JOC Article

Design, Synthesis, and Evaluation of 6-Carboxyalkyl and 6-Phosphonoxyalkyl Derivatives of 7-Oxo-8-ribitylaminolumazines as Inhibitors of Riboflavin Synthase and Lumazine Synthase

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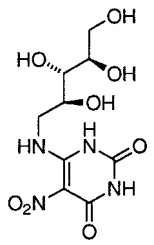
A series of 6-carboxyalkyl and 6-phosphonoxyalkyl derivatives of 7-oxo-8-D-ribityllumazine were synthesized as inhibitors of both *Escherichia coli* riboflavin synthase and *Bacillus subtilis* lumazine synthase. The compounds were designed to bind to both the ribitylpurine binding site and the phosphate binding site of lumazine synthase. In the carboxyalkyl series, maximum activity against both enzymes was observed with the 3′-carboxypropyl compound **22**. Lengthening or shortening the chain linking the carboxyl group to the lumazine by one carbon resulted in decreased activity. In the phosphonoxyalkyl series, the 3′-phosphonoxypropyl compound **33** was more potent than the 4′-phosphonoxybutyl derivative **39** against lumazine synthase, but it was less potent against riboflavin synthase. Molecular modeling suggested that the terminal carboxyl group of 6-(3′-carboxypropyl)-7-oxo-8-D-ribityllumazine (**22**) may bind to the side chains of Arg127 and Lys135 of the enzyme. A hypothetical molecular model was also constructed for the binding of 6-(2′-carboxyethyl)-7-oxolumazine (**15**) in the active site of *E. coli* riboflavin synthase, which demonstrated that the active site could readily accommodate two molecules of the inhibitor.

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 nucleophilic attack of the ribitylamino group of **7** on the ketone affords the carbinolamine **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,



9

icosahedral, β_{60} *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-

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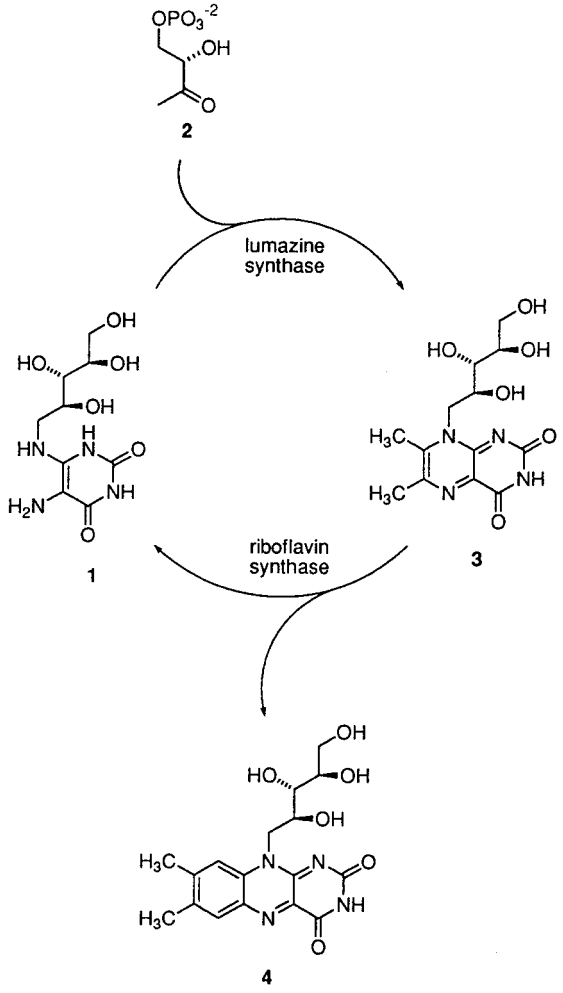
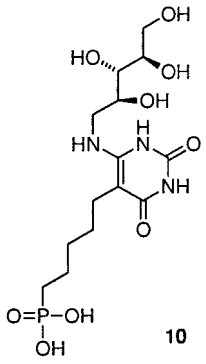
SCHEME 1

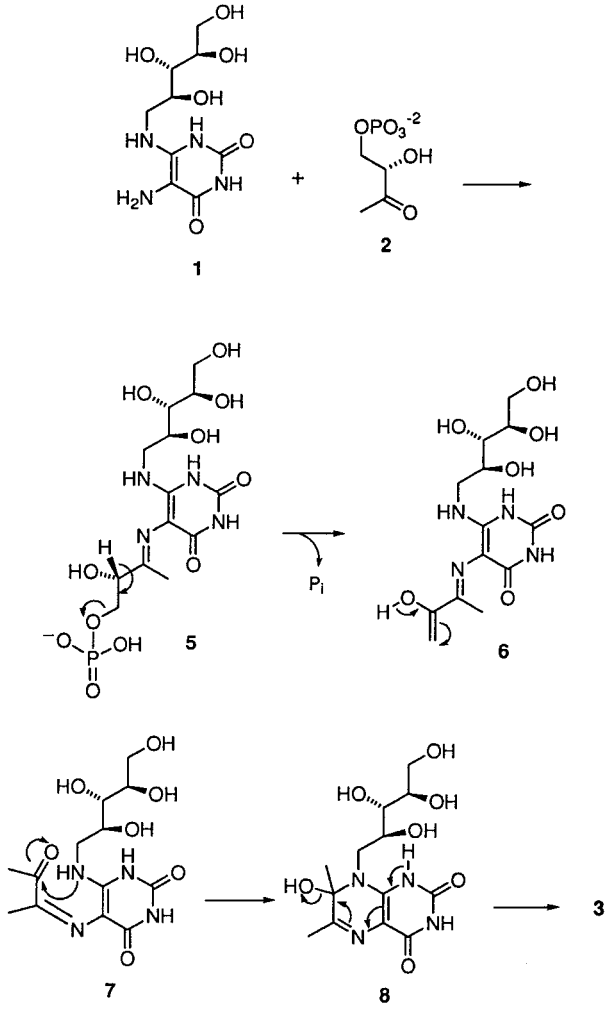


containing side chain is held roughly as shown in structure **5**. This model has been supported by the X-ray structure of a complex of *Saccharomyces cerevisiae* lumazine synthase with a metabolically stable analogue **10**



