Introduction

The last two steps in the biosynthesis of riboflavin are catalyzed by lumazine synthase and riboflavin synthase (Scheme 1).\(^1\)\(^-\)\(^5\) Lumazine synthase catalyzes the penultimate step, in which the four-carbon phosphate \(2\) condenses with the pyrimidinedione \(1\) to form 6,7-dimethyl-8-D-ribityllumazine (3). In an unusual dismutation reaction, riboflavin synthase catalyzes the transfer of a four-carbon unit between two molecules of 3, resulting in the formation of riboflavin 4 and the lumazine synthase substrate 1, which can then be recycled.

A mechanism for the lumazine synthase-catalyzed reaction is outlined in Scheme 2. Schiff base formation involving the primary amino group of 1 and the ketone of 2 leads to the imine 5. Elimination of phosphoric acid from 5 produces the enol 6, which tautomerizes to the ketone 7. Intramolecular nucelophilic attack of the ribitylamino group of 7 on the ketone affords the carbinalmine 8, which dehydrates to yield the product 3.\(^6\)\(^-\)\(^7\)

A hypothetical model of the phosphate 5 bound in the active site of lumazine synthase has been proposed on the basis of the X-ray structure of a complex formed between the substrate analogue 9 and reconstituted, icosahedral, \(\beta\)\(\text{lo}\) Bacillus subtilis lumazine synthase capsid.\(^8\) By overlapping the phosphate group of 5 with a solvent-derived, inorganic phosphate molecule in the X-ray structure of the complex, a model of bound 5 could be constructed in which the conformation of the phosphate-
containing side chain is held roughly as shown in structure 5. This model has been supported by the X-ray structure of a complex of Saccharomyces cerevisiae lumazine synthase with a metabolically stable analogue 10 of the hypothetical Schiff base intermediate 5.9,10 In this structure, the end of the phosphate-containing side chain is distant from the nucleophilic ribitylamino group, which implies that the enol group of 6 would also be generated away from the nucleophilic amine. To cyclize, the end of the chain would have to swing toward the nucleophilic amine, and the enol would have to isomerize to a ketone. The details of this structural reorganization remain to be determined.10 Although an E-imine is shown in structure 5, the imine geometry in the actual intermediate is not known, and both Z- and E-imines can be modeled to fit the active site of lumazine synthase. However, a Z-imine is required in intermediate 7 for cyclization to occur.

A variety of Gram-negative bacteria and yeasts have been shown to lack an efficient riboflavin uptake system and are consequently dependent on endogenous synthesis.11–15 The enzymes involved in the biosynthesis of riboflavin are therefore rational targets for antibiotic drug development.

The reaction catalyzed by riboflavin synthase is thought to be initiated by a nucleophilic attack of the lumazine anion 11, formed at the acceptor site of the enzyme, on C-6 of the lumazine derivative 12, formed at the donor

(11) Bandrin, S. V.; Beburov, M. Y.; Rabinovich, P. M.; Stepanov, A. I. Genetika 1979, 15, 2063–2065.
site by addition of water to substrate 3,6,16-20. The riboflavin synthase inhibitors 13,21-23 and 1418 were designed and synthesized as analogues of the anion 11 formed at the acceptor site. The 7-oxolumazine derivative 15 was also synthesized as part of a program to prepare affinity chromatography columns for riboflavin synthase purification.21-23 Both 13 and 15 were effective inhibitors of baker’s yeast riboflavin synthase.24 Although 15 (K, 10 μM) was less potent than 13 (K, 0.5 μM), the fact that 15 inhibited riboflavin synthase indicates a degree of tolerance for larger substituents at C-6 on the lumazine ring system. It is possible that lumazine derivatives related to 15 might also function as lumazine synthase inhibitors because the carboxylate anion could conceivably occupy the phosphate binding site of 10 and the presumed phosphate binding site of 5. To test this hypothesis, the 7-oxolumazine 15 and some of its chain-extended carboxyalkyl homologues have been synthesized. Several analogues incorporating phosphates instead of carboxylates at the end of the chain have also been prepared and tested as inhibitors of both B. subtilis lumazine synthase and Escherichia coli riboflavin synthase. A long-range goal of this research has been to obtain potential antibiotics that inhibit both lumazine synthase and riboflavin synthase, since it would be more difficult for microorganisms to develop resistance to drugs that target both enzymes.

