Articles

Synthesis of Epimeric 6,7-Bis(trifluoromethyl)-8-ribityllumazine Hydrates. Stereoselective Interaction with the Light Riboflavin Synthase of *Bacillus subtilis*

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Reaction of perfluorobutane-2,3-dione with 5-amino-6-(D-ribitylamino)pyrimidine-2,4(1*H*,3*H*)-dione hydrochloride (**13**) gave a mixture of the epimeric covalently hydrated fluorolumazines **14a** and **14b**, which were separated by preparative HPLC. The epimer that eluted first using a methanol–ammonium formate buffer mobile phase (epimer A) interacts stereospecifically with light riboflavin synthase of *Bacillus subtilis* as shown by ^{19}F NMR spectroscopy, which provided evidence for the formation of three enzyme-bound species that exchange with the free ligand on the NMR time scale. Dissociation constants of 13.0 and 16.2 μM were obtained from NMR and equilibrium dialysis experiments, respectively. Both methods showed the binding of one ligand molecule per protein subunit. Epimer A is a competitive inhibitor of the enzyme (K_I = 38 μM at pH 6.8). Epimer B did not bind to the enzyme.

Introduction

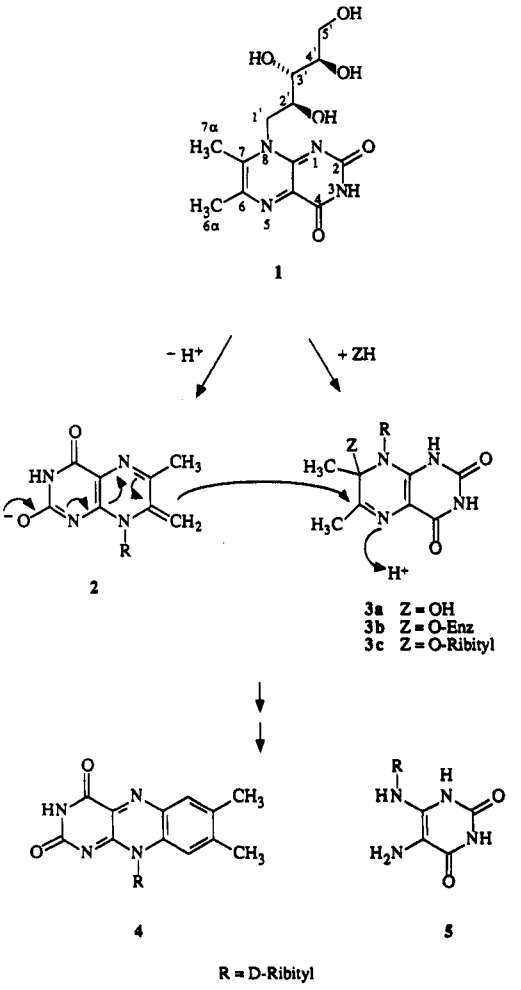
Inhibition of the biosynthesis of riboflavin provides a strategy for the development of therapeutically useful antibiotics. While pathogenic microorganisms must synthesize their own riboflavin, mammals obtain this vitamin through dietary sources. Thus, toxicity to the pathogen without affecting the host can, in principle, be achieved by interrupting this biosynthetic pathway via the inhibition of riboflavin synthase.

Much work has been devoted to the understanding of the biosynthesis of riboflavin, and several reviews have been written.[1-5] The direct precursor of the vitamin, 6,7-dimethyl-8-ribityllumazine (**1**), was first observed in flavogenic ascomycetes.[6,7] The dismutation of lumazine **1** (Scheme I) to yield riboflavin (**4**) and 5-amino-6-(ribitylamino)pyrimidine-2,4(1*H*,3*H*)-dione (**5**) is catalyzed by the enzyme riboflavin synthase,[8,9] but can also proceed nonenzymatically at elevated temperature.[10-12]

Riboflavin synthase has been observed in a variety of microorganisms and in plants.[2,3] The enzyme from yeast has been studied in considerable detail by Plaut and his co-workers.[3,13] *Bacillus subtilis* contains an enzyme consisting of three identical α subunits (light riboflavin synthase)[14] whose primary structure has been determined recently.[15] Moreover, the microorganism contains a lumazine synthase–riboflavin synthase complex consisting of three α subunits and 60 β subunits (heavy riboflavin synthase).[16] The structure of this unusual protein has been studied in some detail.[17-19]

Mechanisms for the dismutation of the lumazine have been proposed on the basis of the acidity of the 7-methyl protons and the regiochemistry observed in the nonenzymatic and enzymatic dismutation of the 7-deuteriomethyl and 6-deuteriomethyl analogues of the substrate, respectively.[20,21] A four-carbon unit consisting of C-6α, C-6, C-7, and C-7α is removed from the "donor" molecule and transferred to the "acceptor" molecule. New carbon–carbon bonds are formed between C-7α of the acceptor