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

SCHEME 2



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

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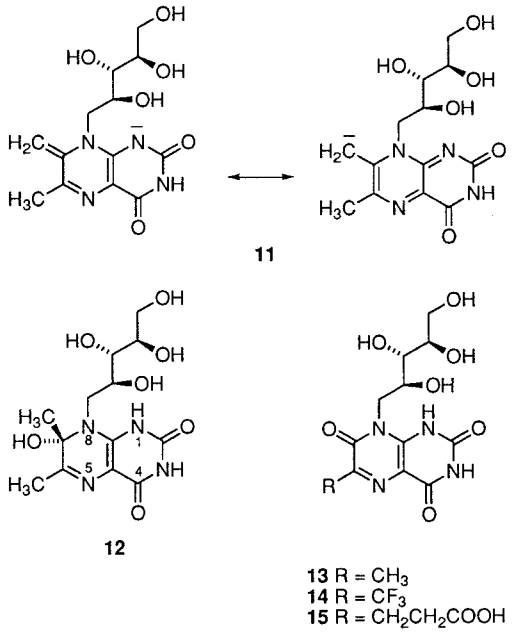


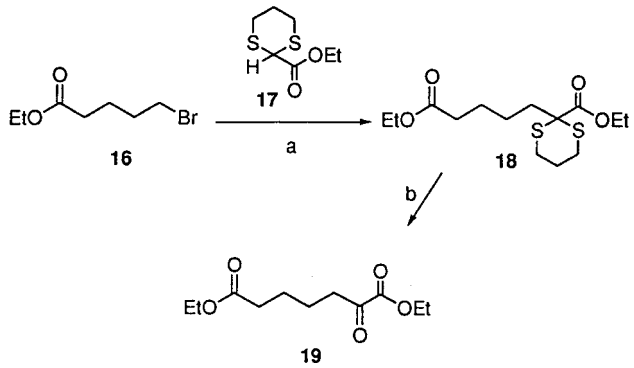
11

12

13 R = CH_3
14 R = CF_3
15 R = CH_2CH_2COOH

site by addition of water to substrate **3**.[3,16−20] The riboflavin synthase inhibitors **13**[21−23] and **14**[18] 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_i 10 μM) was less potent than **13** (K_i 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 phosphonate 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.

SCHEME 3[a]



[a] Reagents and conditions: (a) (1) ethyl 1,3-dithiane-2-carboxylate, *n*-BuLi, THF, −78 °C (30 min), (2) ethyl 5-bromovalerate, −78 to 23 °C; (b) NBS, $AgNO_3$, 2,6-lutidine, aq CH_3CN, 0−23 °C (30 min).

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-bromovalerate (**16**) to provide the product **18**. Hydrolysis 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-D-ribityllumazines **15**, **22**, and **23** as well as the lactone **25** are outlined in Scheme 4. Catalytic hydrogenation of 5-nitro-6-D-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-oxoadipic 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-D-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

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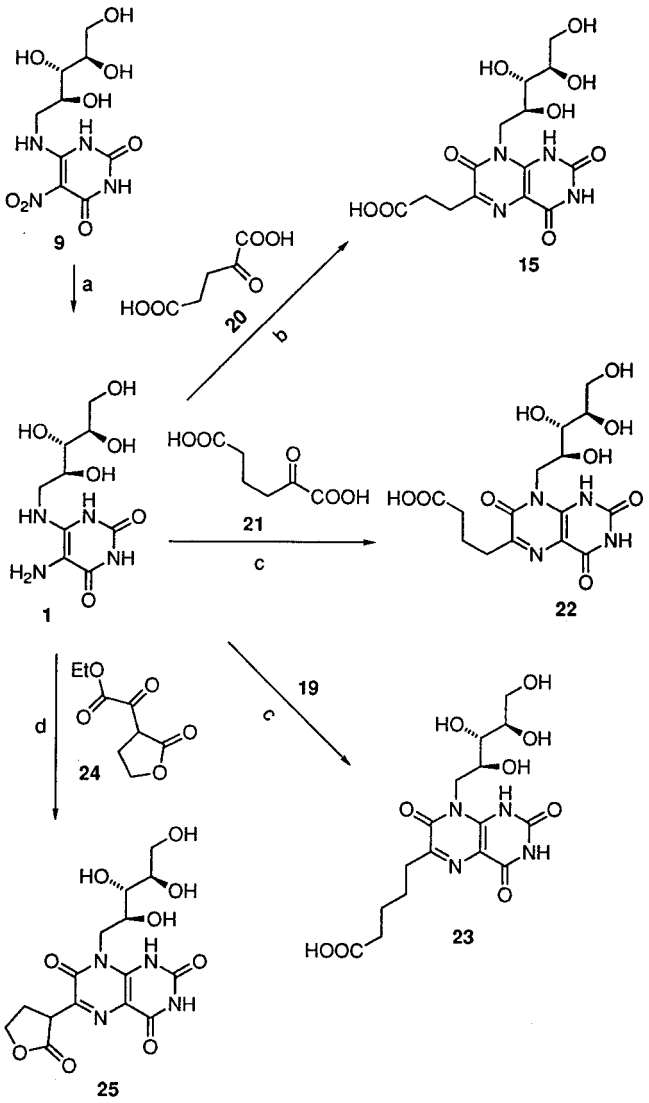
SCHEME 4[a]

