Design and Synthesis of (Ribitylamino)uracils Bearing Fluorosulfonyl, Sulfonic Acid, and Carboxylic Acid Functionality as Inhibitors of Lumazine Synthase

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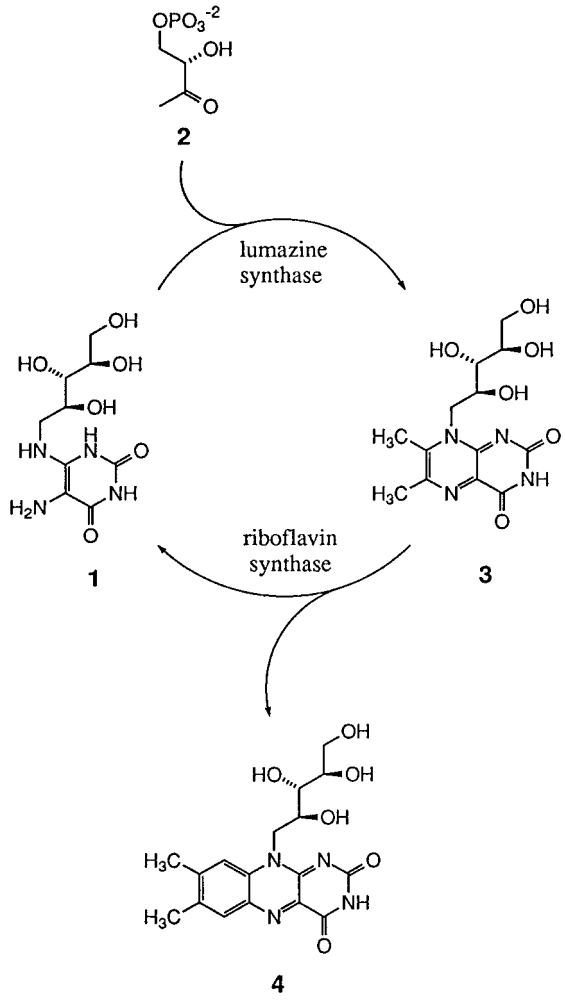
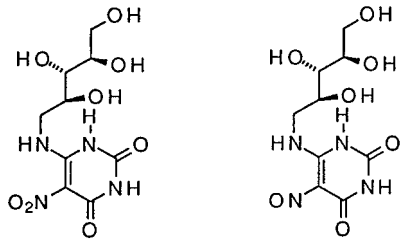
The last step in the biosynthesis of riboflavin is catalyzed by riboflavin synthase, an enzyme which has been observed in a variety of plants and microorganisms.[1−7] In *Bacillus subtilis*, the enzyme occurs as a complex with lumazine synthase, and the structure of the complex has been studied in great detail.[8−13] The complex consists of an inner core of three α subunits (riboflavin synthase) and an outer icosahedral capsid of 60 β subunits (lumazine synthase).[14,15] The β-subunits catalyze the formation of 6,7-dimethyl-8-(D-ribityl)lumazine **(3)** from 5-amino-6-(D-ribitylamino)-2,4-(1*H*,3*H*)-pyrimidinedione **(1)**, and the novel four-carbon carbohydrate, L-3,4-dihydroxy-2-butanone 4-phosphate **(2)**[16] (Scheme 1).[16−18] The α-subunits catalyze the dismutation of two molecules of 6,7-dimethyl-8-(D-ribityl)lumazine **(3)** to form one molecule of riboflavin **(4)** and one molecule of **1**. The pyrimidinedione **1** formed by the catalytic action of the α subunits can then be reutilized in the reaction catalyzed by the β subunits.