Scheme I



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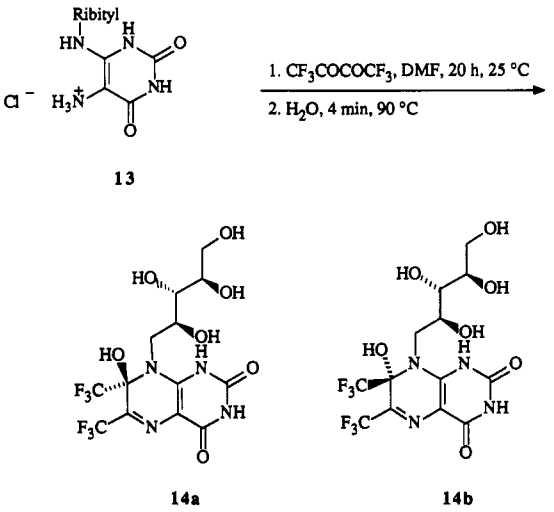
molecule and C-6 of the donor molecule and between C-7 of the donor molecule and C-6α of the acceptor molecule. Deprotonation of the acceptor molecule gives the 7-exo methylene anion **2**. The donor molecule undergoes protonation of N-1 and attack by a nucleophile "Z" to give intermediate **3**. Attack at C-6 of **3** by anion **2** begins a series of events resulting in the formation of riboflavin (**4**) and the pyrimidine **5**.

In line with this hypothesis, riboflavin synthase from yeast has been shown to display two different binding sites for the substrate **1**, a "donor site" and an "acceptor site".[22] Similarly, it was shown that each α subunit of light riboflavin synthase from *B. subtillis* can bind two substrate molecules.[23] However, only one molecule of the product, riboflavin, and the substrate analogue, 5-nitroso-6-(ribitylamino)pyrimidine-2,4(1*H*,3*H*)-dione (**11**), can be bound per α subunit. Optical spectra of enzyme-bound 7-methyl-8-ribityllumazine are consistent with the existence of intermediates such as **2** or **3**.[23]

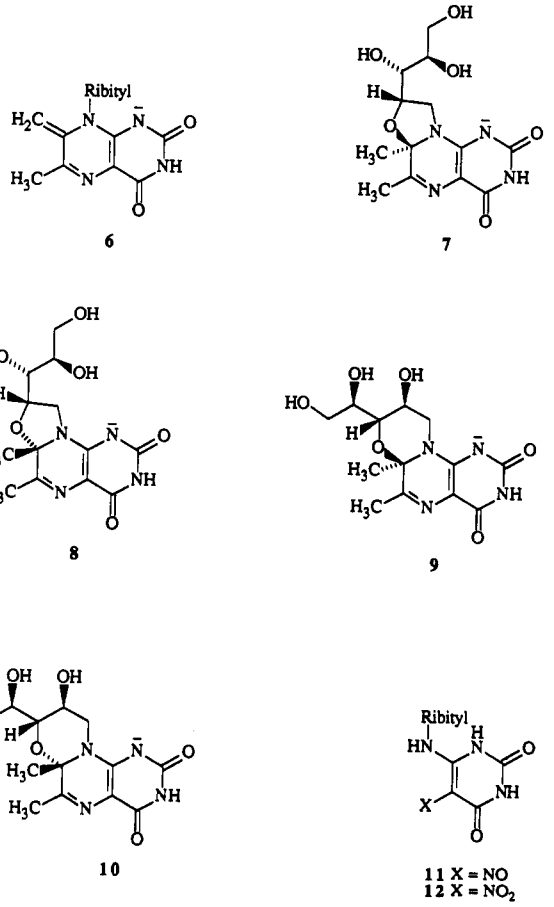
The identity of the nucleophile Z is unknown. A hydroxyl group from water,[20] a nucleophilic group provided by the enzyme,[21] and the 2′-hydroxyl group of the ribityl side chain[24] have all been proposed at various times to produce **3a**, **3b**, and the five-membered ring ether **3c**, respectively. The abstraction of a proton from the lumazine **1** yields an equilibrium mixture of five anionic species as shown by ^1H and ^{13}C NMR analysis: the 7-exo methylene anion **6** (17% of the mixture), the epimeric five-membered ring anions **7** and **8** (40 and 22%), and the epimeric six-membered ring anions **9** and **10** (14 and 7%).[25-27] The

Scheme II



13 **14a** **14b**

covalent hydrate of the lumazine, compound **3a**, was not observed in the NMR time frame under either neutral or basic conditions.



6 **7** **8** **9** **10**

11 X = NO
12 X = NO_2

The goal of the present research is the synthesis of 6,7-bis(trifluoromethyl)lumazine analogues of the natural substrate of riboflavin synthase as potential inhibitors of the enzyme. The generality of the condensation of perfluorobutane-2,3-dione (PFBD) with an ortho diamine to produce a bis-trifluoromethylated, fused pyrazine system, and the extent to which covalent hydrates thereof are stabilized, have been investigated previously in our laboratories.[28,29] It was envisioned that the electronegativity

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of the trifluoromethyl groups of the product would stabilize the epimers of 14 to the extent that the neutral species could be isolated.

