Design, Synthesis, and Biological Evaluation of Homologous Phosphonic Acids and Sulfonic Acids as Inhibitors of Lumazine Synthase

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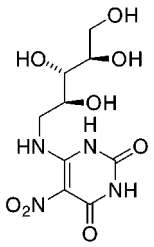
Three phosphonic acid inhibitors of lumazine synthase were synthesized in which the phosphorus atom was separated from the pyrimidinedione ring by polymethylene linker chains containing four, five, and six carbon atoms. Three analogous sulfonic acids were also synthesized. The inhibitors were designed as metabolically stable analogues of a hypothetical intermediate in the reaction catalyzed by lumazine synthase, and the design process was supported by the results of computer graphics molecular modeling of the inhibitors within the active site of the enzyme. The most potent of the new inhibitors, 6-(6-D-ribitylamino-2,4-dihydroxypyrimidine-5-yl)-1-hexylphosphonic acid, inhibited recombinant lumazine synthase β_{60} capsids of *Bacillus subtilis* with a K_i of 130 μM, making it the most potent inhibitor of lumazine synthase reported to date.

The last two steps in the biosynthesis of riboflavin involve the lumazine synthase catalyzed condensation of 5-amino-6-D-ribitylamino-2,4(1*H*,3*H*)pyrimidinedione **(1)** with 3,4-dihydroxy-2-butanone-4-phosphate **(2)** to form 6,7-dimethyl-8-D-ribityllumazine **(3)** and the riboflavin synthase catalyzed dismutation reaction of two molecules of **3** to form one molecule of riboflavin **(4)** and one molecule of **1**, which can then be recycled (Scheme 1).[1,2] Because various pathogenic *Enterobacteriaceae* lack a transport system for the absorption of extracellular riboflavin,[3] the inhibition of enzymes involved in riboflavin biosynthesis provides a rational strategy for antibiotic drug design.

The structure elucidation of the four-carbon unit **2**[4] has made it feasible to assay potential lumazine synthase inhibitors. In addition, the mechanism of the condensation of the phosphate **2** with the ribitylaminopyrimidine **1** to form the lumazine **3** has been studied in some detail.[5] The reaction pathway is thought to involve Schiff base formation between the ketone of **2** and the primary amino group of **1** to afford intermediate **5**, which can then eliminate phosphoric acid to generate the enol **6** (Scheme 2). Tautomerization of **6** generates the ketone **7**, which on nucleophilic attack by the ribitylamino group affords intermediate **8**. Dehydration of **8** yields the lumazine **3**.

The design of potential inhibitors of lumazine synthase is aided considerably by the recent determination of the X-ray structure of reconstituted, icosahedral lumazine synthase capsids complexed with the substrate analogue 5-nitro-6-(D-ribitylamino)-2,4(1*H*,3*H*)-pyrimidinedione **(9)**.[6,7] 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[8] **(10)** is also available.[9] These structures have provided a



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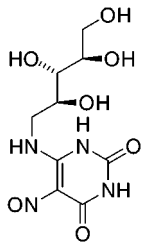

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detailed description of the active site of lumazine synthase, and they have also allowed the creation of a hypothetical model for the binding of the Schiff base **5** to the enzyme.[7] The model was constructed by allowing the ribitylaminopyrimidine portion of **5** to overlap with the structure of **9** in the active site. The phosphate group of **5** was then placed in a region occupied by buffer-derived inorganic phosphate, which crystallizes with the complex of the ligand **9** and the enzyme. The model of bound **5** suggests that a potential inhibitor bearing an appropriate functional group that could occupy the space of the phosphate group of enzyme-bound **5** but would be more stable then **5** when bound to the enzyme might in fact function effectively as an inhibitor. Accordingly,

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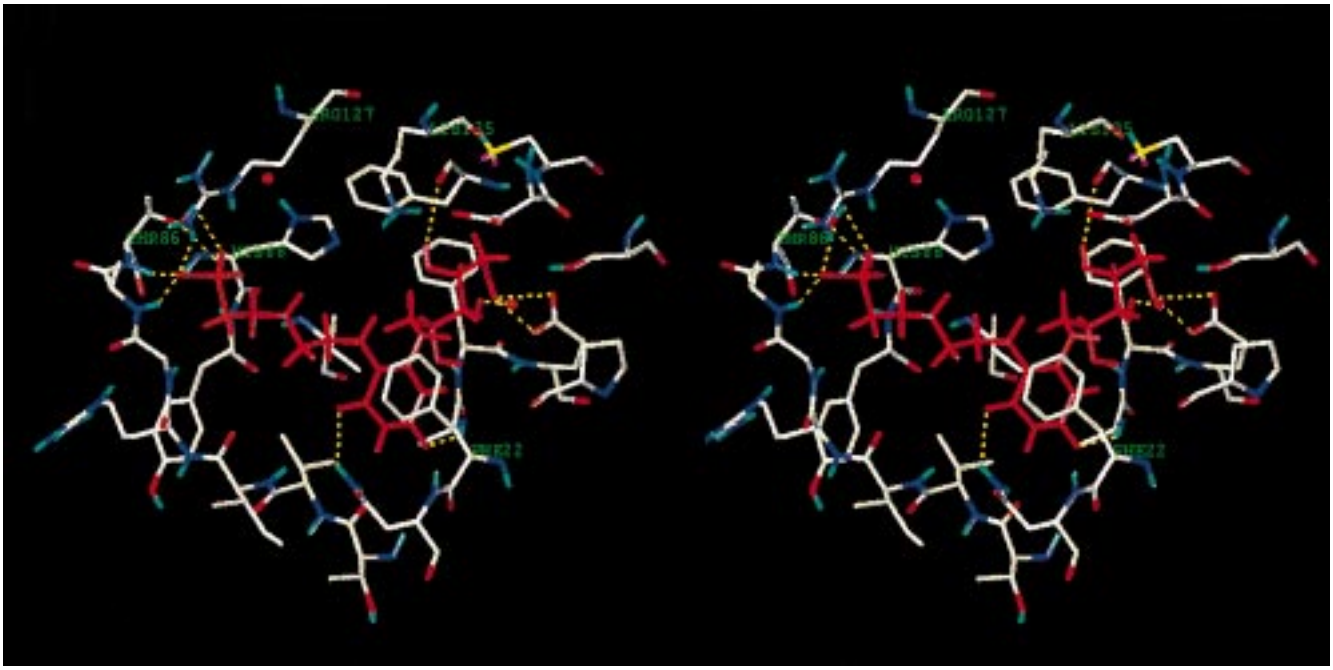
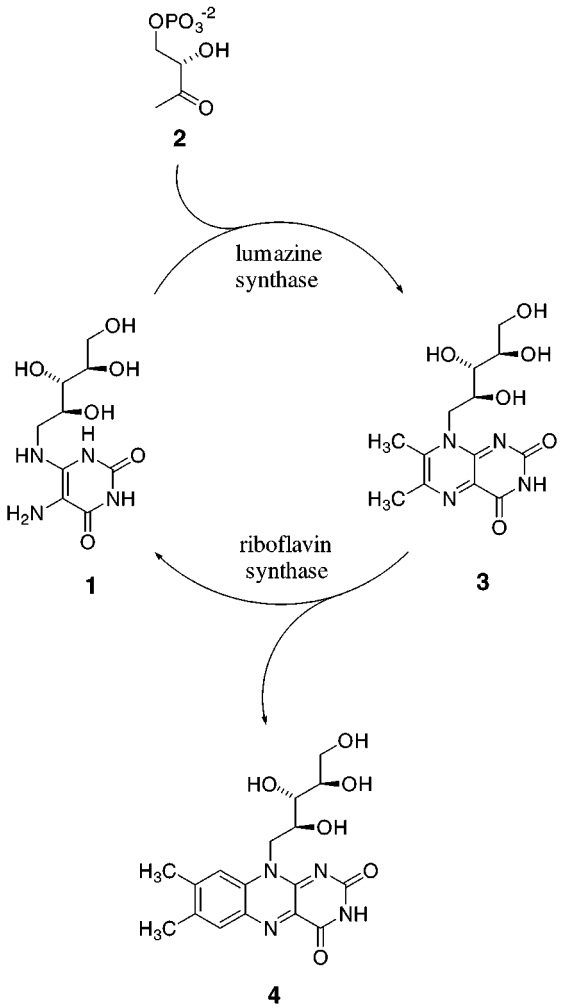