Results and Discussion

An α-ketoester 19 required as an intermediate for the synthesis of one of the desired 7-oxolumazines 23 was prepared by modification of a procedure reported by Whitman et al. (Scheme 3).25 Deprotonation of ethyl 1,3-dithiane-2-carboxylate 17 with n-butyllithium in THF afforded the expected lithium anion, which was alkylated with ethyl 5-bromoacetate 16 to provide the product 18. Cyclization of the dithiane in aqueous acetonitrile in the presence of silver nitrate, NBS, and 2,6-lutidine afforded the desired compound 19.

Syntheses of three homologous 6-carboxyalkyl-7-oxo-8-ribityllumazines 15, 22, and 23 as well as the lactone 25 are outlined in Scheme 4. Catalytic hydrogenation of 5-nitro-6-ribitylaminouracil (9)26 over palladium on charcoal afforded the unstable intermediate 1. Since 1 is very easily oxidized when it is present as the free base, resulting in complex mixtures of products, acetic acid and 2-ketoglutaric acid (20) were added directly to the hydrogenation reaction mixture, and the subsequent transformation was carried out at elevated temperature to yield the product 15. Similarly, addition of ethanolic HCl and 2-oxoacetic acid (21) to the hydrogenation reaction mixture containing 1, followed by heating at reflux, resulted in the expected oxolumazine derivative 22. In a comparable procedure, intermediate 1 was reacted with diethyl 2-oxopimelate (19) to produce compound 23. Reaction of 1 with α-ethoxalylbutyrolactone (24)27 in hot acetonitrile in the presence of acetic acid led to the oxolumazine 25, having a lactone substituent in the 6-position.

The synthesis of the 7-oxo-8-ribityllumazine analogue 33, having a phosphate connected to the lumazine ring system by a three-carbon linker, is outlined in Scheme 5. Deprotonation of γ-butyrolactone (26) with sodium
ethoxide and reaction of the resulting anion with diethyl oxalate (27) afforded α-ethoxalylbutyrilactone (24).28 Reaction of 24 with HBr in acetic acid yielded 5-bromo-2-oxovaleric acid (28), which was converted to the corresponding ester, methyl 5-bromo-2-oxovalerate (29). Treatment of intermediate 29 with trimethyl orthoforate provided the ketal 30. Displacement of the bromide 30 with dibenzyl phosphate gave the protected phosphate derivative 36. Hydrolysis of the dithiane 36 resulted when it was subjected to NBS, silver nitrate, and 2,6-lutidine in aqueous acetone. Debenzylation of 37 was effected using palladium hydroxide on carbon, and the resulting product 38 yielded the substituted lumazine 39 after reaction with the lumazine synthase substrate 1.

Since we were somewhat unsure about the optimal distance between the lumazine ring system and the phosphate for enzyme inhibitory activity, a synthesis of the higher homologue 39 of 33 was carried out, as outlined in Scheme 6. Deprotonation of ethyl 1,3-dithiane-2-carboxylate (17) with n-butyllithium afforded the anion, which was alkylated with 1,4-dibromobutane (34), resulting in intermediate 35. Displacement of the bromide with dibenzylphosphate led to the protected phosphate derivative 36. Hydrolysis of the dithiane 36 resulted when it was subjected to NBS, silver nitrate, and 2,6-lutidine in aqueous acetone. Debenzylation of 37 was effected using palladium hydroxide on carbon, and the resulting product 38 yielded the substituted lumazine 39 after reaction with the lumazine synthase substrate 1.