The covalently hydrated fluorolumazines 14 are attractive as potential inhibitors of riboflavin synthase. Such compounds could possibly act as transition-state analogue inhibitors by mimicking the proposed intermediate **3a**.

Results

5-Amino-6-(ribitylamino)pyrimidine-2,4(1*H*,3*H*)-dione hydrochloride (**13**, Scheme II) was prepared by catalytic hydrogenation of 5-nitro-6-(ribitylamino)pyrimidine-2,4-(1*H*,3*H*)-dione (**12**)[30] or the 5-nitroso analogue **11**[31] in aqueous HCl. The crude lyophilized product was reacted with PFBD in DMF at room temperature. This reaction and all subsequent steps in the isolation of the products were performed in the dark or in subdued lighting, due to the photolability of 8-ribityllumazines.[7] A minimum of 5 equiv of PFBD was required for this reaction due to the reaction of PFBD with the ribityl hydroxyl groups. After the removal of DMF under high vacuum, the hydrolysis of the reaction mixture in water at 90 °C for 4 min was necessary to regenerate the free hydroxyl groups. The analytical HPLC tracing of the hydrolyzed reaction mixture showed the presence of two major products in nearly equal amounts. The very difficult and tedious separation of the two major products was achieved by preparative HPLC on a C-18 column, eluting isocratically with 25% methanol in aqueous ammonium formate buffer (pH 3.7) or 17.5% acetonitrile in aqueous 0.1% TFA. Analytical HPLC tracings of the isolated products indicated that the order of elution of the two products with the latter mobile phase was opposite to that observed with the former mobile phase. This was confirmed by the ^{19}F NMR spectra of the isolated products. In all subsequent discussion, the two products are designated as "epimer A" and "epimer B" according to the order of their elution using the methanol–ammonium formate mobile phase. The UV, ^{19}F NMR, and FAB mass spectra of the two products support the covalent hydrate structures **14a** and **14b**. The 470-MHz ^{19}F NMR spectrum in DMSO-d_6 of epimer A contained quartets at δ 12.47 and −7.65 (J = 6.8 Hz, trifluoroacetic acid as external standard), while the spectrum of epimer B contained quartets at δ 12.65 and −6.59 (J = 6.8 Hz). The downfield and upfield signals are consistent with trifluoromethyl groups attached to sp^2 and sp^3 hybridized carbon atoms, respectively.[28,29] The UV spectrum of each product in 0.1 N HCl contained absorbance maxima near 340 and 267 nm. The FAB mass spectrum of each product contained an MH^+ peak at m/e 453, consistent with a molecular weight of 452. While the ^{19}F NMR and UV data are also consistent with neutral fluorinated cyclic ether structures analogous to **7–10**, the mass spectral data are inconsistent with such structures, each of which would have a molecular weight of 434. The absolute configurations of the epimeric products at C-7 have yet to be determined. Efforts to obtain crystals suitable for X-ray analysis have been unfruitful.

A UV-monitored titration indicated pK_a values of 4.3 and 7.3 for epimer A and 4.1 and 7.3 for epimer B. The chemical shifts of the ^{19}F NMR signals of epimer A in aqueous media varied with pH. The upfield quartet appeared between δ −7.9 and −8.4 over the pH range 6.0–9.0. No additional NMR signals were observed to appear at any pH, and similar effects of pH on the ^{19}F NMR chemical shifts were observed in the spectra of epimer B. These effects are due to deprotonation at higher pH. In the case of epimer B, the high-field ^{19}F NMR signal shifted from δ −6.88 to −8.15 as the pH was raised from 6.0 to 9.0. The downfield quartet appeared between δ 11.9 and 12.3 over the pH range 6.0–9.0. The absence of separate signals for the mono- and dianionic species in ^{19}F NMR spectra of **14a** and **14b** as the pH is raised from 6 to 9 is consistent with rapid deprotonation–reprotonation. The ^{19}F NMR monitored titration experiments with both epimers over this pH range provided a pK_a of 7.7 for epimer A and 7.4 for epimer B. The fact that no cyclic anions of **14** related to **7–10** have been observed by NMR, as well as the fact that no equilibration of the two epimers has been detected by HPLC, is evidence of the high stability of the bond between C-7 and the adjacent oxygen atom. For either situation to occur, **14** must first undergo dehydration to a lumazine species that is sp^2 hybridized at position 7.