Figure 1. Hypothetical model for the binding of compound **16c** to lumazine synthase. The figure is programmed for walleyed viewing. The ligand is colored in red.

Scheme 1



models were constructed of the binding of potential phosphonate and sulfonate inhibitors of the enzyme in which the phosphonate and sulfonate groups were attached to the pyrimidinedione ring by a polymethylene linker chain. The model of bound phosphonate **16c** is shown in Figure 1.

Because the distance from the phosphorus atom to the pyrimidine ring in the hypothetical intermediate **5** is five atoms, a phosphonate connected to the pyrimidine ring by a polymethylene chain containing five atoms is a rational choice for the synthesis of a potential inhibitor. This would place the phosphonate approximately in the region of space thought to be occupied by the phosphate of **5**, and the phosphonate would be stable to the enzyme-catalyzed elimination of phosphate (conversion of **5** to **6**) seen with the natural substrate. In addition, the distance between the phosphonate and the pyrimidine ring could be modulated by making the linker chain four atoms and six atoms in length.

The syntheses of the three phosphonates having four-, five-, and six-carbon linker chains between the phosphonate and the pyrimidine ring are outlined in Scheme 3. Treatment of 6-chloro-2,4-dimethoxypyrimidine with *n*-butyllithium in THF at −78 °C resulted in the lithiated species **12**.[10,11] Intermediate **12** reacted with 1,4-diiodobutane, 1,5-diiodopentane, and 1,6-diiodohexane to afford the desired iodides **13a**, **13b**, and **13c**. Treatment of diethyl phosphite with sodium hydride in DMF at room temperature afforded the corresponding anion, which reacted with the iodides **13a**, **13b**, and **13c** at 95 °C to yield the diethyl phosphonates **14a**, **14b**, and **14c**. The two methyl groups and the two ethyl groups were removed from each of **14a**, **14b**, and **14c** with trimethylsilyl iodide in methylene chloride to give **15a**, **15b**, and **15c**.[12] Reaction of the chlorides **15a**, **15b**, and **15c** with

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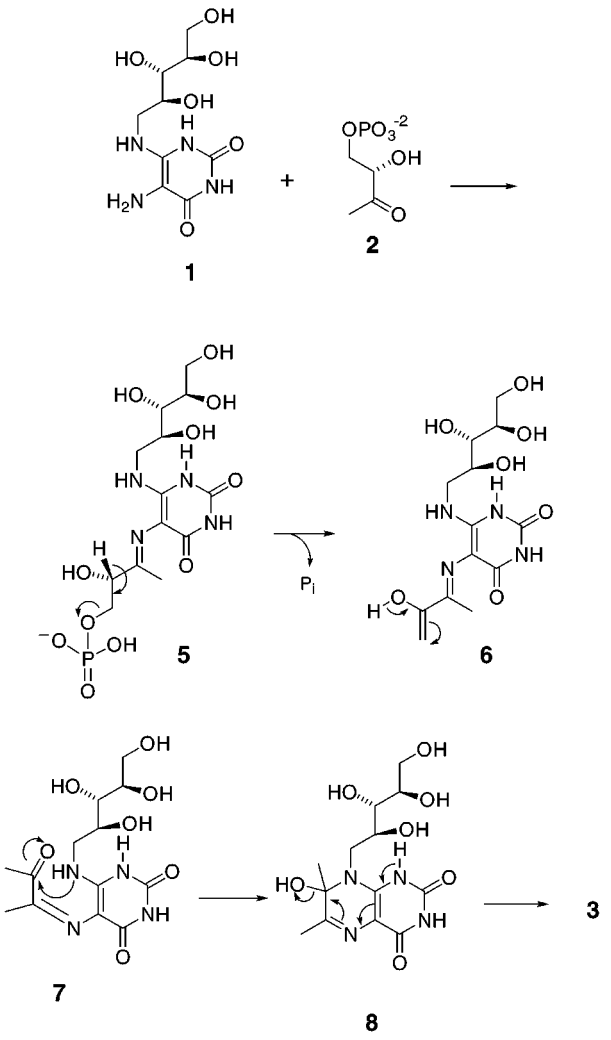
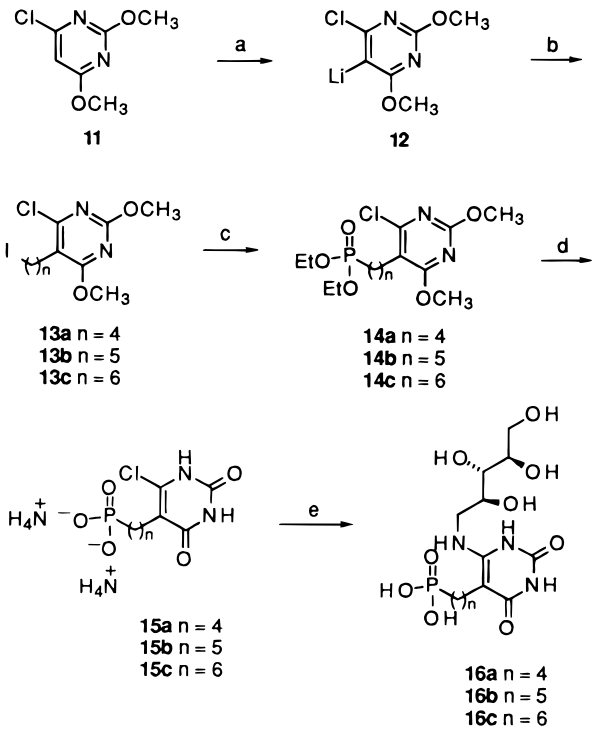
Scheme 2

