Synthesis and Biochemical Evaluation of Bis(6,7-dimethyl-8-D-ribityllumazines) as Potential Bisubstrate Analogue Inhibitors of Riboflavin Synthase

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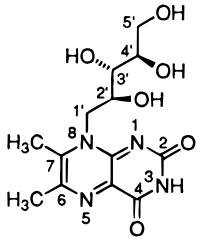
The reaction catalyzed by riboflavin synthase utilizes two identical 6,7-dimethyl-8-D-ribityllumazine substrate molecules. Three bis(6,7-dimethyl-8-D-ribityllumazines) were, therefore, synthesized in which the two lumazine moieties were connected through their N-3 nitrogen atoms by polymethylene linker chains containing three, four, and five carbon atoms. The compounds with three and five carbon linkers were found to be very weak inhibitors of riboflavin synthase, having inhibition constants of 320 and >1000 μM, respectively. In contrast, the bis(lumazine) with a four-carbon linker was much more potent, with an inhibition constant of 37 μM. These results have potential implications for understanding the distance between the donor and acceptor sites of riboflavin synthase and the orientations of the two 6,7-dimethyl-8-D-ribityllumazine substrate molecules which occupy these two sites.

Introduction

Riboflavin synthase (E.C. 2.5.1.9) catalyzes an unusual dismutation reaction, involving the transfer of a four-carbon unit from one molecule of 6,7-dimethyl-8-(D-ribityl)lumazine (**1**) bound at the donor site of the enzyme



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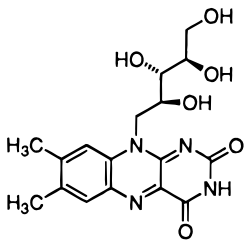

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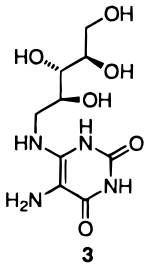

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to a second molecule of **1** bound at the acceptor site of the enzyme to form one molecule of riboflavin (**2**) and one molecule of the pyrimidinedione **3**.[1−3] A working hypothesis concerning the reaction mechanism has been advanced as shown in Scheme 1.[4−6] Addition of an unidentified nucleophile to **1** yields **4** at the donor site, which then undergoes nucleophilic attack by an anion **5**, formed by deprotonation of the 7-methyl group of lumazine **1** bound at the acceptor site. A 1,2-elimination in **6** followed by a 1,6-elimination in **7** yields a conjugated triene system in **8**, which undergoes a 3,3-sigmatropic rearrangement to afford intermediate **9**. Intermediate **9** then aromatizes by 1,2-elimination to yield the final products, riboflavin (**2**) and the pyrimidinedione **3**. Although the overall transformation is mechanistically complex, Wood and co-workers were able to demonstrate that riboflavin (**2**) forms in the absence of enzyme when the lumazine **1** was boiled in phosphate buffer at neutral pH.[7,8] Beach and Plaut showed that the nonenzymatic reaction also occurs under acidic conditions.[9] Both the catalyzed and uncatalyzed reaction are characterized by strict regiospecificity.

The participation of two identical lumazine molecules **1** as enzyme substrates for riboflavin synthase suggests the possibility of bisubstrate inhibitors in which two lumazine moieties are connected by a linker chain. Two critical variables in this strategy for inhibitor design are the points of attachment of the linker chain to the two lumazine moieties and the length of the linker chain. In order for the mechanism proposed in Scheme 1 to operate, the anion **5** generated by deprotonation of the methyl group at C-7 on one lumazine molecule at the acceptor site must attack the C-6 carbon atom of another lumazine