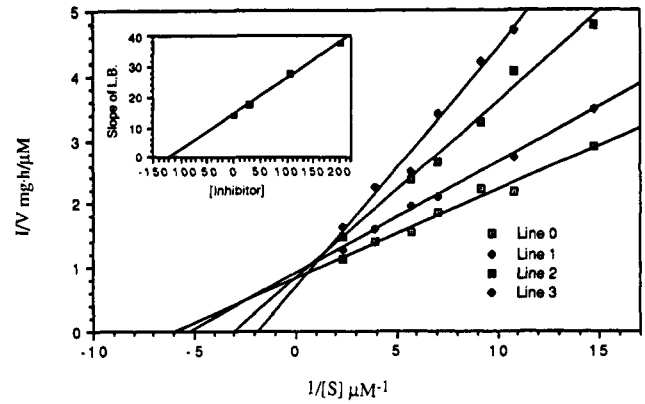

Figure 1. Lineweaver–Burk plot of the kinetic assay of epimer A against light riboflavin synthase of *B. subtilis*. These determinations were performed in a 170 mM phosphate buffer (pH 7.4) containing 10 mM sodium sulfite and 10 mM EDTA at 37 °C. Inhibitor concentration: line 0, 0 μM; line 1, 30 μM; line 2, 105 μM; line 3, 200 μM. Insert: slope versus inhibitor concentration of each line of Lineweaver–Burk plot.

Both epimers of **14** were assayed against the light riboflavin synthase of *B. subtilis*. The enzyme (α subunit trimer) was purified to a specific activity of about 45 000 units/mg by published procedures.[15] Steady-state kinetic measurements were performed at 37 °C, as described under methods. Epimer A is a competitive inhibitor of riboflavin synthase with K_I = 120 μM at pH 7.4 (Figure 1) and K_I = 38 μM at pH 6.8 (K_M = 14 μM). Weak inhibition (apparent K_I = 500 μM) was observed with epimer B (data not shown).

Equilibrium dialysis with epimer A at 4 °C gave a linear Scatchard plot (Figure 2), indicating the binding of 2.9 molecules of ligand per mole of α subunit trimer, i.e., one molecule of ligand per α subunit. The dissociation constant had a value of 13 μM. Epimer B did not bind detectably in equilibrium dialysis experiments.

The interaction of both epimers with riboflavin synthase was studied by ^{19}F NMR spectroscopy at 24 °C. Figure 3 shows a titration of enzyme with epimer A. In the presence of enzyme, the free ligand produces signals that appear at δ 12.25 and −8.03 with a broadened width of approximately 60 Hz. Five additional signals representing bound ligand are observed at δ 15.37, 14.37, 13.50, −5.22, and −5.96. These signals are characterized by a width of about 110 Hz. The spectra also show some spurious peaks that are sharp. It is therefore obvious that the broad

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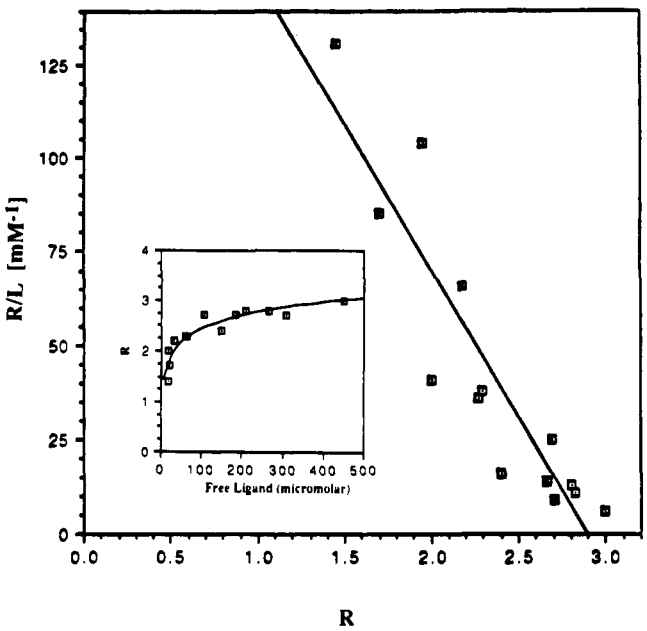

Figure 2. Scatchard plot of the equilibrium dialysis of light riboflavin synthase of *B. subtilis* and epimer A of 14 in 170 mM phosphate buffer (pH 6.8) containing 10 mM sodium sulfite at 4 °C. Insert: hyperbolic plot of the binding data before transformation by Scatchard analysis.

appearance of the signals observed for epimer A is not due to sample viscosity. It should be noted that the spurious signals assigned to impurities are somewhat exaggerated because they appear sharp relative to the signals of epimer A.

All ^{19}F NMR signals attributed to enzyme-bound epimer A were completely quenched by the addition of the tightly binding ligand, 5-nitroso-6-(ribitylamino)pyrimidine-2,4-(1*H*,3*H*)-dione (11) (Figure 4). The signals of the enzyme-bound ligand were reduced by the addition of riboflavin, but did not disappear completely. However, due to the poor solubility of riboflavin, the total concentration of this ligand was only 0.2 mM.