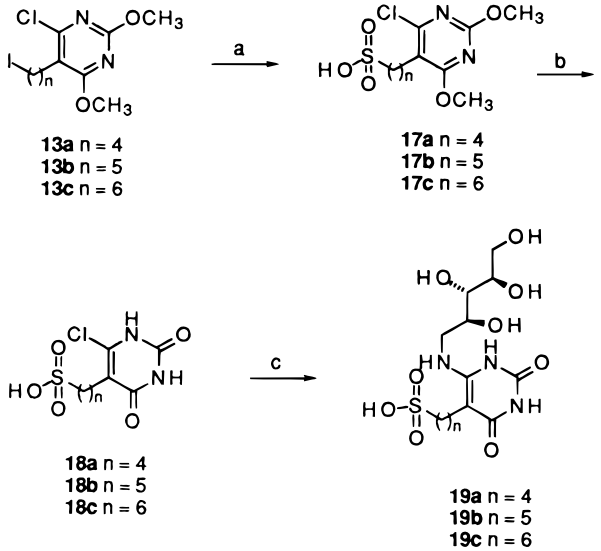


Scheme 3[a]



[a] Reagents and conditions: (a) *n*-BuLi, THF, −78 °C (15 min). (b) Diiodoalkane, −78 °C to rt (12 h). (c) (1) NaH, $(EtO)_2P(O)H$, DMF, rt, (45 min); (2) intermediate **13**, rt (30 min), 95 °C (4 h). (d) TMSI, CH_2Cl_2, rt (23 h). (e) D-Ribitylamine, $CH_3OCH_2CH_2OH$, reflux (23 h).

Scheme 4[a]



[a] Reagents and conditions: (a) Na_2SO_3, aq Me_2CO, reflux (24 h). (b) HCl, AcOH, reflux (2.5 h). (c) D-Ribitylamine, CH_3OCH_2-CH_2OH, reflux (24 h).

D-ribitylamine[13] in 2-methoxyethanol at reflux resulted in Michael addition of the amine to the α,β-unsaturated carbonyl moiety, followed by chloride elimination, to afford the desired products **16a**, **16b**, and **16c**.

In addition to the phosphonates, sulfonates were also considered as potential lumazine synthase inhibitors. Previous work had shown that certain benzenesulfonic acids function as moderately active inhibitors.[14] In the present case, nonaromatic sulfonic acids, which are analogous to the phosphonic acids discussed above, were investigated. As shown in Scheme 4, reaction of the three iodides **13a**, **13b**, and **13c** with sodium sulfite in refluxing acetone resulted in the three sulfonic acids **17a**, **17b**, and **17c**. Demethylation of the two methyl ethers was accomplished with hydrochloric acid in refluxing acetic acid to afford the pyrimidinedione derivatives **18a**, **18b**, and **18c**. Michael addition of D-ribitylamine[13] to the α,β-unsaturated carbonyl moiety present in **18a**, **18b**, and **18c**, followed by chloride elimination, afforded the desired products **19a**, **19b**, and **19c**.

The new phosphonic acids and sulfonic acids were tested for inhibition of lumazine synthase β_{60} capsids of *Bacillus subtilis* and recombinant riboflavin synthase from *Escherichia coli* (Table 1). Lineweaver−Burk plots for the inhibition of lumazine synthase by the phosphonates **16a**−**c** and the sulfonates **19a**−**c** are presented in Figure 2. The kinetic data were fitted with a nonlinear regression method using the program DynaFit from P. Kuzmic.[15] The previously reported lumazine synthase

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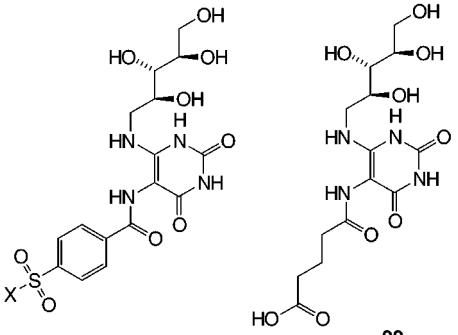
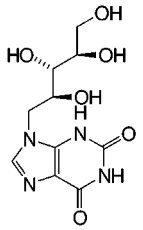
Table 1. Inhibition Constants vs Lumazine Synthase and Riboflavin Synthase

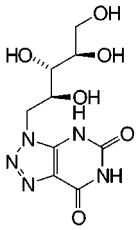
	lumazine synthase[a]		riboflavin synthase[c]	
compound	K_i (μM)	K_{is} (μM)[b]	K_i (μM)	inhibition type
16a	440 ± 220	640 ± 300	>1000	mixed
16b	180 ± 88	350 ± 22	>1000	mixed
16c	130 ± 33	140 ± 15	>1000	mixed
19a	290 ± 120		>1000	competitive
19b	690 ± 290	1500 ± 640	>1000	mixed
19c	290 ± 130	580 ± 170	>1000	mixed
20	200		40[d]	
21	360		135[d]	
22	430		650[d]	
23	470		540	
24	330		390	

[a] Recombinant β_{60} capsids from *B. subtilis*. [b] K_{is} is the equilibrium constant for the reaction EI + S ⇌ EIS. [c] Recombinant riboflavin synthase from *E. coli*. [d] Not previously reported.

inhibitors **20**−**24** are included in Table 1 for comparison.[14,16] On the basis of the five-atom connection of the







phosphorus of the phosphate to the pyrimidinedione ring in the hypothetical intermediate **5**, one might expect that **16b**, having a pentamethylene linker chain connecting the phosphonate to the pyrimidine, would be significantly more potent than **16a** or **16c**. As the data in Table 1 show, this expectation was not realized. In proceeding from the C-4 to the C-5 to the C-6 linkers in **16a**, **16b**, and **16c**, respectively, the K_i values decreased from 440 to 180 to 130 μM. Phosphonates **16b** and **16c** are more potent than any of the previously reported inhibitors of lumazine synthase **20**−**24** listed in Table 1.

Figure 1 shows the hypothetical structure of a potential phosphonate inhibitor **16c** bound to lumazine synthase. The model was constructed by overlapping the ribitylaminopyrimidine fragment of **16c** with the X-ray structure of bound **5**, with the phosphate group of **16c** occupying the space where buffer-derived inorganic phosphate is found in the X-ray structure.[6,7] The structures of **5** and inorganic phosphate were then removed, the protein was "frozen", and the energy was minimized while allowing bound **16c** to move. This procedure was carried out using Sculpt 2.5 software (Interactive Simulations, Inc.). The resulting structure was then displayed using Sybyl software (Tripos, Inc.). According to this structure, the proposed inhibitor **16c** fits nicely within the active site of lumazine synthase, with the phosphate group positioned near the guanidino group of Arg127 and the side chain hydroxyl group and backbone carbonyl of Thr86, and the pyrimidine ring of the inhibitor stacked with Phe22.

In conclusion, the phosphonate **16c**, having a K_i of 130 μM, is the most potent lumazine synthase inhibitor reported to date. The activity of this compound and the molecular modeling of its binding to lumazine synthase provide insight which may be useful in the design and synthesis of even more potent inhibitors of lumazine synthase for potential therapeutic use as antibiotic agents. We also expect that the X-ray structure of **16c** or a related ligand bound to the enzyme may be useful in the future to gain information about the binding of the hypothetical intermediate **5**.

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 that produces MeV fission fragments. The interaction of the fission fragments with the sample produces ions that are mass analyzed with a time-of-flight mass spectrometer.[17] 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 17 kV. Elemental analyses were performed by the Purdue Microanalytical Laboratory.