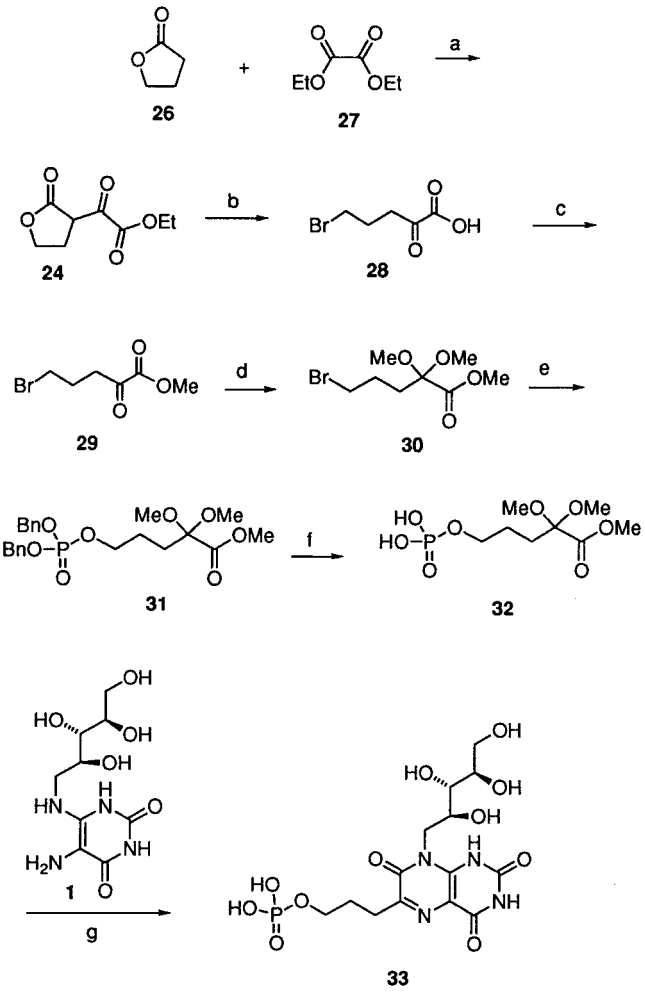


[a] Reagents and conditions: (a) H_2, Pd/C, H_2O (12 h); (b) AcOH, 90−100 °C (4 h); (c) HCl, EtOH, reflux (12 h); (d) AcOH, CH_3CN, 80 °C (2 h).

ethoxide and reaction of the resulting anion with diethyl oxalate (**27**) afforded α-ethoxalylbutyrolactone (**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 orthoformate provided the ketal **30**. Displacement of the bromide **30** with dibenzyl phosphate gave the expected substitution product **31**. Deprotection of the phosphate by hydrogenolysis over palladium hydroxide on carbon afforded the expected phosphate **32**, which was reacted with the lumazine synthase substrate **1** to provide the desired lumazine analogue **33**.

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

SCHEME 5[a]



[a] Reagents and conditions: (a) NaOEt, EtOH, diethyl oxalate, 0 °C (1 h); (b) HBr, AcOH, 120 °C (4 h); (c) MeOH, 23 °C (12 h); (d) $(MeO)_3CH$, H_2SO_4, 23 °C (12 h); (e) $AgOPO(OBn)_2$, $C_6H_5CH_3$, reflux (12 h); (f) H_2, $Pd(OH)_2$/C, ethanol (5 h); (g) HCl, EtOH, reflux (12 h).

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 β_{60} 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

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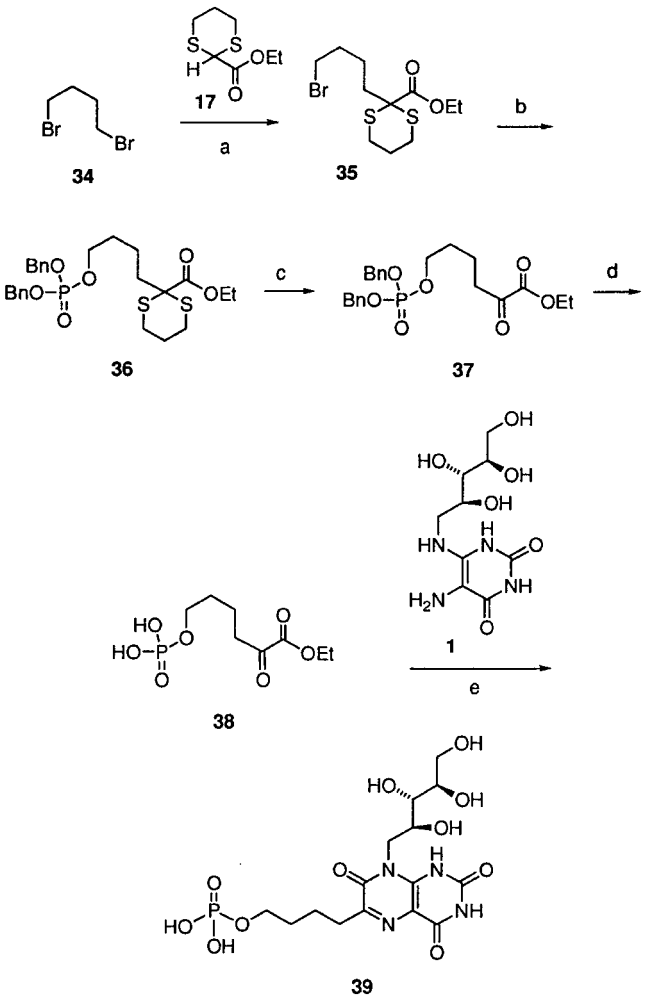
SCHEME 6[a]



[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) $AgOPO(OBn)_2$, $C_6H_5CH_3$, reflux (5 h); (c) NBS, $AgNO_3$, 2,6-lutidine, aq acetone, 0−23 °C (30 min); (d) H_2, $Pd(OH)_2$, 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**.[9,30]