The ^{19}F NMR data were used to determine a Scatchard plot of the binding of epimer A of 14 to the enzyme as shown in Figure 5. This indicated a dissociation constant K_D = 16.2 μM, which is in very good agreement with the value of 13.0 μM determined by equilibrium dialysis. The x-intercept at 3.05 indicates three molecules of ligand bound per α subunit trimer, also in excellent agreement with the value of 2.90 obtained from equilibrium dialysis.

Epimer B gave no evidence of binding in ^{19}F NMR experiments. No additional signals appeared upon addition of protein to the ligand. The signals of both trifluoromethyl groups were not broadened, and their chemical shifts were not changed by the presence of the protein (data not shown).

Discussion

This paper describes for the first time the interaction of riboflavin synthase with substrate analogues carrying a stable sp^3-hybridized carbon atom at position 7. Binding studies by NMR and equilibrium dialysis indicated a marked stereochemical preference of the protein for the A epimer of 14. On the other hand, both epimers inhibit the enzyme albeit the B epimer is less potent and has an apparent K_I of about 0.5 mM. The active site of riboflavin synthase must accommodate two substrate molecules in close proximity to allow the transfer of a four-carbon moiety. It is conceivable that the presence of the substrate 1 at the acceptor site increases the affinity of the donor

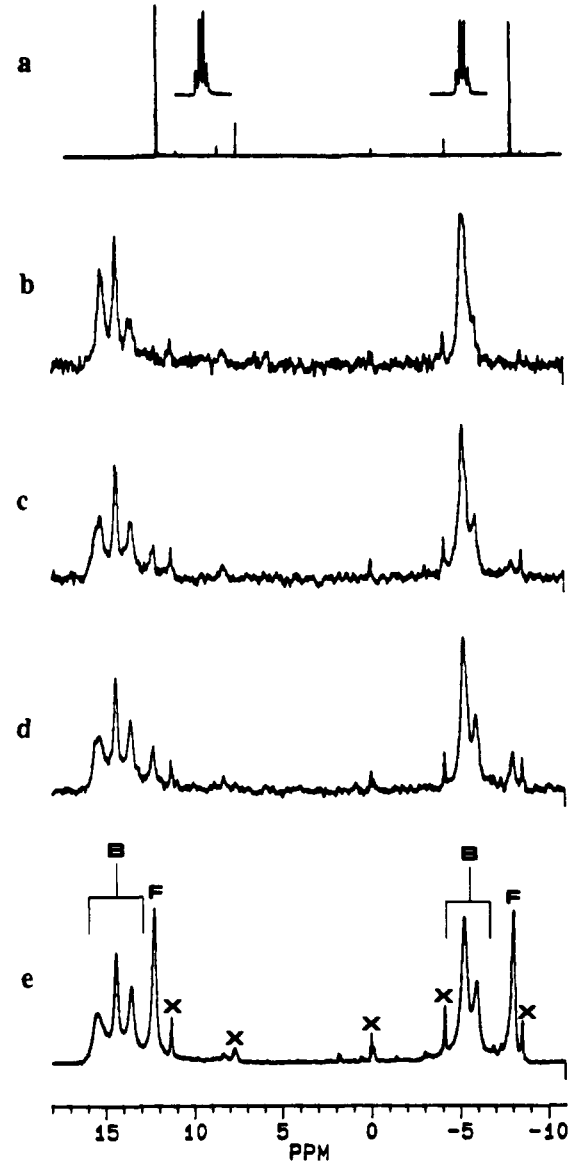

Figure 3. 338-MHz ^{19}F NMR spectrum of light riboflavin synthase of *B. subtilis* (0.225 mM) plus epimer A of 14 (0.955 mM) in 170 mM phosphate buffer (pH 6.8) containing 10 mM sodium sulfite and 5% D_2O at 24 °C. The signals are designated B (bound ligand), F (free ligand), and X (spurious peaks due to impurities). A line broadening of 20 Hz was applied to traces b through e: (a) no enzyme; (b) 345 μM fluorolumazine; (c) 664 μM fluorolumazine; (d) 872 μM fluorolumazine; (e) 950 μM fluorolumazine.

site for epimer B. The binding of epimer B would then depend upon the presence of 1 at the acceptor site, which could result in the observed weak inhibition of enzyme activity. However, an NMR experiment with the enzyme, the ligand 7-methyl-8-ribityllumazine, which has been demonstrated to bind to the acceptor and donor sites of the enzyme,[23] and epimer B did not result in the detection of any bound fluorolumazine. It is conceivable that a minor, undetected impurity present in epimer B is responsible for the observed enzyme inhibition.

NMR data obtained with epimer A bound to riboflavin synthase emphasize the complex nature of the binding site. Three enzyme-bound species can be distinguished on the basis of their respective chemical shifts.