Scheme 1



The X-ray structure has recently been determined of reconstituted, icosahedral lumazine synthase capsids complexed with the substrate analogue 5-nitro-6-(D-ribitylamino)-2,4-(1*H*,3*H*)pyrimidinedione **(5)**, and information is now available about the enzyme-catalyzed reaction.[5,10,13] The crystal structure of the icosahedral β_{60} capsid of heavy riboflavin synthase complexed with 5-nitroso-6-(D-ribitylamino)-2,4-(1*H*,3*H*)pyrimidinedione **(6)**[19] is also available.[8] These structures have



provided a detailed description of the active site of lumazine synthase. In addition, the elucidation of the structure of the four-carbon precursor **2** has made it feasible to assay potential lumazine synthase inhibitors.[16] With the structures of both of the substrates **1** and **2** now

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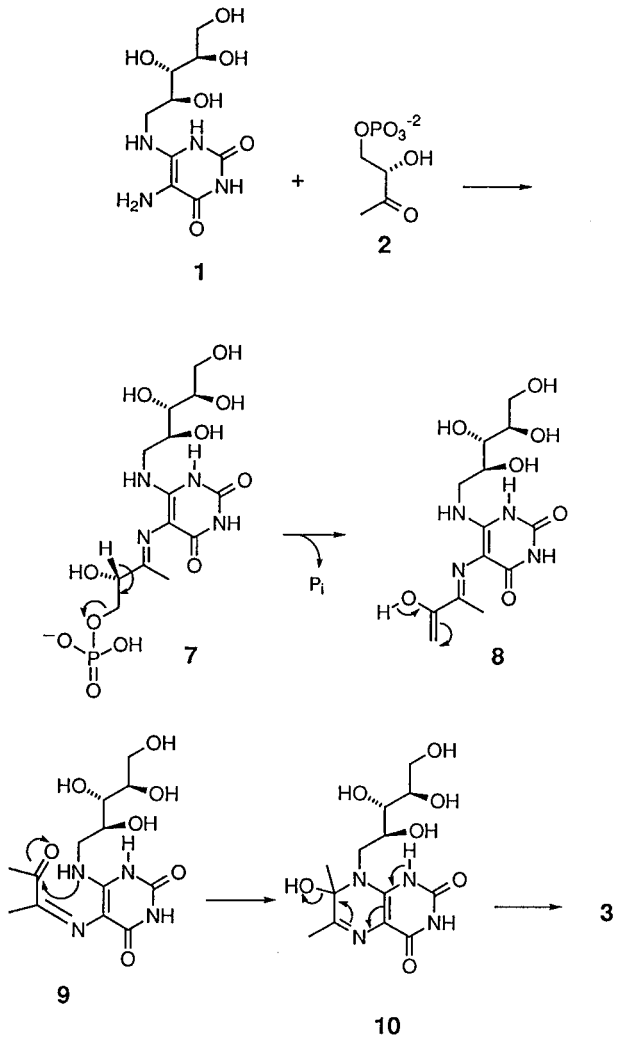
Scheme 2



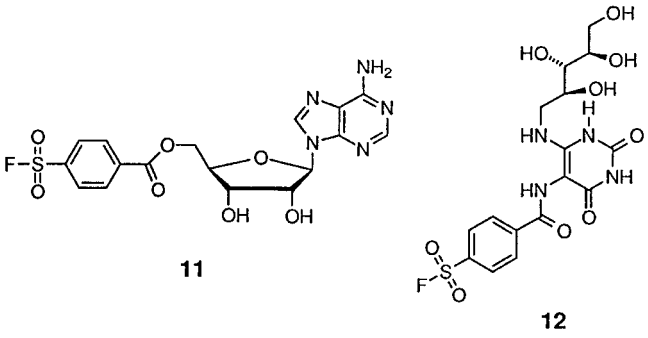
in hand, detailed steady-state enzyme kinetic parameters for recombinant β_{60} capsids devoid of α subunits have been measured,[6,7] and inhibition constants of potential lumazine synthase inhibitors can now be determined.

The mechanism proposed for the lumazine synthase-catalyzed reaction is outlined in Scheme 2.[6] Schiff base formation between the substrates **1** and **2** would afford the imine **7**. Phosphate elimination from **7** yields the enol **8**, which tautomerizes to the corresponding ketone **9**. Ring closure involving the attack of the ribitylamino group on the ketone provides the carbinolamine **10**, which eliminates water to form the product **3**.