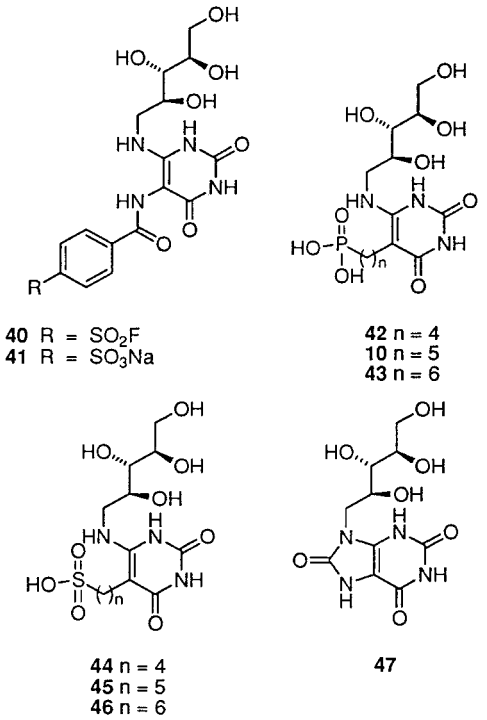
The most potent inhibitor of lumazine synthase in the present series was the 6-(3′-carboxypropyl)lumazine derivative **22** (K_i 84 μM), followed closely by the corresponding 6-(2′-carboxyethyl)lumazine analogue **15** (K_i 94 μM). Both of these compounds were more potent than the 6-(4′-carboxybutyl)lumazine homologue **23** (K_i 200 μM) and the lactone **25** (K_i 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** (K_i 120 μM) and **39** (K_i 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** (K_i 440 μM), **43** (K_i 180 μM), and **10** (K_i 130 μM), as well as the sulfonates **44** (K_i 130 μM), **45** (K_i 690 μM), and **46** (K_i 290 μM), in which

TABLE 1. Inhibition Constants vs Lumazine Synthase and Riboflavin Synthase

compd	lumazine synthase[a] K_i (μM)	riboflavin synthase[b] K_i (μM)
10	180 ± 88 (K_i, mixed inhib) 350 ± 22 (K_{is})[c]	>1000
15	94 ± 28 (mixed inhib) 180 ± 35 (K_{is})[c] 30.1 ± 2.1 (k_{cat}) 29 ± 5.9 (K_s)	650 ± 140 (comp inhib[d]) 87 ± 17 (K_s) 210 ± 22 (k_{cat})
22	84 (uncomp inhib) 49 ± 6.7 (K_s) 84 ± 8.5 (K_{is})[c]	20 ± 6.4 (comp inhib)
23	200 ± 69 (mixed inhib) 57 ± 9.5 (K_s) 93.3 ± 6.4 (k_{cat}) 270 ± 75 (K_{is})[c]	56 ± 6.9 (comp inhib) 18 ± 2.4 (K_{is}) 192 ± 5.7 (k_{cat})
25	220 ± 93 (partial inhibition) 45 ± 8.9 (K_s) 78.7 ± 5.9 (k_{cat}) 230 ± 60 (K_{is})[c]	170 ± 26 (comp inhib) 160 ± 45 (K_s) 290 ± 5 (k_{cat})
33	120 (uncomp inhib) 100 ± 21 (K_s) 150 ± 20 (k_{cat}) 120 ± 24 (K_{is})[c]	>1600
39	160 ± 32 (comp inhib) 27 ± 4.8 (K_s) 73.1 ± 3.7 (k_{cat})	150 ± 46 (comp inhib) 130 ± 14 (k_{cat})
40	200	
41	360	
42	440 ± 200 (K_i, mixed inhib) 640 ± 300 (K_{is})[c]	>1000
43	130 ± 33 (K_i, mixed inhib) 140 ± 15 (K_{is})[c]	>1000
44	290 ± 120 (K_i, comp inhib)	
45	690 ± 290 (K_i, mixed inhib) 1500 ± 640 (K_{is})[c]	>1000
46	290 ± 130 (K_i, mixed inhib) 580 ± 170 (K_{is})	>1000
47	46 ± 5 (K_i, partial inhib) 250 ± 42 (K_{is})[c]	0.61 ± 0.5 (K_i, comp inhib) 9.6 ± 0.9 (K_s) 39.9 ± 0.7 (k_{cat})

[a] Recombinant β_{60} capsids from *B. subtilis*. [b] Recombinant riboflavin synthase from *E. coli*. [c] K_{is} is the equilibrium constant for the reaction EI + S ⇌ EIS. [d] Competitive inhibition.



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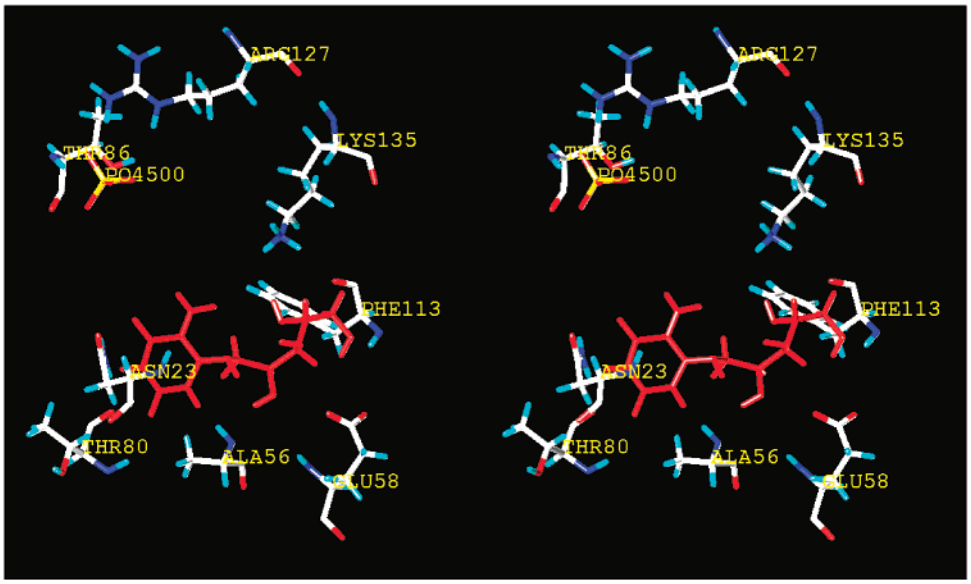

FIGURE 1. Binding of 5-nitro-6-ribitylaminouracil to *B. subtilis* lumazine synthase. The figure was constructed using published X-ray coordinates and is programmed for walleyed viewing.