The NMR signals of the free ligand are substantially broadened by the presence of the enzyme. This is not due to increased viscosity since several spurious signals that are due to minor fluorine-containing impurities are sharp. This indicates that the protein-bound species exchange

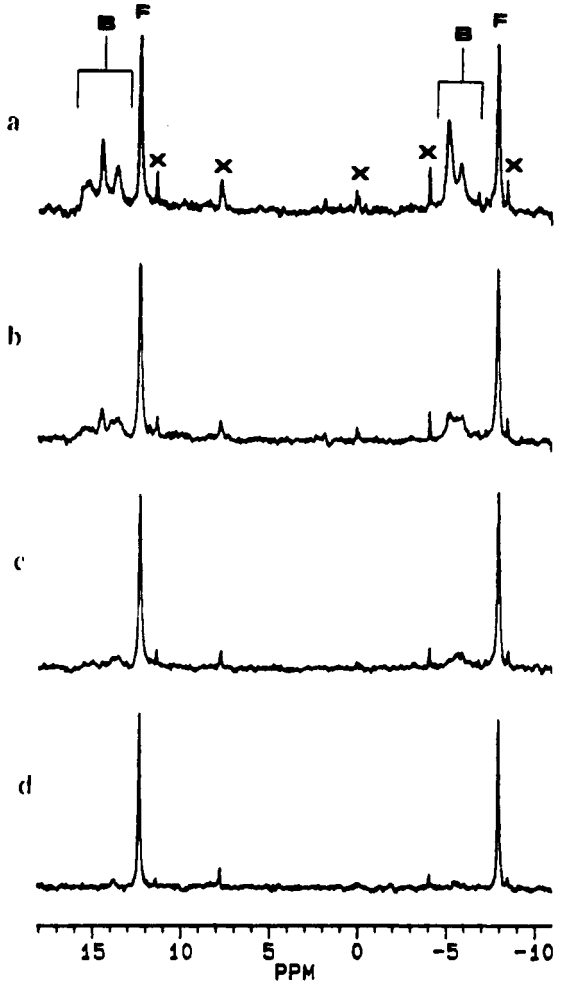

Figure 4. 338-MHz ^{19}F NMR spectrum of the titration of a solution of epimer A of **14** (0.65 mM) plus the light riboflavin synthase of *B. subtilis* (0.07 mM) with 5-nitroso-6-(ribitylamino)pyrimidine-2,4(1*H*,3*H*)-dione (**11**). The signals are designated B (bound ligand), F (free ligand), and X (spurious peaks due to impurities): (a) no nitrosopyrimidine; (b) titration to 0.37 mM nitrosopyrimidine; (c) titration to 1.08 mM nitrosopyrimidine; (d) titration to 2.40 mM nitrosopyrimidine. The line broadening was 20 Hz.

with the free ligand on the NMR time scale.

The Scatchard plot of the equilibrium dialysis experiment indicated that the inhibitor binds at only one of two sites per subunit. All bound fluorolumazine molecules are displaced by the strong inhibitor, 5-nitroso-6-(ribitylamino)pyrimidine-2,4(1*H*,3*H*)-dione (**11**), which binds only at the donor site.[23] Binding of the fluorolumazine primarily at the donor site might be expected since this covalently hydrated molecule resembles **3**, a hypothetical intermediate at the donor site, as opposed to **2**, which is thought to be involved at the acceptor site.

The selective binding of one epimer of **14** by the enzyme is quite intriguing. This strongly suggests that the enzyme is sensitive to the C-7 configuration of intermediate **3** and that one epimer of **3** is involved in the enzymatic reaction. The binding of one epimer of **14**, a 6,7-bis(trifluoromethyl) analogue of proposed intermediate **3a**, to the enzyme, could be argued as evidence for the existence of this intermediate. Very little direct evidence for the existence of **3a** has been found.

Experimental Section

Measurements with riboflavin synthase were performed in Wilmad 535 tubes at 24 °C. The aqueous solutions contained 5% D_2O, 170 mM phosphate (pH 6.8), 10 mM sodium sulfite, 5–20 mg of protein per mL, and ligand as indicated. Experimental parameters for ^{19}F NMR measurements of samples in aqueous solutions were as follows: pulse angle, 30°; pulse time, 2 μs; repetition rate, 0.5 s. The FAB mass spectra were determined using a 0.5 M HCl–glycerol matrix. All pH values are uncorrected glass electrode readings.