The 6-substituted 7-oxo-8-ribityllumazines 15, 22, 23, 25, 33, and 39 were evaluated as inhibitors of recombinant B. subtilis lumazine synthase β60 capsids and recombinant E. coli riboflavin synthase, and the results are listed in Table 1. Several additional inhibitors which were previously designed and synthesized to target both the phosphate binding site and the ribitylpyrimidine binding site of lumazine synthase are also listed in Table 1 for comparison purposes. These include the benzamides 40 and 41,29 the homologous phosphonates 10, 42, and
SCHEME 6

Derivatives of 7-Oxo-8-ribitylaminolumazines

a Reagents and conditions: (a) 1. n-BuLi, ethyl 1,3-dithiane-2-carboxylate, -78 to 23 °C, (2) 1,4-dibromobutane, 23 °C (3 h); (b) AgOPO(OBu)₂, C₆H₅CH₃, reflux (5 h); (c) NBS, AgNO₃, 2,6-lutidine, aq acetone, 0–23 °C (30 min); (d) H₂, Pd(OH)₂, EtOH (5 h); (e) EtOH, reflux (12 h).

43,9 as well as the homologous sulfonates 44, 45, and 46,9 and the purinetrione 47.3,9,10

The most potent inhibitor of lumazine synthase in the present series was the 6-(3′-carboxypropyl) lumazine derivative 22 (Kᵢ 84 μM), followed closely by the corresponding 6-(2′-carboxyethyl) lumazine analogue 15 (Kᵢ 94 μM). Both of these compounds were more potent than the 6-(4′-carboxybutyl) lumazine homologue 23 (Kᵢ 200 μM) and the lactone 25 (Kᵢ 220 μM). Evidently, the optimal distance between the carboxyl group and the lumazine ring system is afforded by a three-carbon linker.

Turning to the phosphates 33 (Kᵢ 120 μM) and 39 (Kᵢ 160 μM), we can see that connection of the phosphate to the lumazine ring system by a three-carbon linker results in a more potent inhibitor of lumazine synthase than a four-carbon connection. However, both of these phosphates were less potent than the carboxylates 15 and 22. It should be noted that the phosphate 33 was also more potent than the phosphonates 42 (Kᵢ 440 μM), 43 (Kᵢ 180 μM), and 10 (Kᵢ 130 μM), as well as the sulfonates 44 (Kᵢ 130 μM), 45 (Kᵢ 690 μM), and 46 (Kᵢ 290 μM), in which...
the phosphonates or sulfonates are connected through a linker chain to a pyrimidine ring system instead of a lumazine ring system.

A 2.4 Å resolution X-ray structure is available of the substrate analogue 9 complexed with *B. subtilis* lumazine synthase (Figure 1). The related 1.85 Å resolution X-ray structure of the phosphonate inhibitor 10 (Ki 180 μM vs that of *B. subtilis* lumazine synthase) bound to *Saccharomyces cerevisiae* lumazine synthase has also been published. These structures allow the construction of a hypothetical model of the binding of 22 to *B. subtilis* lumazine synthase (Figure 2), which was produced by overlapping the structure of 22 with the structure of 9 in the active site of *B. subtilis* lumazine synthase. The structure of 9 was then removed, and the energy of the complex was minimized using the MMFF94 force field while allowing the ligand and the protein structure contained within a 6 Å sphere surrounding the ligand to remain flexible with the remainder of the protein structure frozen. The resulting Figure 2 was constructed by displaying the amino acid residues involved in hydrogen bonding of the protein with ligand 22, using a maximum hydrogen bonding distance of 2.8 Å. The calculated distances between the hydrogen bond donors and acceptors in the complex of 22 with the protein are provided in Figure 3 (top), along with the corresponding distances derived from the X-ray structure of the complex of the protein with 9 (bottom). Figure 3 also shows the distances for hydrogen bondings of inorganic phosphate with lumazine synthase, which were derived from the X-ray structure of the complex of the protein with 9.