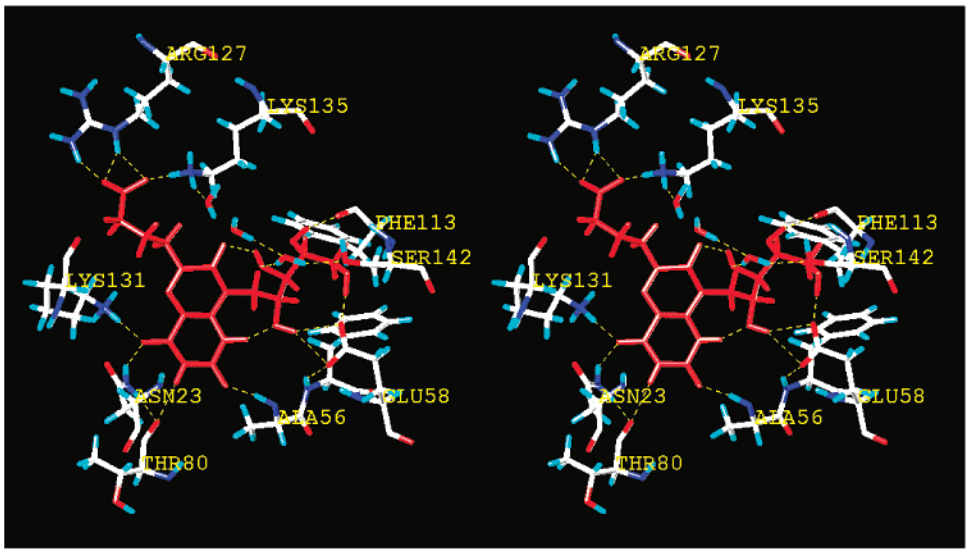

FIGURE 2. Hypothetical model of the binding of compound **22** to *B. subtilis* lumazine synthase. The figure is programmed for walleyed viewing.

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).[8] The related 1.85 Å resolution X-ray structure of the phosphonate inhibitor **10**[9] (K_i 180 μM vs that of *B. subtilis* lumazine synthase) bound to *Saccharomyces cerevisiae* lumazine synthase has also been published.[10] These structures allow the construction of a hypothetical model of the binding of **22** to 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 the nitropyrimidine **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

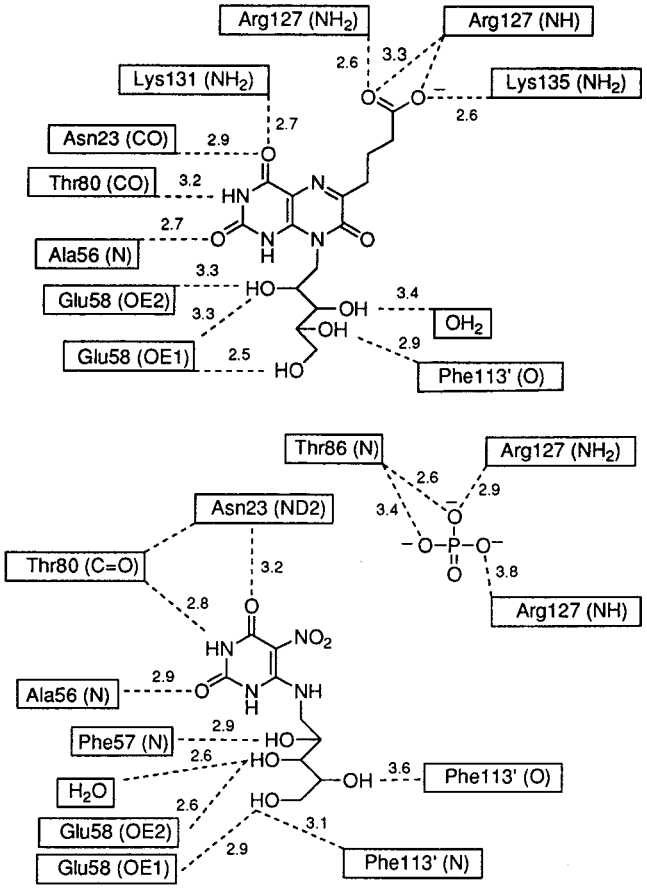

FIGURE 3. Top: hydrogen bonds and distances in the calculated model of inhibitor **22** bound in the active site of *B. subtilis* lumazine synthase. Bottom: the corresponding diagram based on the X-ray structure of the complex of **9** with the enzyme.

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_i 20 μM). Either shortening or lengthening the linker chain connecting the carboxyl group to the lumazine ring resulted in less active compounds (**15**, K_i 650 μM; **23**, K_i 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_i > 1600 μM). However, lengthening the linker chain by one carbon atom resulted in the riboflavin synthase inhibitor **39** (K_i 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-D-ribityllumazine (**3**).[31] According to the model, as well as NMR studies of the structure of the N-terminal domain of riboflavin synthase,[32] 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.[31] 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**.[33] 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.[31] 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 ^{1}H NMR spectra were determined at 300 MHz, and the ^{13}C 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-D-ribityllumazine (15). 5-Nitro-6-(D-ribityl)aminouracil (**9**) (0.5 g, 1.6 mmol) was dissolved in water (20 mL) and hydrogenated under H_2 atmosphere for 12 h in the presence of Pd on activated carbon (50 mg, 10%). Acetic acid (5 mL) and 2-ketoglutaric acid (**20**) (1.0 g, 6.8 mmol)