6-Chloro-5-(4-iodobut-1-yl)-2,4-dimethoxypyrimidine (13a). 6-Chloro-2,4-dimethoxypyrimidine **(11)** (1.5 g, 8.59 mmol) was dissolved in anhydrous THF (25 mL) under argon. This solution was cooled to −78 °C, and a 1.6 M solution of *n*-butyllithium in hexanes (5.42 mL, 8.68 mmol) was added dropwise while the temperature was maintained below −70 °C. After 15 min of stirring at −78 °C, 1,4-diiodobutane (2.5 mL, 18.9 mmol) was added quickly, and the solution was allowed to slowly warm to room temperature and stir overnight. Brine (20 mL) was then added, and the entire solution was extracted with ethyl acetate and dried over sodium sulfate. After concentration, the remaining oil was purified by flash chromatography (2 cm × 30 cm column of SiO_2, 230−400 mesh), eluting with hexane/ethyl acetate 10:1. Similar fractions were pooled and concentrated to give **13a** (2.56 g, 84%) as a white solid: mp 51 °C; IR 2954, 1589, 1548, 1482, 1462, 1204, 1082, 1036 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.97 (s, 3 H), 3.95 (s, 3 H), 3.19 (t, J = 9 Hz, 2 H), 2.61 (t, J = 9 Hz, 2 H), 1.85 (quintet, J = 9 Hz, 2 H), 1.59 (quintet, J = 9 Hz, 2 H); PDMS (MH^+) m/z 357. Anal. Calcd for $C_{10}H_{14}N_2O_2ClI$: C, 33.68; H, 3.96; N 7.86. Found: C, 34.02; H, 3.91; N, 8.15.

6-Chloro-5-(5-iodopent-1-yl)-2,4-dimethoxypyrimidine (13b). 6-Chloro-2,4-dimethoxypyrimidine **(11)** (0.200 g, 1.14 mmol) was dissolved in anhydrous THF (20 mL) under argon. This solution was cooled to −78 °C, and a 1.6 M solution of *n*-butyllithium in hexanes (0.71 mL, 1.14 mmol) was added

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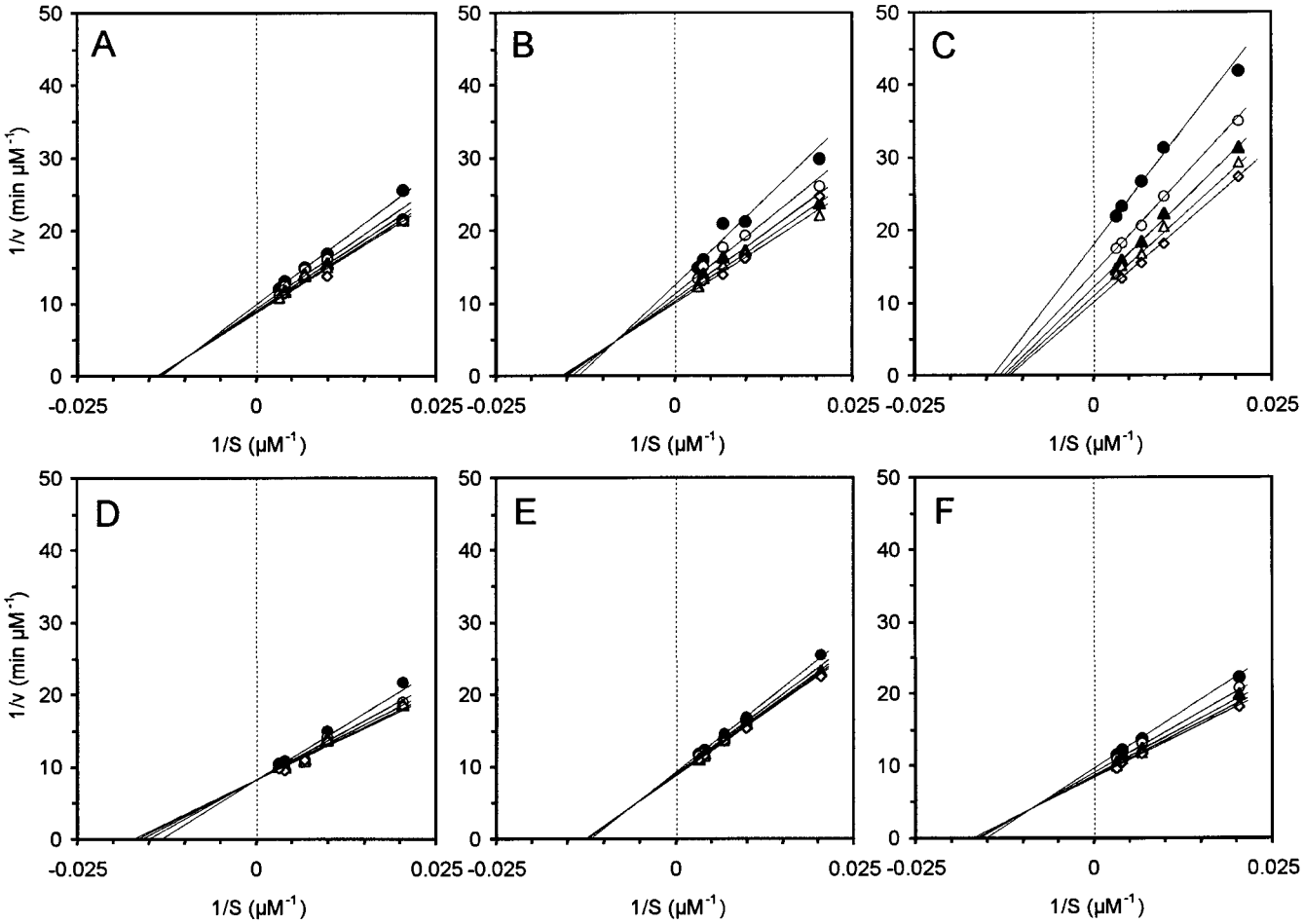

Figure 2. Lineweaver−Burk plots of the inhibition of lumazine synthase by phosphonic and sulfonic acids. A, **16a**; B, **16b**; C, **16c**; D, **19a**; E, **19b**; F, **19c**. The concentrations of the substrate ranged from 49.1 to 310.3 μM. Inhibitor concentrations: ◇, 0 μM; △, 8.6 μM; ▲, 21.6 μM; ○, 43.1 μM; ●, 86.2 μM. The initial velocity of lumazine formation, *v*, was determined at steady-state conditions with varying amounts of substrate, *s*, and inhibitor. The kinetic data were fitted with a nonlinear regression method using the program DynaFit from P. Kuzmic.[15] Different kinetic models were considered. The most likely inhibition mechanisms found were mixed-type inhibition, A, B, C, E, and F, and competitive inhibition, D.

dropwise while the temperature was maintained below −70 °C. After 15 min of stirring at −78 °C, 1,5-diiodopentane (1 mL, 6.72 mmol) was added quickly, and the solution allowed to slowly warm to room temperature and stir overnight. Brine (20 mL) was then added, and the entire solution was extracted with ethyl acetate and dried over sodium sulfate. After concentration, the remaining oil was purified by flash chromatography (2 cm × 30 cm column of SiO_2, 230−400 mesh), eluting with hexane/ethyl acetate 10:1. Similar fractions were pooled and concentrated to give **13b** (0.344 g, 81%) as a clear oil: IR 2934, 1590, 1548, 1481, 1462, 1377, 1212, 1082, 1034 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 3.97 (s, 3 H), 3.95 (s, 3 H), 3.17 (t, J = 8 Hz, 2 H), 2.58 (t, J = 9 Hz, 2 H), 1.85 (quintet, J = 9 Hz, 2 H), 1.42 (m, 4 H); PDMS (MH^+) *m/z* 370.7 Anal. Calcd for $C_{11}H_{16}N_2O_2ClI$: C, 35.65; H, 4.35; N 7.56. Found: C, 35.98; H, 4.35; N, 7.76.