The calculated structure of 22 with lumazine synthase indicates that it is likely that the terminal carboxylate of the side chain occupies a site that is near to, but not...
Derivatives of 7-Oxo-8-ribitylaminolumazines

**Figure 3.** Top: hydrogen bonds and distances in the calculated model of inhibitor 22 bound in the active site of B. subtilis lumazine synthase. Bottom: the corresponding diagram based on the X-ray structure of the complex of 9 with the enzyme.

identical with, the site occupied by inorganic phosphate in the X-ray structure of the complex of 9 with lumazine synthase. Although Arg127 both is involved in binding to inorganic phosphate and is calculated to bind to the carboxylate of 22, the second residue calculated to be involved in hydrogen bonding is different in each case. With phosphate, the backbone nitrogen of Thr86 is involved in bonding to phosphate, whereas, in the calculated structure of 22, it is Lys135. Comparison of the location of Lys135 in the X-ray structure (Figure 1) with that of the hypothetical structure in Figure 2 suggests that the side chain of Lys135 may undergo a conformational change in which the terminal nitrogen moves toward Arg127, resulting in a binding pocket that is ideally suited for binding to the carboxylate. In the X-ray structure (Figure 1), the distance from the side chain amino nitrogen of Lys135 to the central carbon atom of the guanidine moiety of Arg127 is 9.7 Å, while, in the model of the binding of 22, it is 6.1 Å.

Considering inhibition of riboflavin synthase, the data presented in Table 1 show that the most potent riboflavin synthase inhibitor in the present series is also the 6-(3-carboxypropyl)lumazine derivative 22 (K < 20 μM). Either shortening or lengthening the linker chain connecting the carboxyl group to the lumazine ring resulted in less active compounds (15, K > 650 μM; 23, K > 56 μM). A three-carbon linker attaching the carboxyl group to the lumazine ring therefore seems to be optimal for inhibition of both riboflavin synthase and lumazine synthase. Interestingly, the replacement of the carboxylate of 22 with a phosphate resulted in compound 33, which was inactive against riboflavin synthase (K > 1600 μM). However, lengthening the linker chain by one carbon atom resulted in the riboflavin synthase inhibitor 39 (K < 150 μM).

An X-ray structure of E. coli riboflavin synthase, which contains three identical subunits, has been published along with a hypothetical model of bound 6,7-dimethyl-8-α-ribityllumazine (39). According to the model, as well as NMR studies of the structure of the N-terminal domain of riboflavin synthase, the active site of the enzyme is derived from the intermolecular juxtaposition of residues belonging to both of the barrels in the C-terminal and N-terminal domains of two monomer units, with one substrate molecule bound to each barrel. A model was constructed for the binding of inhibitor 15 to E. coli riboflavin synthase by manually positioning its structure, as suggested by the published model. The initial orientations of the ligand molecules in the active site were also consistent with the X-ray structure of monomeric Schizosaccharomyces pombe riboflavin synthase complexed with two molecules of 15. Energy minimization then led to the structure of the enzyme–inhibitor complex displayed at the top of Figure 4. According to this hypothetical structure of the inhibitor–enzyme complex, one molecule of 15 (magenta) binds to the C-barrel (orange residues), while another molecule of the inhibitor (white) binds to the N-barrel (blue-green residues). The empty active site at the bottom of Figure 4 was constructed from the published X-ray coordinates. There are 18 water molecules included in or near the cavity that constitutes the active site, and many of them are involved in hydrogen bonding to the protein and the ligand molecules in the occupied active site.

An antibiotic that would be active against both riboflavin synthase and lumazine synthase would be less likely to lead to resistant strains, since the pathogen would have to select two different resistant mutant enzymes simultaneously to become resistant. The present series of lumazine derivatives features several compounds that are active against both enzymes.

**Experimental Section**

**General.** Melting points were determined in capillary tubes and are uncorrected. The 1H NMR spectra were determined at 300 MHz, and the 13C NMR spectra were recorded at 75.5 MHz. Microanalyses were performed at the Purdue Microanalysis Laboratory, and all values were within 0.4% of the calculated compositions. Silica gel used for column chromatography was 230–400 mesh.