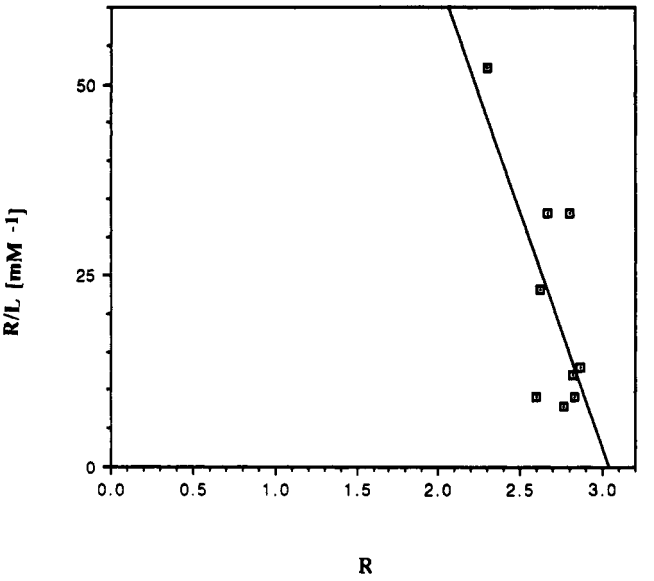

Figure 5. Scatchard plot from ^{19}F NMR data of the binding of light riboflavin synthase of *B. subtilis* and epimer A of **14** in 170 mM phosphate buffer (pH 6.8) containing 10 mM sodium sulfite and 5% D_2O at 24 °C.

Materials. 6,7-Dimethyl-8-ribityllumazine,[33] 5-nitroso-6-(ribitylamino)pyrimidine-2,4(1*H*,3*H*)-dione,[30,31] 5-nitro-6-(ribitylamino)pyrimidine-2,4(1*H*,3*H*)-dione,[31] and perflurobutane-2,3-dione[34] were prepared by published procedures.

7-Hydroxy-8-D-ribityl-6,7-bis(trifluoromethyl)-7,8-dihydropteridine-2,4(1*H*,3*H*)-dione (14), Epimer A. 5-Nitroso-6-D-(ribitylamino)pyrimidine-2,4(1*H*,3*H*)-dione (**11**, 918.5 mg, 3.17 mmol) and 10% palladium on charcoal (200 mg) were suspended in 1 N HCl (30 mL). The mixture was hydrogenated at 1 atm at room temperature for 5.5 h. The UV spectrum at 4.5 h showed a λ_{max} at 267.5 nm and disappearance of absorbance of starting material. From this point, all work was done in the dark or in subdued lighting. The catalyst was filtered off (Celite 545), and the yellow solution was lyophilized (with minimal exposure to light or to the atmosphere) to a glass. To the lyophilized material was added perfluorobutane-2,3-dione (4.375 g, 22.6 mmol, 7.11 equiv) in DMF (15 mL). The mixture was stirred in the dark under nitrogen for ca. 20 h. DMF was evaporated at 0.5 mmHg. The oily residue was diluted with water and stirred on a steam bath for 4 min. The pH of this solution was raised to >3 by filtering through neutral alumina (Brockmann grade I) and then diluting with formate buffer. The two major products were isolated on an Econosphere C18 column (5 micron), 2.2 × 35 cm, eluting with methanol (25% v/v) in 0.1 M ammonium formate/formic acid buffer (pH 3.75). The mobile phase flow rate was 5.0 mL/min. Separation was monitored by UV detection (340 nm). A total of 44 injections were made, with retention times of 46 and 50 min for the two major products. Fractions were collected corresponding to the two major products and the unresolved area between them and lyophilized. The fractions were analyzed on an Econosphere C18 column (5 μm), 4.6 × 250 mm, eluting with methanol (20% v/v) in 0.1 M ammonium formate/formic acid buffer (pH 3.75) at 1.0 mL/min. The combined fractions corresponding to epimer A were virtually free of epimer B. The combined fractions corresponding to epimer B contained less than 3% of the first eluting epimer. The combined unresolved fractions were a 15:85 mixture of epimers A and B, respectively. This mixture was separated in 11 injections using the previously

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stated preparative HPLC conditions, except that the methanol concentration was 22.5%. All fractions corresponding to epimer A were combined and lyophilized to a very pale yellow powder (127 mg, 8.9%): mp >250 °C dec; IR (KBr) 3600–2600 (broad), 1705, 1615, 1600, 1570, 1510, 1385, 1320, 1230, 1220, 1170, 1120, 1020, 990, 770 cm^{-1}; λ_{max} (0.1 N aqueous HCl) 339 (ϵ 7850), 267 (13700) nm; λ_{max} (0.1 N aqueous NaOH) 343 (ϵ 9280), 274 (9990), 249.0 (16200) nm; ^{19}F NMR (470 MHz, DMSO-d_6, CF_3COOH as external standard) δ 12.47 (q, 3 F, J = 6.8 Hz) and -7.65 (q, 3 F, J = 6.8 Hz); FABMS (glycerol–0.5 M HCl matrix) m/e (relative intensity) 453 (100, MH^+); high-resolution FABMS calcd for $C_{13}H_{15}F_6N_4O_7$ m/e 453.0845 (MH^+), found 453.0844.