6-Chloro-5-(6-iodohex-1-yl)-2,4-dimethoxypyrimidine (13c). 6-Chloro-2,4-dimethoxypyrimidine **(11)** (0.551 g, 3.16 mmol) was dissolved in anhydrous THF (25 mL) under argon. This solution was cooled to −78 °C, and a 1.6 M solution of *n*-butyllithium in hexanes (1.99 mL, 3.19 mmol) was added dropwise while the temperature was maintained below −70 °C. After 15 min of stirring at −78 °C, 1,5-diiodohexane (1.30 mL, 7.89 mmol) was added quickly, and the solution allowed to slowly warm to room temperature and stir overnight. Brine (20 mL) was then added, and the entire solution was extracted with ethyl acetate (30 mL) and dried over sodium sulfate. After concentration, the remaining oil was purified by flash chromatography (2 cm × 30 cm column of SiO_2, 230−400 mesh), eluting with hexane/ethyl acetate 10:1. Similar fractions were pooled and concentrated to give **13c** (1.02 g, 84%) as a clear oil: IR 2931, 1590, 1548, 1481, 1459, 1210, 1082, 1028 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 3.97 (s, 3 H), 3.95 (s, 3 H), 3.17 (t, J = 6 Hz, 2 H), 2.57 (t, J = 6 Hz, 2 H), 1.79 (quintet, J = 6 Hz, 2 H), 1.43 (m, 6 H); FABMS (MH^+) *m/z* 384.8. Anal. Calcd for $C_{12}H_{18}N_2O_2Cl$: C, 37.47; H, 4.72; N 7.28. Found: C, 37.80; H, 4.76; N, 7.37.

Diethyl 4-(6-Chloro-2,4-dimethoxypyrimidin-5-yl)-1-butylphosphonate (14a). Sodium hydride (33.7 mg, 1.40 mmol) was suspended in dry DMF (10 mL) under an atmosphere of argon. Diethyl phosphite (0.180 g, 1.34 mmol) was added to the suspension, and the entire mixture was stirred at room temperature for 45 min. After H_2 liberation ceased, a solution of 6-chloro-5-(4-iodobut-1-yl)-2,4-dimethoxypyrimidine **(13a)** in dry DMF (1 mL) was added, and the mixture was allowed to stir at room temperature for 30 min and then at 95 °C for 4 h. Excess DMF was removed under reduced pressure, and the residue was dissolved in ethyl acetate (35 mL), washed with water and then brine, dried over Na_2SO_4, and concentrated to give a yellow oil. This oil was further purified by flash chromatography (2 cm × 30 cm column of SiO_2, 230−400 mesh), eluting with ethyl acetate/hexane 2:1. Similar fractions were pooled and concentrated. The remaining oil contained unreacted diethyl phosphite along with compound **14a**. This impurity was easily removed by Kugelrohr distillation, giving 0.204 g (40%) of compound **14a** as a light yellow oil: IR 2954, 1591, 1548, 1483, 1463, 1212, 1022 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 4.07 (m, 4 H), 3.96 (s, 3 H), 3.95 (s, 3 H), 2.59 (t, J =

9 Hz, 2 H), 1.80−1.60 (m, 4 H), 1.30 (t, J = 9 Hz, 6 H); ^{31}P NMR (121 MHz, $CDCl_3$) δ 31.92; FABMS (MH^+) m/z 367. Anal. Calcd for $C_{14}H_{24}N_2O_5PCl$: C, 45.85; H, 6.60; N 7.64. Found: C, 45.80; H, 6.46; N, 7.51.

Diethyl 5-(6-Chloro-2,4-dimethoxypyrimidin-5-yl)-1-pentylphosphonate (14b). Sodium hydride (44 mg, 1.83 mmol) was suspended in dry DMF (10 mL) under an atmosphere of argon. Diethyl phosphite (0.181 g, 1.31 mmol) was added to the suspension, and the entire mixture was stirred at room temperature for 45 min. After H_2 liberation ceased, a solution of 6-chloro-5-(5-iodopentan-1-yl)-2,4-dimethoxypyrimidine (**13b**) (0.536 g, 1.43 mmol) in dry DMF (1 mL) was added, and the mixture was allowed to stir at room temperature for 30 min and then at 95 °C for 4 h. Excess DMF was removed under reduced pressure, and the residue was dissolved in ethyl acetate (35 mL), washed with water and then brine, dried over Na_2SO_4, and concentrated to give a yellow oil. This oil was further purified by flash chromatography (2 cm × 30 cm column of SiO_2, 230−400 mesh), eluting with ethyl acetate/hexane 2:1. Similar fractions were pooled and concentrated to give **14b** (0.385 g, 55%) as a light yellow oil: IR 2938, 1591, 1546, 1482, 1459, 1209, 1028 (cm^{-1}); ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.05 (m, 4 H), 3.96 (s, 3 H), 3.94 (s, 3 H), 2.57 (t, J = 9 Hz, 2 H), 1.32−1.60 (m, 4 H), 1.46 (m, 4 H), 1.30 (t, J = 9 Hz, 6 H);^{31}P NMR (121 MHz, $CDCl_3$) δ 32.20; FABMS (MH^+) m/z 381. Anal. Calcd for $C_{15}H_{26}N_2O_5PCl$: C, 47.31; H, 6.88; N 7.36. Found: C, 47.56; H, 6.90; N 7.17.

Diethyl 6-(6-Chloro-2,4-dimethoxypyrimidin-5-yl)-1-hexylphosphonate (14c). Sodium hydride (29.5 mg, 1.23 mmol) was suspended in dry DMF (10 mL) under an atmosphere of argon. Diethyl phosphite (0.168 g, 1.22 mmol) was added to the suspension, and the entire mixture was stirred at room temperature for 45 min. After H_2 liberation ceased, a solution of 6-chloro-5-(4-iodohex-1-yl)-2,4-dimethoxypyrimidine (**13c**) (0.468 g, 1.22 mmol) in dry DMF (1 mL) was added, and the mixture was allowed to stir at room temperature for 30 min and then at 95 °C for 4 h. Excess DMF was removed under reduced pressure, and the residue was dissolved in ethyl acetate (35 mL), washed with water and then brine, dried over Na_2SO_4, and concentrated to give a yellow oil. This oil was further purified by flash chromatography (2 cm × 30 cm column of SiO_2, 230−400 mesh) eluting with ethyl acetate/hexane 2:1. Similar fractions were pooled and concentrated. The remaining oil contained unreacted diethyl phosphite along with compound **14c**. This impurity was easily removed by Kugelrohr distillation giving compound **14c** (0.1211 g, 25%) as a light yellow oil: IR 2930, 1591, 1547, 1482, 1460, 1216, 1027 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.07 (m, 4 H), 3.96 (s, 3 H), 3.95 (s, 3 H), 2.56 (t, J = 9 Hz, 2 H), 1.36−1.76 (m, 10 H), 1.30 (t, J = 9 Hz, 6 H); ^{31}P NMR (121 MHz, $CDCl_3$) δ 28.12; FABMS (MH^+) m/z 395.3. Anal. Calcd for $C_{16}H_{28}N_2O_5PCl$: C, 48.67; H, 7.15; N 7.09. Found: C, 48.98; H, 7.24; N, 7.12.