6-(2-Carboxylethyl)-7-oxo-8-ribityllumazine (15), 5-Nitro-6-(o-ribityl)aminouracil (9) (0.5 g, 1.6 mmol) was dissolved in water (20 mL) and hydrogenated under H2 atmosphere for 12 h in the presence of Pd on activated carbon (50 mg, 10%). Acetic acid (5 mL) and 2-ketogluartic acid (20) (1.0 g, 6.8 mmol)

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were added, and the mixture was heated at 90–100 °C for 4 h. After cooling, the catalyst was removed by filtration and the filtrate was concentrated under reduced pressure. The residue was precipitated from EtOH to afford 15 (0.31 g, 50%) as an amorphous solid: 1H NMR (300 MHz, D2O) δ 4.38 (m, 1 H), 4.25 (m, 1 H), 4.02 (m, 1 H), 3.72 (m, 1 H), 3.65 (m, 2 H), 3.58 (m, 1 H), 2.91 (t, J = 6 Hz, 2 H), 2.70 (t, J = 6 Hz, 2 H); 13C NMR δ 178.45, 163.23, 157.49, 151.85, 151.71, 145.02, 109.80, 73.67, 73.06, 70.16, 63.23, 46.29, 30.95, 28.41. Anal. Calcd for C14H18N4O9·H2O: C, 41.59; H, 4.99; N, 13.86. Found: C, 41.64; H, 4.94; N, 13.75.

Ethyl 2-(4-Ethoxycarbonylbutyl)-1,3-dithiane-2-carboxylate (18).

n-Butyllithium (6.5 mL, 1.6 M in hexane) was added to a solution of ethyl 1,3-dithiane-2-carboxylate (17) (1.92 g, 0.01 mol) in THF (20 mL) at −78 °C under N2 atmosphere. The reaction mixture was stirred for 30 min, and then, ethyl 5-bromovalerate (16) (2.09 g, 0.01 mol) was added. The mixture was warmed to room temperature and stirred for a further 30 min. It was diluted with diethyl ether and washed with 1 N HCl and H2O, dried, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography, eluted with hexanes–ethyl acetate (5:1–2:1) to provide 18 as a colorless oil (2.4 g, 75%): 1H NMR (300 Hz, CDCl3) δ 4.15 (q, J = 7.1 Hz, 2 H), 4.05 (q, J = 7.2 Hz, 2 H), 3.2 (m, 2 H), 2.55 (m, 2 H), 2.1–1.5 (m, 10 H), 1.26 (t, J = 7.1 Hz, 3 H), 1.18 (t, J = 7.2 Hz, 3 H).

Diethyl 2-Oxopimelate (19).

A solution of 18 (1.2 g, 3.7 mmol) in acetonitrile (3 mL) was added quickly, dropwise, to a solution of N-bromosuccinimide (4.27 g, 0.024 mol), silver nitrate (2.72 g, 0.016 mol), and 2,6-lutidine (0.86 g, 0.008 mol) in acetonitrile–water (40:10) that was cooled in an ice bath. The ice bath was removed, and the mixture was stirred for 30 min at room temperature. The suspension was diluted with diethyl ether (100 mL), washed with saturated NH4Cl, saturated aqueous NaSO3, and saturated aqueous NaCl, dried, and concentrated. The crude product was purified by silica gel column chromatography and eluted with hexanes–ethyl acetate (5:1–2:1) to provide 19 as a colorless oil (0.35 g, 41%): 1H NMR (300 Hz, CDCl3) δ 4.43 (q, J = 7.1 Hz, 2 H), 4.15 (q, J = 7.2 Hz, 2 H), 2.88 (m, 2 H), 2.34 (m, 2 H), 1.68 (quintet, J = 3.4 Hz, 4 H), 1.38 (t, J = 7.1 Hz, 3 H), 1.26 (t, J = 7.2 Hz, 3 H).

6-(3-Carboxylpropyl)-7-oxo-8-(D-ribityl)lumazine (22).