7-Hydroxy-8-D-ribityl-6,7-bis(trifluoromethyl)-7,8-dihydropteridine-2,4(1*H*,3*H*)-dione (14), Epimer B. All fractions corresponding to epimer B were combined and lyophilized to a very pale yellow powder (117.2 mg, 8.2%): mp >250 °C dec; IR (KBr) 3600–2600 (broad), 1620, 1510, 1390, 1325, 1240, 1230, 1170, 1115, 1020, 940, 885, 775 cm^{-1}; λ_{max} (0.1 N aqueous HCl) 341 (ϵ 7120), 338 (7120), 268 (13400) nm; λ_{max} (0.1 N aqueous NaOH) 345.0 (ϵ 8940), 274.0 (9650), 248.5 (14900) nm; ^{19}F NMR (470 MHz, DMSO-d_6, CF_3COOH as external standard) δ 12.65 (q, 3 F, J = 6.4 Hz) and -6.59 (q, 3 F, J = 6.4 Hz); FABMS (glycerol–0.5 M HCl matrix) m/e (relative intensity) 453 (74.4, MH^+), 185 (100, glycerol); high-resolution FABMS calcd for $C_{13}H_{15}F_6N_4O_7$ m/e 453.0845 (MH^+), found 453.0855.

Enzyme. Light riboflavin synthase was purified from the derepressed mutant H94 of *B. subtilis* by published procedures.[15]

Equilibrium Dialysis. Experiments were performed at 4 °C using microdialysis cells from Dianorm and Visking dialysis tubing. The buffer contained 170 mM phosphate (pH 6.8) and 10 mM sodium sulfite. The protein concentration was 2.7 mg/mL. The dialysis cells were allowed to equilibrate for 5 h at 4 °C under slow rotation. Protein was precipitated by the addition of trichloroacetic acid to a final concentration of 5%. Subsequently, the ligand concentration was determined by HPLC analysis using a column of Nucleosil RP18 (4 × 250 mm) with an eluent containing 25% methanol in 0.1% trifluoroacetic acid. The effluent was monitored photometrically (365 nm). The retention times of epimers A and B were 5 and 6 min, respectively. The flow rate was 2 mL/min.

Enzyme Assay. Riboflavin synthase activity was assayed at 37 °C according to published procedures.[32] Assay mixtures for the determination of initial rates contained 0.1 M phosphate (pH 7.4), 10 mM sodium sulfite, 10 mM EDTA, 5–45 μM 6,7-dimethyl-8-ribityllumazine, 30–200 μM enzyme inhibitor, and protein. Aliquots were removed at 1-min intervals and quenched by the addition of trichloroacetic acid to a final concentration of 5%. The concentration of riboflavin was monitored by HPLC on a column of Nucleosil RP 18, 4 × 250 mm. The eluent was 100 mM ammonium formate containing 35% methanol. For fluorensence detection, excitation was at 470 nm and emission was at 530 nm.

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Supplementary Material Available: HPLC analyses of each epimer of 14 (2 pages). Ordering information is given on any current masthead page.

New Nucleoside Phosphoramidites and Coupling Protocols for Solid-Phase RNA Synthesis

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The 5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(trialkylsilyl)ribonucleoside 3′-*O*-(2-cyanoethyl *N*,*N*-diethylphosphoramidites) **3**, **5**, **7**, and **9**, modified monomers for RNA synthesis, were prepared from 2-cyanoethyl *N*,*N*-diethylchlorophosphoramidite (**1**). In conjunction with newly developed coupling protocols for automated solid-phase synthesis, they afforded synthetic oligoribonucleotides up to 74 base units in length. The performance of the new compounds was compared to the analogous 5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(trialkylsilyl)ribonucleoside 3′-*O*-(2-cyanoethyl *N*,*N*-diisopropylphosphoramidites) **4**, **6**, **8**, and **10**. Complete removal of benzoyl groups from N^2-benzoylguanosine, which was incorporated into some of the synthetic oligoribonucleotides, was demonstrated. Purification procedures by reverse phase HPLC and PAGE methods are also presented.

Introduction

There is a continuing interest in refining techniques for the solid-phase chemical synthesis of RNA. The well-established internucleotide coupling protocols that have been developed for DNA synthesis, including methyl phosphoramidites, 2-cyanoethyl phosphoramidites, and H-phosphonates,[1] have been proven to be much less satisfactory for RNA. The necessity of protecting the 2′-hydroxyl group of RNA[2] introduces additional steric bulk that reduces the efficiency of internucleotide phosphate bond formation. This problem has led to the use of relatively reactive methyl *N*,*N*-diisopropylphosphoramidites (MAs),[3,4] which results in rapid and efficient coupling (>96% within 2 min). Unfortunately, subsequent oxidation of the resultant phosphinate generates a methyl phosphate triester intermediate that is a potent alkylating agent that has been shown to methylate thymidine and guanosine residues during DNA synthesis.[5,6] It is therefore likely

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