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(32) Truffault, V.; Coles, M.; Diercicks, T.; Abelmann, K.; Eberhardt, S.; Lüttgen, H.; Bacher, A.; Kessler, H. *J. Mol. Biol.* **2001**, *309*, 949−960.
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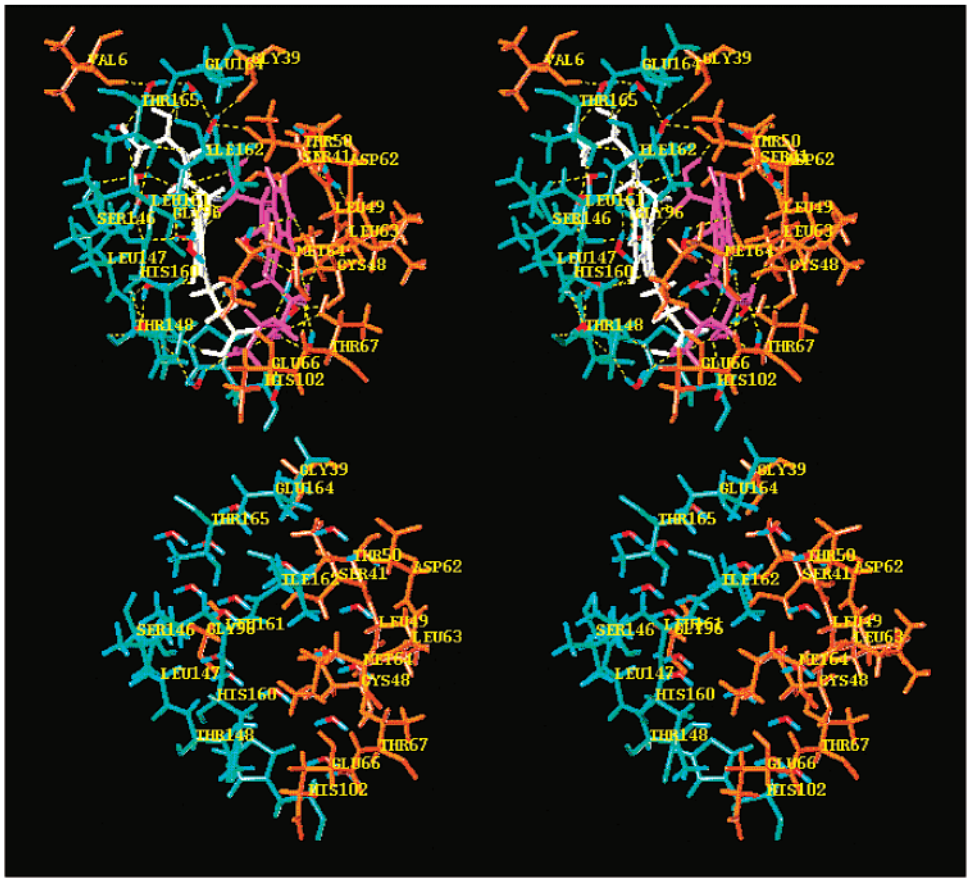

FIGURE 4. Top: hypothetical model of two molecules of compound **15** bound in the active site of *E. coli* riboflavin synthase. Magenta: compound **15** bound to the N-barrel residues. Green: C-barrel residues. White: compound **15** bound to the C-barrel residues. Orange: N-barrel residues. Red and green: water molecules. Yellow: hydrogen bonds. Both ligand molecules are viewed approximately from within the planes of the lumazine rings, which are stacked. Bottom: X-ray structure of the empty active site of *E. coli* riboflavin synthase.

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: ^{1}H NMR (300 MHz, D_2O) 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); ^{13}C 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 $C_{14}H_{18}N_4O_9 \cdot 1.0(H_2O)$: 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 N_2 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 H_2O, 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**[25] as a colorless oil (2.4 g, 75%): ^{1}H NMR (300 Hz, $CDCl_3$) 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 NH_4Cl, saturated aqueous $NaSO_3$, 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**[25] as a colorless oil (0.35 g, 41%): ^{1}H NMR (300 Hz, $CDCl_3$) 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 H_2 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: ^{1}H NMR (500 MHz, D_2O) 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), 2.25 (t, J = 6 Hz, 2 H), 1.80 (quintet, J = 6 Hz, 2 H); ESIMS (negative ion mode) m/z 399 $(M - H)^-$. Anal.

Calcd for $C_{15}H_{20}N_4O_9 \cdot 0.6(AcOEt) \cdot 0.4(HCO_2H)$: C, 45.33; H, 5.47; N, 11.88. Found: C, 45.38; H, 5.40; N, 11.81.

6-(4-Carboxylbutyl)-7-oxo-8-(D-ribityl)lumazine (23). 5-Nitro-6-(D-ribityl)aminouracil (**9**) (0.35 g, 1.1 mmol) was dissolved in water (15 mL) with a few drops of triethylamine and hydrogenated under H_2 atmosphere for 12 h in the presence of Pd on activated carbon (100 mg, 10%). A solution of **19** (0.35 g, 1.5 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 an anion-exchange resin column (Dowex 1 × 2−400) and eluted with water followed by 5% formic acid. The corresponding fractions were combined and concentrated. The residue was triturated with a small amount of acetonitrile to afford **23** (350 mg, 77%) as an amorphous solid: ^{1}H NMR (300 Hz, D_2O) δ 4.34 (m, 1 H), 4.23 (m, 1 H), 4.02 (m, 1 H), 3.75−3.65 (m, 3 H), 3.57 (m, 1 H), 2.62 (m, 2 H), 2.29 (m, 2 H), 1.52 (m, 4 H). Anal. Calcd for $C_{16}H_{22}N_4O_9 \cdot 0.1(H_2O) \cdot 0.5(HCO_2H)$: C, 45.12; H, 5.32; N, 12.76. Found: C, 45.10; H, 5.25; N, 12.86.