5-Nitro-6-(D-ribityl)aminouracil (9) (0.5 g, 1.6 mmol) was dissolved in water (15 mL) with a few drops of triethylamine and hydrogenated under H2 atmosphere for 12 h in the presence of Pd on activated carbon (100 mg, 10%). A solution of 2-oxoadipic acid (21) (0.5 g, 2.1 mmol) in ethanol (10 mL) and 2 N HCl (2 mL) was added, and the mixture was heated at reflux for 12 h. After cooling, the catalyst was removed by filtration and the filtrate was concentrated under reduced pressure. The residue was dissolved in a solution of LiOH (0.1 g) in water (10 mL) and stirred at room temperature for 5 h. After removal of the solvent, the residue was applied onto a DEAE cellulose (anion-exchange) column and eluted with water followed by 5% formic acid. The corresponding fractions were combined and concentrated. The residue was precipitated by AcOEt to afford 22 (300 mg, 47%) as an amorphous solid: 1H NMR (500 MHz, D2O) δ 4.30 (m, 1 H), 4.15 (m, 1 H), 3.96 (m, 1 H), 3.71 (m, 1 H), 3.60 (m, 2 H), 3.48 (m, 1 H), 2.60 (t, J = 6 Hz, 2 H), 1.80 (quintet, J = 6 Hz, 2 H), 1.75 (m, 1 H), 1.6 (m, 2 H); ESIMS (negative ion mode) m/z 399 (M–H)−. Anal.
Derivatives of 7-Oxo-8-ribitylaminolumazines

Calcd for C_{15}H_{22}N_{4}O_{9}: 241.31. 30: Methyl 5-bromo-2,2-dimethoxyoxpentaenoate (29). Methyl 5-bromo-2,2-dimethoxyoxpentaenoate (29) (1.0 g, 4.8 mmol), trimethyl-orthooctamethoxy (1.0 g, 9.6 mmol), and concentrated sulfuric acid (2 drops) were stirred under nitrogen at room temperature for 12 h. Sodium bicarbonate (0.5 g) was added, and the mixture was stirred for 0.5 h, filtered, and concentrated to give 30 as a colorless oil (1.0 g, 81%): 1H NMR (300 MHz, CDCl3) δ 7.35 (s, 3 H), 3.30 (t, J = 6.4 Hz, 2 H), 3.22 (s, 6 H), 1.98 (m, 2 H), 1.75 (m, 2 H); 13C NMR (100 MHz, CDCl3) δ 169.17, 102.00, 51.06, 49.68, 32.84, 32.06, 26.47.

Methyl 5-Dibenzylphosphoryl-2,2-dimethoxyxenate (32). A mixture of compound 31 (0.6 g, 1.3 mmol) and Pd(OH)2 on activated carbon (50 mg, 10%) in ethanol (20 mL) was hydrogenated under H2 atmosphere for 5 h. The catalyst was removed by filtration, and the filtrate was concentrated. The residue was purified by silica gel (50 g) column chromatography, eluted with hexane–ethyl acetate (1:1) and concentrated sulfuric acid (3.57 mmol) and silver dibenzylationate (1.5 g, 30 mmol) in toluene (20 mL) was stirred for 0.5 h, filtered, and concentrated to give 32 as a colorless oil (0.35 g, 97%), which was used without further purification. 1H NMR (300 MHz, CD3OD) 3.97 (q, J = 6.4 Hz, 2 H), 3.75 (s, 3 H), 3.22 (s, 6 H), 2.00 (m, 2 H), 1.57 (m, 2 H); 13C NMR (100 MHz, CD3OD) δ 171.17, 103.49, 67.03 (d), 52.94, 50.08, 30.80, 25.57 (d).

7-Oxo-6-(3-phosphonypropyl)-8-(ribityl)lumazine (33). A mixture of 5-nitro-6-(ribityl)aminolaracil (9) (0.35 g, 1.1 mmol) and Pd(OH)2 on activated carbon (50 mg, 10%) in water (15 mL) was hydrogenated under H2 atmosphere for 12 h. A solution of 32 (0.35 g, 1.3 mmol) in ethanol (15 mL) was added, and the mixture was stirred for 0.5 h, filtered, and concentrated to give 33 as a colorless oil (0.35 g, 62%) as an amorphous solid: ESIMS (negative ion mode) m/z 451 (M–H); 1H NMR (300 MHz, CD3OD) δ 4.37 (m, 1 H), 4.23 (m, 1 H), 4.04 (m, 1 H), 3.87 (m, 2 H), 3.8–3.6 (m, 3 H), 3.55 (m, 1 H), 2.52 (m, 2 H); MS ESIMS (negative ion mode) m/z 397 (M–H).