4-(6-Chloro-2,4-dihydroxypyrimidin-5-yl)-1-butylphosphonic Acid Diammonium Salt (15a). Diethyl 4-(6-chloro-2,4-dimethoxypyrimidin-5-yl)-1-butyl-phosphonate **(14a)** (0.189 g, 0.552 mmol) was dissolved in dry dichloromethane (8 mL) under an atmosphere of argon. Trimethylsilyl iodide[12] (0.39 mL, 2.76 mmol) was then added dropwise at room temperature. After 23 h of stirring at room temperature, excess solvent was removed using reduced pressure, and the remaining oil was dissolved in methanol (6 mL). Concentrated ammonium hydroxide was added dropwise until the pH reached 7. A beige precipitate formed and was filtered off, giving 0.159 g (99.8%) of compound **15a**: mp 245 °C; IR (Nujol) 1633, 1599 cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.20 (t, J = 9 Hz, 2 H), 1.2−1.5 (m, 6 H); ^{31}P NMR (121 MHz, DMSO-d_6) δ 21.80; Electrospray MS (MH^+) m/z 283.3 (as the free acid).

5-(6-Chloro-2,4-dihydroxypyrimidin-5-yl)-1-pentylphosphonic Acid Diammonium Salt (15b). Diethyl 5-(6-chloro-2,4-dimethoxypyrimidin-5-yl)-1-pentyl-phosphonate **(14b)** (0.293 g, 0.770 mmol) was dissolved in dry dichloromethane (8 mL) under an atmosphere of argon. Trimethylsilyl iodide (0.55 mL, 3.85 mmol) was then added dropwise at room temperature. After 23 h of stirring at room temperature, excess solvent was removed using reduced pressure, and the remaining oil was dissolved in methanol (6 mL). Concentrated ammonium hydroxide was added dropwise until the pH reached 7. A white precipitate formed and was filtered off, giving 0.231 g (91%) of compound **15b**: mp 254 °C; IR (Nujol) 1667, 1195 cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.12 (t, J = 6 Hz, 2 H), 1.2−1.5 (m, 8 H); ^{31}P NMR (121 MHz, DMSO-d_6) 23.85; Electrospray MS (MH^+) m/z 297.5 (as the free acid).

6-(6-Chloro-2,4-dihydroxypyrimidin-5-yl)-1-hexylphosphonic Acid Diammonium Salt (15c). Diethoxy 6-(6-chloro-2,4-dimethoxypyrimidin-5-yl)-1-hexyl-phosphonate **(14c)** (0.126 g, 0.320 mmol) was dissolved in dry dichloromethane (8 mL) under an atmosphere of argon. Trimethylsilyl iodide (0.23 mL, 1.60 mmol) was then added dropwise at room temperature. After 23 h of stirring at room temperature, excess solvent was removed using reduced pressure, and the remaining oil was dissolved in methanol (6 mL). Concentrated ammonium hydroxide was added dropwise until the pH reached 7. A beige precipitate formed and was filtered off, giving 0.11 g (99.7%) of compound **15c**: IR (Nujol) 1634, 1602 cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.21 (t, J = 9 Hz, 2 H), 1.26 (m, 10 H); ^{31}P NMR (121 MHz, DMSO-d_6) 22.03; Electrospray MS m/z 311 (as the free acid).

4-(6-D-Ribitylamino-2,4-dihydroxypyrimidin-5-yl)-1-butylphosphonic Acid (16a). 4-(6-Chloro-2,4-dihydroxypyrimidin-5-yl)-1-butylphosphonic acid diammonium salt **(15a)** (97.1 mg, 0.336 mmol) and D-ribitylamine (0.500 g, 3.31 mmol) were dissolved in 2-methoxyethanol (10 mL) and heated to reflux for 23 h. Excess solvent was then removed using reduced pressure, and the remaining oily residue was dissolved in water (10 mL). The resulting solution was basified with a solution of sodium hydroxide to pH 10 and then loaded on a column of Dowex 1X2-400 anionic exchange resin (10 g). The column was washed with water (300 mL), and then the product was eluted with a 10% formic acid solution (300 mL). The resulting fractions were concentrated to dryness, dissolved in water (3 mL), and passed through a column of Dowex 50WX2-400 cation-exchange resin (5 g). The filtrate was concentrated, and the remaining residue was dissolved in methanol (2 mL) and then precipitated out with the addition of ether (100 mL). The white solid was filtered under argon, giving **16a** as a hygroscopic beige solid (76.6 mg, 57%): ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.20 (s, 1 H), 10.04 (s, 1 H), 6.47 (m, 1 H), 3.68 (m, 1 H), 3.36−3.58 (m, 5 H), 3.22 (m, 1 H), 2.15 (m, 2 H), 1.50 (m, 4 H), 1.32 (m, 2 H); ^{31}P NMR (121 MHz, DMSO-d_6) 27.36; HRFABMS (MH^+) calcd for $C_{13}H_{24}N_3O_9P$ m/z 398.1328, found 398.1328. Anal. Calcd for $C_{13}H_{24}N_3O_9P\cdot HCOOH$: C, 37.93; H, 5.91; N 9.48. Found: C, 37.91; H, 6.23; N, 9.78.

5-(6-D-Ribitylamino-2,4-dihydroxypyrimidin-5-yl)-1-pentylphosphonic Acid (16b). 5-(6-Chloro-2,4-dihydroxypyrimidin-5-yl)-1-pentylphosphonic acid diammonium salt **(15b)** (100 mg, 0.302 mmol) and D-ribitylamine (0.600 g, 3.97 mmol) were dissolved in 2-methoxyethanol (12 mL) and heated to reflux for 23 h. Excess solvent was then removed using reduced pressure, and the remaining oily residue was dissolved in water (10 mL). The resulting solution was basified with a solution of sodium hydroxide to pH 10 and then loaded on a column of Dowex 1X2-400 anionic exchange resin (8 g). The column was washed with water (300 mL), and then the product was eluted with a 10% formic acid solution (300 mL). The resulting fractions were concentrated to dryness, dissolved in water (3 mL), and passed through a column of Dowex 50WX2-400 cation-exchange resin (5 g). The filtrate was concentrated, and the remaining residue was dissolved in methanol (2 mL) and then precipitated out with the addition of ether (80 mL). The solid was filtered under argon, giving **16b** (93 mg, 75%) as a hygroscopic white solid: ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.21 (s, 1 H), 10.06 (s, 1 H), 6.35 (m, 1 H), 3.68 (m, 1 H), 3.36−3.58 (m, 5 H), 3.22 (m, 1 H), 2.13 (m, 2 H), 1.45 (m, 4 H), 1.28 (m, 4 H); ^{31}P NMR (121 MHz, DMSO-d_6) 27.22; HRFABMS (MH^+) calcd for $C_{14}H_{26}N_3O_9P$ m/z 412.1485, found 412.1485. Anal. Calcd for $C_{14}H_{26}N_3O_9P\cdot HCOOH$: C, 39.39; H, 6.17; N 9.19. Found: C, 39.28; H, 6.04; N, 9.29.