It is assumed that the proposed intermediate **7** binds to the enzyme with the ribitylamino side chain and the pyrimidinedione occupying approximately the same positions as the corresponding groups in the nitro analogue **5**. In addition, the phosphate moiety of **7** likely binds in the same region as a buffer-derived inorganic phosphate molecule which crystallizes with the enzyme along with **5**.[10] This phosphate exists in the region of several basic amino acid residues (Arg127, Lys131, and Lys135), although the lysines are not well defined in electron density so that no precise phosphate−lysine interactions have been determined. The ligand **7** and the phosphate binding sites exist in a region at the interface between two β subunits.

The purpose of the present investigation has been to synthesize an analogue of intermediate **7** containing a reactive group in the approximate location of the phosphate of **7**. Such a reactive group might be positioned to bind covalently to the Arg127, Lys131, or Lys135 groups in the active site of the enzyme. This might provide some insight into the location of the phosphate-containing side chain of **7**. In addition to being a mechanistic probe, such a molecule might also serve as an enzyme inhibitor with potential antibiotic activity. Molecules were also sought which would contain acidic residues in place of the phosphate of **7**, since such compounds could bind to the basic amino acid residues either through electrostatic interactions of their anionic forms with the protonated basic amino acid side chains, as well as through hydrogen bond formation. The protonated forms of the acids could also bind to the unprotonated forms of the basic amino acid side chains.

5′-[*p*-(Fluorosulfonyl)benzoyl]adenosine (FSBA) **(11)** is a well-known affinity reagent that binds covalently to the ATP binding sites of enzymes. It resembles ATP and binds to the ATP binding sites of enzymes with the reactive sulfonyl situated in the region occupied by the γ-phosphate to ATP.[20] This allows covalent bond formation with lysine residues located in that region. Examples include the covalent modification of Lys721 of the EGF receptor,[21] Lys71 of cAMP-dependent protein kinase,[22] and Lys295 of the protein-tyrosine kinase $pp60^{v-src}$.[23] The rationale for the present investigation was that the attachment of a *p*-(fluorosulfonyl)benzoyl group to the 5-amino group of **1** would place the reactive fluorosulfonyl group in the same region occupied by the phosphate of the proposed intermediate **7**, near Lys131 and Lys135.[10]



To evaluate this proposal in more detail, a hypothetical model was constructed of the binding of the proposed inhibitor **12** in the active site of lumazine synthase. This was accomplished by overlapping the (ribitylamino)pyrimidine portion of **12** with **6** in the X-ray structure of the complex of **6** with the enzyme.[8] The structure **6** was then removed and the energy of **12** minimized in the active site while the protein structure was "frozen". These operations were accomplished with Sculpt software (Interactive Simulations, Inc.), resulting in the hypothetical model shown in Figure 1. According to this hypothetical model, the reactive fluorosulfonyl group would be flanked by both Lys131 and Lys135. As with ligand **6**, the pyrimidinedione ring of **12** is stacked with Phe22 of the enzyme.

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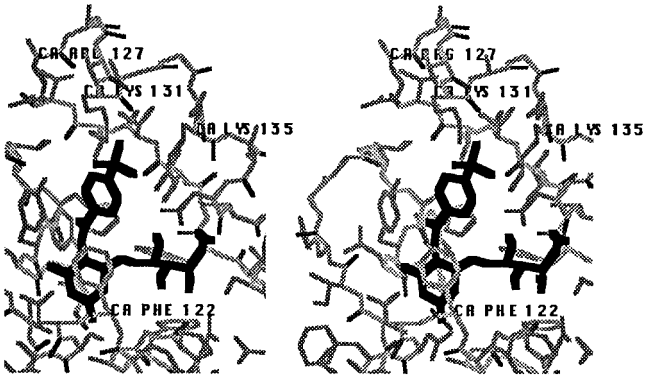
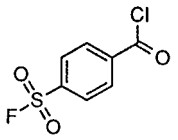


Figure 1. Hypothetical model for the binding of compound **12** to lumazine synthase. The figure is programmed for walleyed viewing.