Methyl 5-Bromo-2,2-dimethoxyxenate (29). Methyl 5-bromo-2,2-dimethoxyxenate (29) (0.4 g, 62%): 1H NMR (300 MHz, CDCl3) δ 7.1 Hz, 0.92 (m, 1 H). Anal. Calcd for C_{16}H_{22}N_{4}O_{9}: C, 45.33; H, 5.24; N, 12.78. Found: C, 45.24; H, 5.09; N, 12.61.

Methyl 5-Bromo-2,2-dimethoxyxenate (29). Methyl 5-bromo-2,2-dimethoxyxenate (29) (0.4 g, 62%): 1H NMR (300 MHz, CDCl3) δ 7.1 Hz, 0.92 (m, 1 H). Anal. Calcd for C_{16}H_{22}N_{4}O_{9}: C, 45.33; H, 5.24; N, 12.78. Found: C, 45.24; H, 5.09; N, 12.61.
heated at reflux for 5 h under N₂. After cooling, the precipitate was removed by filtration and the filtrate was concentrated under reduced pressure. The residue was purified by silica gel column chromatography and eluted with hexanes—ethyl acetate (2:1) to provide 36 as a colorless oil (1.5 g, 62%). ¹H NMR (300 Hz, CDCl₃) δ 7.35 (m, 10 H), 5.05–5.00 (m, 4 H), 4.26 (q, J = 7.1 Hz, 2 H), 4.00 (q, J = 6.4 Hz, 2 H), 3.3 (m, 2 H), 2.65 (m, 2 H), 2.2 (m, 1 H), 1.92 (m, 2 H), 1.85 (m, 2 H), 1.60 (m, 2 H), 1.38 (t, J = 7.1 Hz, 3 H), 0.92 (m, 1 H).

**Ethyl 5-(Dibenzyldiethylene)-2-oxohexanoate (37).** A solution of 36 (2.1 g, 0.004 mol) in acetonitrile (10 mL) was added quickly, dropwise, to a solution of N-bromosuccinimide (4.27 g, 0.024 mol), silver nitrate (2.72 g, 0.016 mol), and 2,6-dibromophenol (3.58 g, 0.016 mol) in acetonitrile–water (40:10) that was cooled in an ice bath. The ice bath was removed, and the mixture was stirred for 30 min at room temperature. The suspension was diluted with diethyl ether (100 mL), washed with saturated aqueous Na₃SO₄, and saturated aqueous NaCl, dried, and concentrated to give 37 (1.7 g, 100%) as an oil. ¹H NMR (300 Hz, CDCl₃) δ 7.57 (m, 10 H), 5.17–4.86 (m, 4 H), 4.24 (q, J = 7.1 Hz, 2 H), 3.33 (m, 2 H), 1.8–1.4 (m, 6 H), 1.26 (t, J = 7.1 Hz, 3 H).

**Ethyl 2-Oxo-6-phosphonyxohexanoate (38).** A mixture of compound 37 (0.9 g, 2.1 mmol) and Pd(OH)₂ on activated carbon (20 mg, 20%) in ethanol (20 mL) was hydrogenated under H₂ atmosphere for 5 h. The catalyst was removed by filtration, and the filtrate was concentrated under reduced pressure to a colorless oil (0.55 g, 99%), which was used without further purification.