6-(6-D-Ribitylamino-2,4-dihydroxypyrimidin-5-yl)-1-hexylphosphonic Acid (16c). 4-(6-Chloro-2,4-dihydroxypyrimidin-5-yl)-1-hexylphosphonic acid diammonium salt **(15c)**

(110 mg, 0.319 mmol) and D-ribitylamine (0.500 g, 3.31 mmol) were dissolved in 2-methoxyethanol (10 mL) and heated to reflux for 23 h. Excess solvent was then removed using reduced pressure, and the remaining oily residue was dissolved in water (10 mL). The resulting solution was basified with a solution of sodium hydroxide to pH 10 and then loaded on a column of Dowex 1X2-400 anionic exchange resin (10 g). The column was washed with water (300 mL), and then the product was eluted with a 10% formic acid solution (300 mL). The resulting fractions were concentrated to dryness, dissolved in water (3 mL), and passed through a column of Dowex 50WX2-400 cation-exchange resin (5 g). The filtrate was concentrated, and the remaining residue was dissolved in methanol (2 mL) and then precipitated out with the addition of ether (100 mL). The white solid was filtered under argon, giving **16c** (72.5 mg, 53%) as a hygroscopic beige solid: ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.19 (s, 1 H), 10.06 (s, 1 H), 6.30 (m, 1 H), 3.68 (m, 1 H), 3.34−3.59 (m, 5 H), 3.22 (dd, J = 6 Hz, J = 15 Hz, 1 H), 2.13 (m, 2 H), 1.49 (m, 4 H), 1.24−1.30 (m, 6 H); ^{31}P NMR (121 MHz, DMSO-d_6) 27.21; PDMS (MH^+) m/z 426.1. Anal. Calcd for $C_{15}H_{28}N_3O_9P\cdot HCOOH$: C, 40.77; H, 6.41; N 8.91. Found: C, 40.39; H, 6.59; N, 9.00.

4-(6-Chloro-2,4-dimethoxypyrimidin-5-yl)butane-1-sulfonic Acid Sodium Salt (17a). 6-Chloro-5-(4-iodobut-1-yl)-2,4-dimethoxypyrimidine **(13a)** (0.455 g, 1.28 mmol) and sodium sulfite (0.322 g, 2.55 mmol) were dissolved in a mixture of acetone (5 mL) and water (5 mL) and heated at reflux for 24 h. The solvents were removed using reduced pressure, and the resulting white solid was partially dissolved in methanol (30 mL) and filtered. The filtrate was concentrated to give a white solid (0.412 g, 97%): ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.94 (s, 3 H), 3.87 (s, 3 H), 2.54 (m, 2 H), 2.38 (t, J = 9 Hz, 2 H), 1.50 (m, 4 H); Electrospray MS $(M + Na)^+$ m/z 355; HREMS $(M + Na)^+$ calcd for $C_{10}H_{14}ClN_2Na_2O_5S$ m/z 355.0107, found 355.0107.

5-(6-Chloro-2,4-dimethoxypyrimidin-5-yl)pentane-1-sulfonic Acid Sodium Salt (17b). 6-Chloro-5-(5-iodopent-1-yl)-2,4-dimethoxypyrimidine **(13b)** (0.300 g, 0.809 mmol) and sodium sulfite (0.204 g, 1.62 mmol) were dissolved in a solution of acetone (5 mL) and water (5 mL) and heated at reflux for 24 h. The solvents were removed using reduced pressure, and the resulting white solid was partially dissolved in methanol (30 mL) and filtered. The filtrate was then concentrated to give a white solid (0.27 g, 96%): ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.93 (s, 3 H), 3.86 (s, 3 H), 2.5 (m, 2 H), 2.37 (t, J = 9 Hz, 2 H), 1.57 (quintet, J = 9 Hz, 2 H), 1.31−1.41 (m, 4 H); HRFABMS (MH^+) calcd for $C_{11}H_{16}N_2O_5SClNa$ m/z 347.0444, found 347.0443.

6-(6-Chloro-2,4-dimethoxypyrimidin-5-yl)hexane-1-sulfonic Acid Sodium Salt (17c). 6-Chloro-5-(6-iodohex-1-yl)-2,4-dimethoxypyrimidine **(13c)** (0.450 g, 1.17 mmol) and sodium sulfite (0.295 g, 2.34 mmol) were dissolved in a solution of acetone (5 mL) and water (5 mL) and heated at reflux for 24 h. The solvents were removed using reduced pressure, and the resulting white solid was partially dissolved in methanol (30 mL) and filtered. The filtrate was then concentrated to give a white solid (0.42 g, 99%): IR (Nujol) 3411, 1591, 1548, 1456, 1199, 1056, 1030 cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.94 (s, 3 H), 3.86 (s, 3 H), 2.52 (m, 2 H), 2.36 (t, J = 9 Hz, 2 H), 1.51 (quintet, J = 9 Hz, 2 H), 1.41 (quintet, J = 9 Hz, 2 H), 1.28 (m, 4 H); HRFABMS (MH^+) calcd for $C_{12}H_{18}N_2O_5SClNa$ m/z 361.0601, found 361.0600.

4-(6-Chloro-2,4-dihydroxypyrimidin-5-yl)butane-1-sulfonic Acid (18a). 4-(6-Chloro-2,4-dimethoxypyrimidin-5-yl)-butane-1-sulfonic acid sodium salt **(17a)** (0.199 g, 0.598 mmol) was dissolved in a solution of concentrated acetic acid (2.0 mL) and concentrated HCl (2 mL) and heated at reflux for 2.5 h. Excess solvent was removed using reduced pressure to give a yellow oil. Methanol (2 mL) and ether (30 mL) were added, and the resulting beige precipitate was filtered to give the product **18a** (0.111 g, 66%): IR (Nujol) 3368, 1694, 1622, 1456, 1162, 1050 cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.82 (s, 1 H), 11.31 (s, 1 H), 2.37 (t, J = 9 Hz, 2 H), 2.26 (t, J = 9 Hz, 2 H), 1.52 (quintet, J = 9 Hz, 2 H), 1.39 (quintet, J = 9 Hz, 2 H).

5-(6-Chloro-2,4-dihydroxypyrimidin-5-yl)pentane-1-sulfonic Acid (18b). 5-(6-Chloro-2,4-dimethoxypyrimidin-5-yl)pentane-1-sulfonic acid disodium salt **(17b)** (0.304, 0.822 mmol) was dissolved in a solution of concentrated acetic acid (2.0 mL) and concentrated HCl (1.5 mL) and heated at reflux for 2.5 h. Excess solvent was removed using reduced pressure to give a yellow oil. Methanol (2 mL) and ether (30 mL) were added, and the resulting beige precipitate was filtered to give the product **18b** (0.198 g, 81%): ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.80 (s, 1 H), 11.29 (s, 1 H), 2.35 (m, 2 H), 2.26 (t, J = 9 Hz, 2 H), 1.54 (quintet, J = 9 Hz, 2 H), 1.30 (m, 4 H); HRFABMS calcd for $C_9H_{13}N_2O_5Cl$ (MH+) m/z 297.0312, found 297.0310.