Table 1. Inhibition Constants vs Lumazine Synthase β_{60} Capsids from *Bacillus subtilis*

compd	inhibition constant (K_i, mM)
12	0.20
15	0.36
16	0.43
17	0.47
18	0.33

The synthesis of the proposed inhibitor was performed by reacting *p*-(fluorosulfonyl)benzoyl chloride **(13)** with the substrate **1** in aqueous acetonitrile in the presence of Proton-Sponge **(14)**. The corresponding sulfonic acid **15** was obtained by carrying out the reaction of **13** with **1** in aqueous methanol while maintaining the pH at 9−10 by addition of sodium hydroxide, followed by acidification. Compound **16**, containing a carboxylic acid, was also prepared. The carboxylic acid moiety of **16** could possibly interact by hydrogen bonding or through electrostatic interactions with Lys131, Lys135, or Arg127. Compound **16** was synthesized by heating the substrate **1** with glutaric anhydride in anisole.



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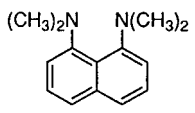

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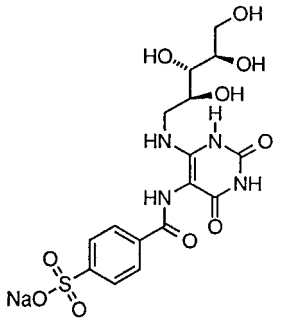

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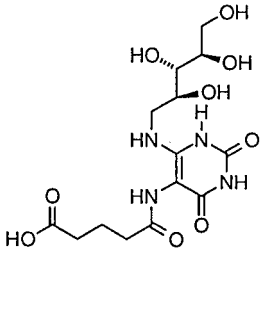

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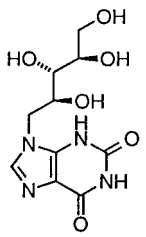
Compounds **12**, **15**, and **16** were tested for their inhibition of lumazine synthase β_{60} capsids from *B. subtilis* (Table 1). The ribitylpurine **17** and the ribitylazapurine **18**, which were synthesized previously in our laboratory, are included in Table 1 for comparison.[24] The fluorosulfonyl compound **12** is the most potent lumazine synthase inhibitor found to date, with an inhibition constant K_i of 0.20 mM. However, there was no evidence of a slow, time-dependent inactivation of the enzyme by **12**, as expected for an affinity reagent acting by covalent bond formation. It may therefore be assumed that **12** occupies the active site of the enzyme but does not react covalently with Lys131 or Lys135. The sulfonic acid derivative **15** was also found to inhibit lumazine synthase and was less potent than **12**, displaying an inhibition constant K_i of 0.36 mM. The least potent of the new compounds proved to be the glutaric acid derivative **16**, which inhibited the enzyme with a K_i of 0.43 mM. The ribitylpurine **17** and the ribitylazapurine **18** were previously determined to have K_i values of 0.47 and 0.33 mM, respectively.[24]



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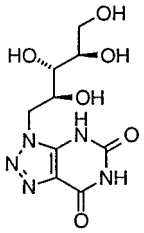

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In summary, the present investigation has provided three additional lumazine synthase inhibitors, one of which (compound **12**) is the most potent inhibitor described to date. This information provides additional insight that may enable the future design of more potent inhibitors.

Experimental Section

Melting points are uncorrected. Nuclear magnetic resonance spectra for proton (^{1}H NMR) were recorded on a 300 MHz spectrometer. The chemical shift values are expressed in ppm (parts per million) relative to tetramethylsilane as internal standard or trifluoroacetic acid as external standard. The plasma desorption mass spectra (PDMS) were determined using a ^{252}Cf ionizing source which produces MeV fission fragments. The interaction of the fission fragments with the sample produces ions which are mass analyzed with a time-of-flight mass spectrometer.[25] The compounds were applied to a nitrocellulose-coated Mylar target and allowed to dry prior to being put into the mass spectrometer, and the acceleration potential was set at 17000 kV. Elemental analyses were performed by the Purdue Microanalytical Laboratory.