**7-Oxo-6-(4-phosphonyxobutyl)-8-(O-ribityl)lumazine (39).** A mixture of 5-ribitylaminoaracil (9) (0.5 g, 1.6 mmol) and Pd on activated carbon (50 mg) in water (20 mL) was hydrogenated under H₂ atmosphere for 12 h. A solution of 38 (0.55 g, 2.1 mmol) in ethanol (15 mL) was added, and the mixture was heated at reflux for 12 h. After cooling, the catalyst was removed by filtration and the filtrate was concentrated under reduced pressure. The residue was treated with ethanol, and the precipitate was removed by filtration. The ethanolic filtrate was concentrated, and the residue was applied onto an anion-exchange resin column (Dowex 1×2, 200–400 mesh). The corresponding fractions were combined and concentrated. The suspension was precipitated with AcOH to afford 39 (300 mg, 36%) as an amorphous solid: ESIMS (negative ion mode) m/z 465 (M – H)⁻; ¹H NMR (500 MHz, D₂O) δ 4.28 (m, 1 H), 4.16 (m, 1 H), 3.96 (m, 1 H), 3.78 (m, 2 H), 3.71 (m, 1 H), 3.64 (m, 2 H), 3.57 (m, 2 H), 3.21 (m, 2 H), 2.92 (m, 1 H), 1.51 (m, 3 H), 1.47 (m, 2 H), 1.20 (m, 6 H); ¹³C NMR (125 MHz, D₂O) δ 162.83, 154.70, 151.02, 143.97, 109.50, 73.64, 73.13, 70.07, 67.29 (d), 63.30, 46.54, 33.15, 30.15 (d), 23.23. Anal. Calcd for C₂H₅NO₂H₂O₃P·0.3(AcOEt)·0.5-(HCOOH): C, 38.89; H, 5.16; N, 10.86. Found: C, 38.77; H, 5.11; N, 10.96.

**Molecular Modeling on Lumazine Synthase.** Using Sybyl (Tripos, Inc., version 6.5, 1998), the X-ray crystal structure of E. coli riboflavin synthase (1I8D) was downloaded and two molecules of the ligand 15 were docked and oriented as suggested by the published model of the binding of two molecules of substrate 3 in the active site. The C- and N-terminal groups were changed to neutral carboxylic acid and amino groups, and hydrogens were added to the protein structure and to the oxygens of the water molecules. MMFF94 charges were loaded, and the energy of the complex was minimized using the Powell method to a termination gradient of 0.05 kcal/mol while employing the MMFF94 force field. The remaining portion of the protein was held ridged using the aggregate function during energy minimization. Figure 3 was constructed by displaying the amino acid residues in the C- and N-barrels surrounding the two ligand molecules. The maximum distance between donor and acceptor atoms contributing to the hydrogen bonds shown in Figure 3 was set to 2.8 Å.

**Lumazine Synthase Assay.** Reaction mixtures contained 100 mM potassium phosphate, pH 7.0, 5.5 mM EDTA, 5 mM dithiothreitol, inhibitor (0–86 μM), 170 μM 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione (1), and lumazine synthase (30 μg, specific activity 12.5 μmol mg⁻¹ h⁻¹) in a total volume of 560 μL. The solution was incubated at 37 °C, and the reaction was started by the addition of a small volume (20 μL) of L-3,4-dihydroxy-2-butanoate 4-phosphate to a final concentration of 50–310 μM. The velocity–substrate data were fitted for all inhibitor concentrations with a nonlinear regression method. Different inhibition models were considered for the calculation. Kᵢ values ± standard deviations were obtained from the fit under consideration of the most likely inhibition model.

**Riboflavin Synthase Assay.** Reaction mixtures contained buffer (100 mM potassium phosphate, 10 mM EDTA, 10 mM sodium sulfite), inhibitor (0–87 μM), and riboflavin synthase (30 μg, specific activity 50 μmol mg⁻¹ h⁻¹). After precubation, the reactions were started by the addition of various amounts of 6,7-dimethyl-8-ribityllumazine (3) (20–200 μM) to a total volume of 570 μL. The formation of riboflavin (4) was measured online with a computer controlled photometer at 470 nm (ε₉₁₀ = 9100 M⁻¹ cm⁻¹). The Kᵢ evaluation was performed in the same manner as described above.

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