α-Ethoxalylbutyrolactone (24).[27] Sodium (3.7 g, 0.16 mol) was cautiously added to absolute ethanol (80 mL) cooled by an ice bath. Diethyl oxalate (22.0 g, 0.15 mol) was added to the resultant solution of sodium ethoxide. Then, a solution of butyrolactone (13.0 g, 0.15 mol) in ethanol (20 mL) was dropwise added over 30 min, and the reaction mixture was stirred at ice temperature for 1 h. Upon removal of the cooling bath, the mixture was further stirred overnight at room temperature. The solvent was removed under reduced pressure, and the pasty residue was partitioned between ether and water. The aqueous layer was acidified with 2 N HCl, saturated with NaCl, and extracted with dichloromethane (4 × 100 mL). The extracts were combined, dried, and concentrated to yield the desired compound **24**[34] (27 g, 96%) as a colorless oil: ^{1}H NMR (300 Hz, $CDCl_3$) δ 10.8 (br s, 1 H, OH), 4.43 (t, J = 7.6 Hz, 2 H), 4.29 (q, J = 7.1 Hz, 2 H), 3.21 (t, J = 7.6 Hz, 2 H), 1.31 (t, 7.1 Hz, 3 H).

6-(γ-Butyrolacton-5-yl)-7-oxo-8-(D-ribityl)lumazine (25). 5-Nitro-6-(D-ribityl)aminouracil (**9**) (0.5 g, 1.6 mmol) was dissolved in water (20 mL) and hydrogenated under H_2 atmosphere for 12 h in the presence of Pd on activated carbon (100 mg, 10%). Acetic acid (1 mL) and α-ethoxalylbutyrolactone (**24**)[27] (1.0 g, 5.4 mmol) in CH_3CN (10 mL) were added, and the mixture was heated at 80 °C for 2 h. After cooling, the catalyst was removed by filtration and the filtrate was concentrated under reduced pressure. The residue was crystallized from EtOH to afford **25** (0.4 g, 62%): ^{1}H NMR (300 MHz, D_2O) δ 4.55 (m, 1 H), 4.40 (m, 1 H), 4.30 (m, 2 H), 4.15 (m, 1 H), 4.02 (m, 1 H), 3.75 (m, 1 H), 3.65 (m, 2 H), 3.56 (m, 1 H), 2.52 (m, 2 H); MS ESIMS (negative ion mode) m/z 397 ($M - H)^-$. Anal. Calcd for $C_{15}H_{18}N_4O_9 \cdot 1.2(H_2O)$: C, 42.90; H, 4.90; N, 13.34. Found: C, 42.74; H, 4.99; N, 13.58.

Methyl 5-Bromo-2-oxopentanoate (29). α-Ethoxalylbutyrolactone (**24**) (9.0 g, 48 mol) was dissolved in a solution of 30% HBr in acetic acid (30 mL). The reaction mixture was heated at 120 °C for 1 h. Then additional HBr−AcOH solution (20 mL) was added, and the mixture was stirred at 120 °C for 3 h. The solution was concentrated, and residue **28**[27] was taken into methanol (50 mL) and stirred overnight at room temperature. The solvent was evaporated, and the residue was dissolved in Et_2O (100 mL), washed with water, and dried over Na_2SO_4. After removal of the solvent, the residue was purified by silica gel column chromatography (ethyl acetate−hexane, 2:1) to afford **29**[35] as a colorless oil (6.5 g, 48%): ^{1}H NMR (300 Hz, $CDCl_3$) δ 3.80 (s, 3 H), 3.44 (t, J = 6.3 Hz, 2 H), 3.05 (t, J = 7.0 Hz, 2 H), 2.16 (m, 2 H); ^{13}C NMR ($CDCl_3$) δ 192.70, 160.86, 52.89, 37.47, 32.25, 25.59.

Methyl 5-Bromo-2,2-dimethoxypentanoate (30). Methyl 5-bromo-2-oxopentanoate (**29**) (1.0 g, 4.8 mmol), trimethylorthoformate (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**[35] as a colorless oil (1.0 g, 81%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.75 (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); ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.17, 102.00, 51.06, 49.68, 32.84, 32.06, 26.47.

Methyl 5-Dibenzylphosphonoxy-2,2-dimethoxypentanoate (31). A mixture of **30** (1.0 g, 3.9 mmol) and silver dibenzylphosphate (1.5 g, 3.9 mmol) in toluene (20 mL) was heated to reflux for 12 h. After cooling, the precipitate 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 (1:1−1:2), to give a colorless oil **31** (0.6 g, 34%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.37 (m, 10 H), 5.08−4.97 (m, 4 H), 3.99 (q, J = 6.4 Hz, 2 H), 3.77 (s, 3 H), 3.22 (s, 6 H), 1.92 (m, 2 H), 1.60 (m, 2 H). Anal. Calcd for $C_{22}H_{29}O_8P$: C, 58.40; H, 6.46. Found: C, 58.23; H, 6.43.

Methyl 5-Phosphonoxy-2,2-dimethoxypentanoate (32). A mixture of compound **31** (0.6 g, 1.3 mmol) and $Pd(OH)_2$ on activated carbon (50 mg, 20%) in ethanol (20 mL) was hydrogenated under H_2 atmosphere for 5 h. The catalyst was removed by filtration, and the filtrate was concentrated under reduced pressure to give **32** as a colorless oil (0.35 g, 97%), which was used without further purification. ^{1}H NMR (300 MHz, CD_3OD) 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); ^{13}C NMR (100 MHz, CD_3OD) δ 171.17, 103.49, 67.03 (d), 52.94, 50.08, 30.80, 25.57 (d).