5-[4-(Fluorosulfonyl)benzamido]-6-(D-ribitylamino)uracil (12). A solution of 5-nitro-6-(ribitylamino)uracil **(5)**[26] (0.50 g, 1.63 mmol) in 2 N HCl (50 mL) was hydrogenated in the presence of 10% Pd−C (0.05 g) at 30 psi for 4 h. The catalyst was removed by filtering the reaction mixture through Celite. 5-Amino-6-(D-ribitylamino)uracil hydrochloride, obtained as a pink solid upon lyophilizing the filtrate, was dissolved in water (10 mL), cooled to 0 °C, and neutralized with 1 N NaOH. 4-(Fluorosulfonyl)benzoyl chloride **(13)** (0.54 g, 2.44 mmol) and a solution of Proton-Sponge (0.523 g, 2.445 mmol), dissolved in acetonitrile (10 mL), were added to the amine solution at 0 °C. The yellow suspension was stirred at 0 °C for 4 h, after which a mixture of hexanes (50 mL) was added to the suspension. The mixture was shaken vigorously, transferred to a separatory

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funnel, and allowed to stand. The organic layer containing the Proton-Sponge was separated, and the aqueous layer was once again extracted with hexanes (50 mL). This aqueous solution (pH 5) was loaded onto a cation-exchange column (2.5 cm × 58.4 cm, Dowex-50Wx2-400, 20 g) and eluted with water (200 mL). Fractions corresponding to the product were pooled and lyophilized. A light-yellow solid thus obtained was suspended in hexanes (100 mL), filtered, and washed with hexanes (100 mL), ethyl acetate (100 mL), and finally ether (50 mL). The solid was dried under argon to afford pure compound **12** (0.25 g, 33%): PDMS *m*/*z* 463 (MH^+); fast atom bombardment mass spectrum (FABMS), negative ion (DTT/DTE) 461 $(M - 1)^-$; ^{1}H NMR (Me_2SO-d_6) δ 3.12−3.67 (m, 7 H), 6.79 (t, 1 H), 8.26 (s, 4 H), 9.27 (s, 1 H), 10.36 (s, 1 H), 10.47 (s, 1 H); ^{19}F NMR (DMSO-d_6; CF_3COOH external std) −17.60 (s, 1 F). Anal. Calcd for $C_{16}H_{19}N_4O_9SF \cdot 0.2NaCl \cdot 1.8H_2O$: C, 37.94; H, 4.5; F, 3.75; N, 11.06; S, 6.33. Found: C, 37.58; H, 4.18; F, 3.56; N, 11.11; S, 6.16.

5-[4-(Hydroxysulfonyl)benzamido]-6-(D-ribitylamino)-uracil (15). A solution of 5-nitro-6-(D-ribitylamino)uracil **(5)**[26] (0.96 g; 3.13 mmol) in 2 N HCl (10 mL) was hydrogenated in the presence of 10% Pd−C (0.2 g) at 30 psi for 4 h. The catalyst was removed by filtering the reaction mixture through Celite. 5-Amino-6-(ribitylamino)uracil hydrochloride, obtained as a pink solid upon lyophilizing the filtrate, was dissolved in water (10 mL), cooled to 0 °C and neutralized with 1 N NaOH. 4-(Fluorosulfonyl)benzoyl chloride **(13)** (1.04 g; 4.69 mmol) was added to the solution, followed by dropwise addition of 1 N NaOH until pH 10−11. MeOH (5 mL) was added to dissolve the benzoyl chloride, and the mixture was stirred at 0 °C for 2 h. The reaction mixture was then acidified with 2 N HCl to pH 2. The MeOH was evaporated under reduced pressure, and the aqueous acid solution was loaded onto a cation-exchange column (2.5 cm × 58.4 cm, Dowex-50Wx2-400, 20 g), which was eluted with water (200 mL). Fractions corresponding to the product were pooled, concentrated, and filtered, and the filtrate was lyophilized. The resulting yellow solid was suspended in absolute ethanol and filtered. The ethanol-insoluble residue corresponding to the product was washed repeatedly with ethanol to remove all impurities and dried to afford **15** (0.09 g, 6.2%): R_f 0.35 ($CHCl_3$:MeOH; 2:1; silica gel); mp 245−250 °C dec; t_R 18.15 min (Vydac-Protein & Peptide C_{18} column, 0−50−60−50−0% CH_3CN gradient, 5 mL/min, 30 min); PDMS *m*/*z* 460 (M + H); FABMS, negative ion (DTT/DTE) 459 M^-; ^{1}H NMR (Me_2SO-d_6) δ 3.25−3.66 (m, 7 H), 6.53 (bs, 1 H), 7.64 (d, 2 H), 7.88 (d, 2 H), 8.85 (s, 1 H), 10.32 (s, 1 H), 10.42 (s, 1 H). Anal. Calcd for $C_{16}H_{19}N_4O_{10}S \cdot 1.1\ Na \cdot 2.2\ H_2O$: C, 36.65; H, 4.5; N, 10.69; S, 6.12. Found: C, 36.47; H, 4.11; N, 10.32; S, 6.28.

