilized. No attempts were made to crystallize the hexafluororiboflavin 5′-phosphate. The yield of isolated 5′-phosphate was 2.1 μmol (14.1%); 2.7 μmol of the unphosphorylated hexafluoroflavin was recovered. The substance migrated as a single spot in electrophoresis and in all thin layer chromatography systems (Table I). The titration with an excess of apoflavodoxin showed a residual fluorescence of 4.8%. The extinction coefficient at pH 7 was $\varepsilon_{427} = 10200\ M^{-1}\ cm^{-1}$.

*4,5-Bistrifluoromethyl-2-nitro-N-(1′-*D*-arabityl)aniline* (**11**)

D-Arabitylamine (0.15 g, 1.0 mmol) and 1,2-bis(trifluoromethyl)-4,5-dinitrobenzene (**5**, 0.30 g, 1.0 mmol) were added to isoamyl alcohol (8 ml). The stirred solution was heated for 24 h at 90 °C. The reaction mixture was concentrated to yield an orange-yellow solid (0.31 g, 77%): m.p. 182–184 °C; IR (KBr) 3460–3100, 1633, 1570, 1319 cm^{-1}; NMR (acetone-d_6) δ 9.21 (br s, 1H), 8.69 (s, 1H), 7.78 (s, 1H), 3.85 (m, 7H), 3.78 (br s, 4H); mass spectrum, *m/e* (relative intensity) 409 ($M^+ + 1$, 68), 389 (57), 287 (100), 270 (44). Anal. ($C_{13}H_{14}F_6N_2O_6$) C, H, N, F.

*7α,7α,7α,8α,8α,8α-Hexafluoro-*D*-araboflavin* (**13**)

This substance was prepared from compound **11** essentially as described for compound **4** except the reaction time was shortened to 2 days. This gave a yellow solid in 46% yield: m.p. 235 °C (dec); IR (KBr) 3420–3220, 1712, 1697, 1577, 1546, 1500, 1141 cm^{-1}. Anal. ($C_{17}H_{14}F_6N_4O_6$) C, H, N, F.

*4,5-Bistrifluoromethyl-2-nitro-N-(1′-*D*-xylityl)aniline* (**12**)

D-Xylitylamine (0.15 g, 1.0 mmol) and 1,2-bis(trifluoromethyl)-4,5-dinitrobenzene (**5**, 0.30 g, 1.0 mmol) were added to isoamyl alcohol (10 ml). The stirred solution was heated for 24 h at 80 °C. The reaction mixture was concentrated to yield an orange-yellow solid (0.28 g, 70%): m.p. 70–72 °C; IR (KBr) 3500–3200, 1630, 1570, 1310, 1140 cm^{-1}; NMR (acetone-d_6) δ 9.00 (broad s, 1H), 8.54 (s, 1H), 7.78 (s, 1H), 4.08 (br s, 4H), 3.70 (m, 7H); mass spectrum, *m/e* (relative intensity) 409 ($M^+ + 1$, 36), 389 (46), 287 (100), 270 (38), 258 (41). Anal. ($C_{13}H_{14}F_6N_2O_6$) C, H, N, F.

*7α,7α,7α,8α,8α,8α-Hexafluoro-*D*-xyloflavin* (**14**)

The procedure employed for the preparation of **13** yielded **14** from compound **12** as a yellow solid in 49% yield: m.p. 203–205 °C (dec); IR (KBr) 3540–3240, 1721, 1588, 1550, 1510, 1149 cm^{-1}. Anal. ($C_{17}H_{14}F_6N_4O_6 \cdot 1/2\ H_2O$) C, H, N, F.

Determination of the Oxidationreduction potentials

The oxidationreduction potentials of compounds **4**, **13**, and **14** were determined using a Princeton Applied Research model 174A polarographic analyzer operating in the DC mode. It was equipped with a dropping mercury electrode (working electrode), a saturated calomel electrode (reference electrode), a platinum wire (auxiliary electrode), and a model 174/70 drop-timer. A solution of cadmium chloride (10^{-3} M) in potassium chloride (10^{-3} M) was used to check the instrument. This solution has an oxidationreduction potential of −0.60 V. Riboflavin (**1**, 10^{-4} M) in potassium chloride (0.1 M) was used as a reference solution with its oxidationreduction potential being −0.48 V (−0.21 V with respect to the standard hydrogen electrode). Solutions of compounds **4**, **13**, and **14** (10^{-4} M) in potassium chloride (0.1 M) at pH 7 were used to determine the oxidationreduction potentials. The observed value for **4** is −0.25 V, for **13** it is −0.26 V, and for **14**, it is −0.25 V. These correspond to **4**, +0.02 V; **13**, +0.01 V; and **14**, +0.02 V with respect to the standard hydrogen electrode.

Fluorescence titration

Apoflavodoxin was prepared from *M. elsdenii* by the method of Mayhew [26]. Fluorescence titration was performed using a Farrand MK1 spectrofluorometer (excitation 424 nm, emission 507 nm). Hexafluoroflavin 5′-phosphate (**10**) was titrated with an excess of apoflavodoxin to monitor purity. Binding constants were determined by titration of apoflavodoxin with an excess of the flavin phosphate at 23 °C.

Determination of flavodoxin activity

Apoflavodoxin (3.9 μM) was reconstituted with the hexafluoroflavin phosphate (36.7 μM) at pH 7.0.

The flavodoxin activity was measured as described [18].

Determination of luciferase activity

An aerobic solution (0.2 ml) containing 2.9 nM *Photobacterium fisheri* luciferase (Boehringer, Mannheim), 31 mM potassium phosphate, and 0.1 mM dithioerythritol was mixed with 200 µl of a suspension containing 25 µM 1-decanal, 0.01% Triton-X-100, and 30 mM phosphate pH 7.0. The bioluminescence reaction was immediately initiated by the rapid injection of a solution of reduced F_6-FMN. The flavin phosphate was reduced with a slight excess of sodium dithionite. Alternatively, the flavin phosphate was photoreduced in the presence of 10 mM EDTA [27]. Bioluminescence activity was measured at 0 °C with a Biolumat 9500 from Berthold Inc.

Measurement of riboflavin synthase activity

The conversion of 6,7-dimethyl-8-ribityllumazine to riboflavin by light riboflavin synthase from *Bacillus subtilis* was followed photometrically (470 nm) in a thermostated cell (37 °C). The reaction mixture contained 0.2 M phosphate buffer pH 7.0, 237 U of partially purified light riboflavin synthase, 25–75 µM 6,7-dimethyl-8-ribityllumazine and 0–75 µM of the respective flavin in a total volume of 1.0 ml.

Lactobacillus casei growth test

Reagent tubes containing 5 ml of Riboflavin Assay Medium (Difco) and appropriate amounts of flavine were sterilized and inoculated with a dilute suspension of *Lactobacillus casei* ATCC 7469. Turbidity was determined after 24 h. Acid production was determined after 65 h by titration with 0.1 N NaOH using an autotitrator (Radiometer).

Acknowledgements

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