7-Oxo-6-(3-phosphonoxypropyl)-8-(D-ribityl)lumazine (33). A mixture of 5-nitro-6-(D-ribityl)aminouracil (**9**) (0.35 g, 1.1 mmol) and Pd on activated carbon (50 mg, 10%) in water (15 mL) was hydrogenated under H_2 atmosphere for 12 h. A solution of **32** (0.35 g, 1.3 mmol) in ethanol (15 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 ethanol, and the insoluble material was filtered off. The ethanolic filtrate was concentrated, and the residue was applied onto an anion-exchange resin column (Dowex 1 × 2−400) and eluted with water followed by 5% formic acid. The corresponding fractions were combined and concentrated. The residue was precipitated by *i*-PrOH to afford **33** (310 mg, 62%) as an amorphous solid: ESIMS (negative ion mode) m/z 451 ($M - H)^-$; ^{1}H NMR (300 MHz, D_2O) 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), 2.74 (t, J = 6 Hz, 2 H), 1.93 (m, 2 H). Anal. Calcd for $C_{14}H_{21}N_4O_{11}P \cdot 1(H_2O) \cdot 0.1$(*i*-PrOH): C, 36.06; H, 5.04; N, 11.67. Found: C, 36.10; H, 4.95; N, 11.57.

Ethyl 2-(4-Bromobutyl)-1,3-dithiane-2-carboxylate (35). *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 N_2 atmosphere. The solution was warmed to room temperature and added to a solution of 1,4-dibromobutane (4.3 g, 0.02 mol) in THF (30 mL). The reaction mixture was stirred for 3 h at room temperature. It was diluted with diethyl ether, washed with 1 N HCl and H_2O, dried, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography, eluted with hexanes−ethyl acetate (5:1) to provide **35** as a colorless oil (2.5 g, 76%): ^{1}H NMR (300 Hz, $CDCl_3$) δ 4.25 (q, J = 7.1 Hz, 2 H), 3.41 (t, J = 6.5 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), 1.66 (m, 2 H), 1.38 (t, J = 7.1 Hz, 3 H), 0.92 (m, 1 H).

Ethyl 2-(4-Dibenzylphosphonoxybutyl)-1,3-dithiane-2-carboxylate (36). A mixture of **35** (1.5 g, 4.6 mmol) and silver dibenzylphosphate (1.76 g, 4.6 mmol) in toluene (50 mL) was

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(35) Yates, P.; Schwartz, D. A. *Can. J. Chem.* **1983**, *61*, 509−518.

heated at reflux for 5 h under N_2. 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%): ^{1}H NMR (300 Hz, $CDCl_3$) 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-(Dibenzylphosphono)-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-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 NH_4Cl, saturated aqueous $NaSO_3$, and saturated aqueous NaCl, dried, and concentrated to give **37** (1.7 g, 100%) as an oil: ^{1}H NMR (300 Hz, $CDCl_3$) 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-phosphonoxyhexanoate (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 H_2 atmosphere for 5 h. The catalyst was removed by filtration, and the filtrate was concentrated under reduced pressure to give a colorless oil (0.55 g, 99%), which was used without further purification.

7-Oxo-6-(4-phosphonoxybutyl)-8-(D-ribityl)lumazine (39). A mixture of 5-nitro-6-D-ribitylaminouracil (**9**) (0.5 g, 1.6 mmol) and Pd on activated carbon (50 mg, 10%) in water (20 mL) was hydrogenated under H_2 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 × 2−400) and eluted with water followed by 5% formic acid. The corresponding fractions were combined and concentrated. The residue was precipitated by AcOEt to afford **39** (300 mg, 36%) as an amorphous solid: ESIMS (negative ion mode) m/z 465 $(M - H)^-$; ^{1}H NMR (500 MHz, D_2O) 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), 3.50 (m, 1 H), 2.61 (t, J = 6 Hz, 2 H), 1.55 (m, 4 H); ^{13}C NMR (100 MHz, D_2O) δ 162.83, 157.40, 154.21, 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 $C_{15}H_{23}N_4O_{11}P\cdot0.3(AcOEt)\cdot0.5(HCO_2H)$: 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 the complex of 5-nitro-6-ribitylamino-2,4-(1*H*,3*H*)-pyrimidinedione (**9**) and the lumazine synthase of *B. subtilis* (1RVV)[8] was clipped to include information within a 15 Å sphere of one of the 60 equivalent ligand molecules. The residues that were clipped in this cut complex were capped with either neutral amino or carboxyl groups. The structure of inhibitor **22** was overlapped with the structure of 5-nitro-6-ribitylamino-2,4-(1*H*,3*H*)pyrimidinedione (**9**), which was then deleted. Hydrogen atoms were added to the complex. 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. During the minimization of the complex, inhibitor **22** and a 6 Å sphere surrounding it were allowed to remain flexible while the remaining portion of the complex was held ridged using the aggregate function. Figure 1 was constructed by displaying the amino acid residues of the enzyme that are involved in hydrogen bonding with inhibitor **22**. The maximum distance between donor and acceptor atoms contributing to the hydrogen bonds shown in Figure 2 was set to 2.8 Å.

Molecular Modeling on Riboflavin 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.[31] 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 a 6 Å spherical subset including and surrounding the two ligand molecules was then energy 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 4 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 4 was set to 2.8 Å.

Lumazine Synthase Assay.[36] Reaction mixtures contained 100 mM potassium phosphate, pH 7.0, 5 mM EDTA, 5 mM dithiothreitol, inhibitor (0−86 μM), 170 μM 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-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-butanone 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.[37] Different inhibition models were considered for the calculation. K_i values ± standard deviations were obtained from the fit under consideration of the most likely inhibition model.

Riboflavin Synthase Assay.[38] Reaction mixtures contained buffer (100 mM potassium phosphate, 10 mM EDTA, 10 mM sodium sulfite), inhibitor (0−87 μM), and riboflavin synthase (10 μg, specific activity 50 μmol mg^{-1} h^{-1}). After preincubation, 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 ($\epsilon_{Riboflavin}$ = 9100 M^{-1} cm^{-1}). The K_i evaluation was performed in the same manner as described above.

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