5-[Glutaramido]-6-(D-ribitylamino)uracil (16). A mixture of 5-amino-6-(D-ribitylamino)uracil **(1)** (0.51 g, 1.63 mmol) and glutaric anhydride (0.186 g, 1.63 mmol) in anisole (25 mL) was warmed at 75 °C for 20 min. The mixture was cooled to room temperature, the anisole decanted, and the residue washed with ether (25 mL). The sticky residue was dissolved in water (20 mL) and the aqueous mixture was warmed at 70 °C for 1 h. After being cooled to room temperature, the solution was basified with 1 N NaOH. The solution was loaded onto an anion-exchange column (2.5 cm × 58.4 cm, Dowex 1 × 2−400, 20 g) and eluted, sequentially, with H_2O (100 mL) and $HCOONH_4$ buffer pH 4 (200 mL). Fractions corresponding to the product were pooled and loaded onto a cation-exchange column (2.5 cm × 58.4 cm, Dowex-50Wx2-400, 20 g) which was eluted with water (500 mL). Fractions corresponding to the product were pooled and lyophilized to afford **16** (0.065 g, 10.4%): PDMS *m*/*z* 391 (MH^+); FABMS (negative ion) *m*/*z* 389 $(M - 1)^-$; ^{1}H NMR (Me_2SO-d_6) δ 1.51 (m, 2 H), 2.02 (m, 4 H), 3.4−3.7 (m, 7 H), 6.08 (bs, 1 H), 8.04 (s, 1 H), 10.07 (s, 1 H), 10.15 (s, 1 H). Anal. Calcd for $C_{14}H_{22}N_4O_9$: C, 43.08; H, 5.68; N, 14.35. Found: C, 42.79; H, 5.84; N, 14.06.

Lumazine Synthase Assay.[7] Reaction mixtures contained 100 mM potassium phosphate (pH 7.0), 5 mM EDTA, 5 mM dithiothreitol, and recombinant β_{60} lumazine synthase capsids (800 μg, specific activity 12.5 μmol mg^{-1} h^{-1}). The mixtures, also containing various concentrations of 5-amino-6-(ribitylamino)uracil (**1**) (4−400 μM) and inhibitors **12**, **15**, or **16** (30−1000 μM), were preincubated at 37 °C. The reactions were started by adding 400 μM of L-3,4-dihydroxy-2-butanone 4-phosphate **(2)** to a total volume of 100 μL. At intervals, aliquots were taken, and the reactions were quenched by the addition of trichloroacetic acid to a final concentration of 0.4 M. Concentrations of lumazine were determined by HPLC analysis using a reversed-phase column Nucleosil 10 C_{18} (4 × 250 mm). The effluent was monitored fluorimetrically (excitation, 408 nm; emission, 487 nm). With an eluent contanining 7% methanol and 30 mM formic acid, lumazine was eluted with a retention volume of 8.4 mL. A Lineweaver−Burk plot of the initial ratios gave strictly competitive inhibition. The K_i values result from a secondary plot ($1/v$ vs $c_{inhibitor}$).

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