6-(6-Chloro-2,4-dihydroxypyrimidin-5-yl)hexane-1-sulfonic Acid (18c). 6-(6-Chloro-2,4-dimethoxypyrimidin-5-yl)-hexane-1-sulfonic acid sodium salt **(17c)** (0.330 g, 0.915 mmol) was dissolved in a solution of concentrated acetic acid (2.0 mL) and concentrated HCl (2 mL) and heated at reflux for 2.5 h. Excess solvent was removed using reduced pressure to give a yellow oil. Methanol (2 mL) and ether (30 mL) were added, and the resulting beige precipitate was filtered to give the product **18c** (0.210 g, 74%): IR (Nujol) 3410, 1738, 1644, 1454, 1174, 1045 cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.79 (s, 1 H), 11.29 (s, 1 H), 2.36 (m, 2 H), 2.27 (t, J = 9 Hz, 2 H), 1.53 (quintet, J = 9 Hz, 2 H), 1.22−1.37 (m, 6 H); FABMS (MH^+) m/z 310.8.

4-(6-D-Ribitylamino-2,4-dihydroxypyrimidin-5-yl)butane-1-sulfonic Acid (19a). 5-(6-Chloro-2,4-dihydroxypyrimidin-5-yl)butane-1-sulfonic acid **(18a)** (0.0976 g, 0.345 mmol) and D-ribitylamine (0.600 g, 3.97 mmol) were dissolved in 2-methoxyethanol (10 mL) and heated at reflux for 20 h. Excess solvent was removed using reduced pressure, and the resulting oil was dissolved in water (10 mL), basified with a solution of sodium hydroxide to a pH of 12, and then loaded on a column of Dowex 1X2-400 anionic exchange resin (12 g). The column was washed first with water (300 mL) and then with a 10% formic acid solution (200 mL). The product was eluted with 2 N HCl (250 mL), and the resulting solution was concentrated using reduced pressure. The white residue was then dissolved in water (3 mL) and passed through a column of Dowex 50WX2-400 cation-exchange resin (7 g). The product was then eluted with water (200 mL), and the resulting fractions were pooled and concentrated under reduced pressure. The remaining product was dissolved in methanol (2 mL) and then precipitated with the addition of ether (80 mL). The precipitate was filtered under argon, giving **19a** (38.4 mg, 28%) as a white solid: ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.23 (s, 1 H), 10.07 (s, 1 H), 6.52 (m, 1 H), 3.49−3.59 (m, 2 H), 3.38−3.42 (m, 3 H), 3.23 (dd, J = 6 Hz, J = 15 Hz, 1 H), 2.46 (m, 2 H), 2.15 (t, J = 9 Hz, 2 H), 1.57 (quintet, J = 9 Hz, 2 H), 1.33 (quintet, J = 9 Hz, 2 H), HRFABMS calcd for $C_{13}H_{23}N_3O_9S$ (MH^+) m/z 398.1233, found 398.1231. Anal. Calcd for $C_{13}H_{23}N_3O_9S\cdot 1.2H_2O$: C, 39.01; H, 5.94; N 9.75. Found: C, 38.91; H, 5.84; N, 9.95.

5-(6-D-Ribitylamino-2,4-dihydroxypyrimidin-5-yl)pentane-1-sulfonic Acid (19b). 5-(6-Chloro-2,4-dihydroxypyrimidin-5-yl)pentane-1-sulfonic acid **(18b)** (0.0987 g, 0.333 mmol) and D-ribitylamine (0.500 g, 3.31 mmol) were dissolved in 2-methoxyethanol (10 mL) and heated at reflux for 23 h. Excess solvent was removed using reduced pressure, and the resulting oil was dissolved in water (10 mL), basified with a solution of sodium hydroxide to pH 12, and then loaded on a column of Dowex 1X2-400 anionic exchange resin (10 g). The column was washed first with water (300 mL) and then with a 10% formic acid solution (200 mL). The product was eluted with 2 N HCl (250 mL), and the resulting solution was concentrated using reduced pressure. The residue remaining was dissolved in methanol (2 mL) and precipitated out with the addition of ether (80 mL). The precipitate was filtered under argon, giving **19b** (37.1 mg, 27%) as a white solid: ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.22 (s, 1 H), 10.06 (s, 1 H), 6.35 (m, 1 H), 3.82 (s, 4 H), 3.69 (m, 1 H), 3.59−3.47 (m, 2 H), 3.42−3.36 (m, 3 H), 3.24 (dd, J = 7 Hz, J = 14 Hz, 1 H), 2.42 (t, J = 9 Hz, 2 H), 2.12 (t, J = 9 Hz, 2 H), 1.56 (quartet, J = 9 Hz, 2 H), 1.27 (m, 4 H); PDMS (MH^+) m/z 411.9. Anal. Calcd

for $C_{14}H_{25}N_3O_9S$: C, 40.87; H, 6.12; N 10.21. Found: C, 40.54; H, 6.35; N, 9.86.

5-(6-D-Ribitylamino-2,4-dihydroxypyrimidin-5-yl)hexane-1-sulfonic Acid (19c). 6-(6-Chloro-2,4-dihydroxypyrimidin-5-yl)hexane-1-sulfonic acid **(18c)** (0.112 g, 0.360 mmol) and D-ribitylamine (0.500 g, 3.31 mmol) were dissolved in 2-methoxyethanol (12 mL) and heated at reflux for 23 h. Excess solvent was removed using reduced pressure, and the resulting oil was dissolved in water (10 mL), basified with a solution of sodium hydroxide to pH 12, and then loaded on a column of Dowex 1X2-400 anionic exchange resin (12 g). The column was washed first with water (300 mL), and then with a 10% formic acid solution (200 mL). The product was eluted with 2 N HCl (250 mL), and the resulting solution was concentrated using reduced pressure. The residue remaining was dissolved in methanol (2 mL) and then precipitated with the addition of ether (80 mL). The precipitate was then filtered under argon, giving **19c** (47.5 mg, 31%) as a white solid: ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.22 (s, 1 H), 10.07 (s, 1 H), 6.34 (m, 1 H), 4.39 (s, 4 H), 3.69 (m, 1 H), 3.36−3.59 (m, 5 H), 3.24 (dd, J = 7 Hz, J = 14 Hz, 1 H), 2.42 (t, J = 9 Hz, 2 H), 2.13 (m, 2 H), 1.54 (quartet, J = 9 Hz, 2 H), 1.24 (m, 6 H); PDMS (MH^+) m/z 426. Anal. Calcd for $C_{15}H_{27}N_3O_9S\cdot HCOOH$: C, 40.76; H, 6.20; N 8.91. Found: C, 41.00; H, 6.49; N, 8.98.

Lumazine Synthase Assay.[18] 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 using the program DynaFit.[15] 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.[19] Reaction mixtures contained buffer (100 mM potassium phosphate, 10 mM EDTA, 10 mM sodium sulfite), inhibitor (0 to 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 descibed above.

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