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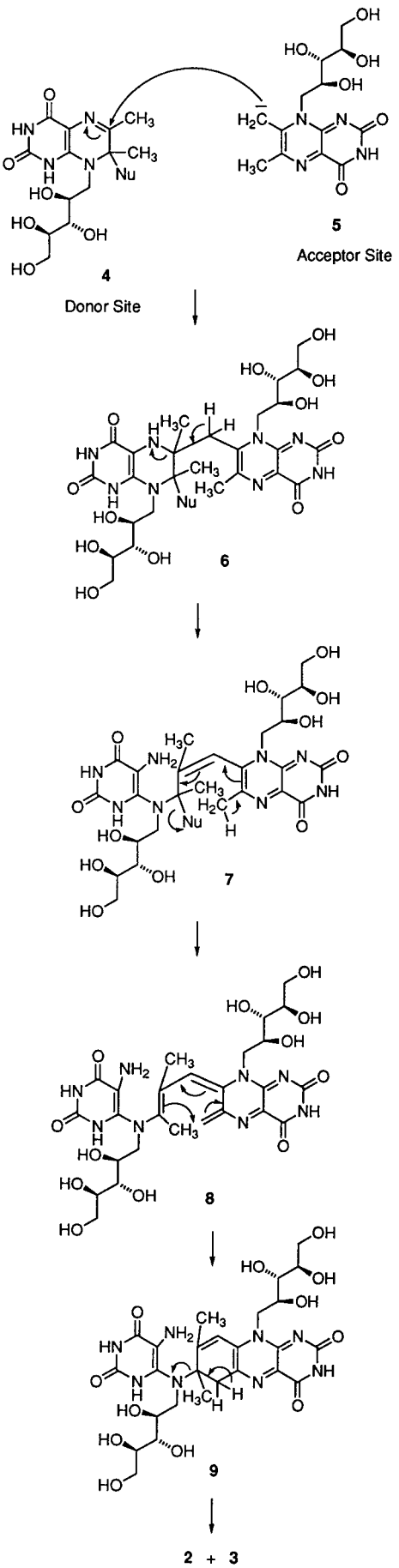
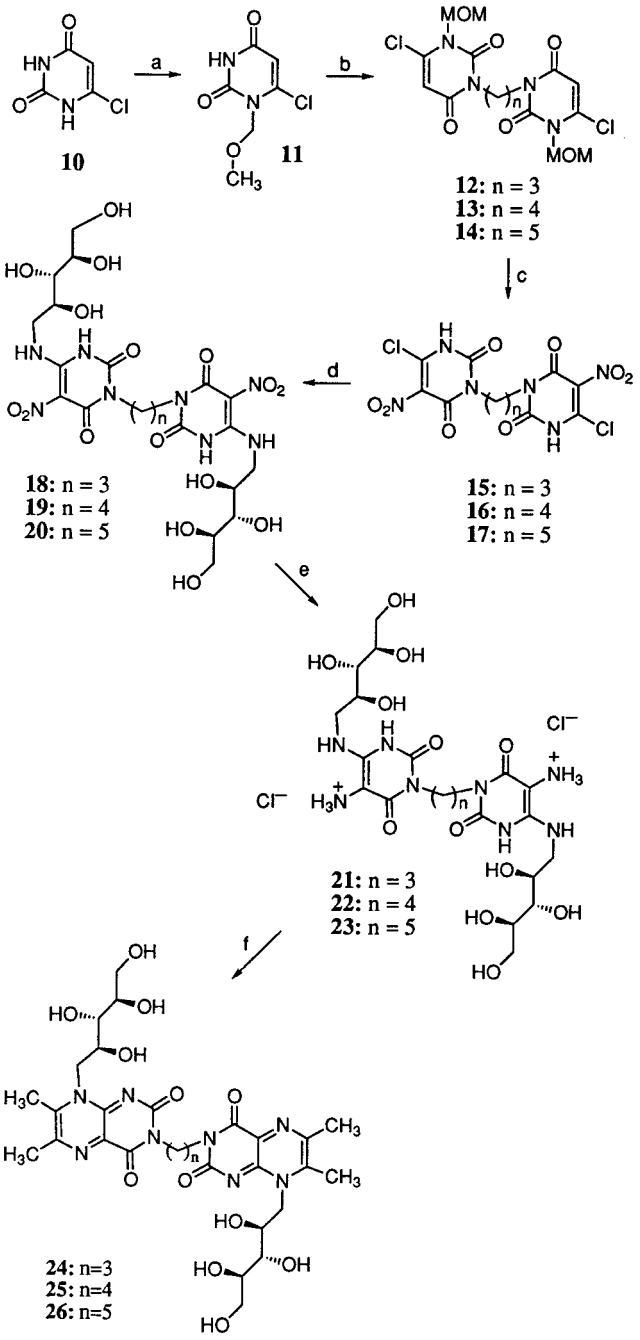
Scheme 1



Scheme 2[a]



[a] Reagents and conditions: (a) (1) LiH, DMF, 0 °C (5 min), (2) $ClCH_2OCH_3$, 0 °C (30 min); (b) (1) LiH, DMF, 0 °C (5 min), (2) dibromoalkane, 23 °C (1.5 h); (c) HNO_3, H_2SO_4, 0 °C (15 min), 23 °C (15 min); (d) D-Ribitylamine, 50% aq EtOH, 23 °C (16 h); (e) H_2, Pd/C, MeOH, HCl, 23 °C (4 h); (f) $CH_3COCOCH_3$, 23 °C (18 h).

molecule bound at the donor site. This might possibly occur, for example, in a stacked arrangement of the two lumazine systems with the ribityl groups pointing in opposite directions as suggested previously.[10] Linker chains of appropriate length to allow stacking of the two lumazine moieties were, therefore, considered. In addition, the attachment of the linker chain to N-3 of the two lumazines could be accomplished with a minimum disturbance to the lumazine systems, and this was also

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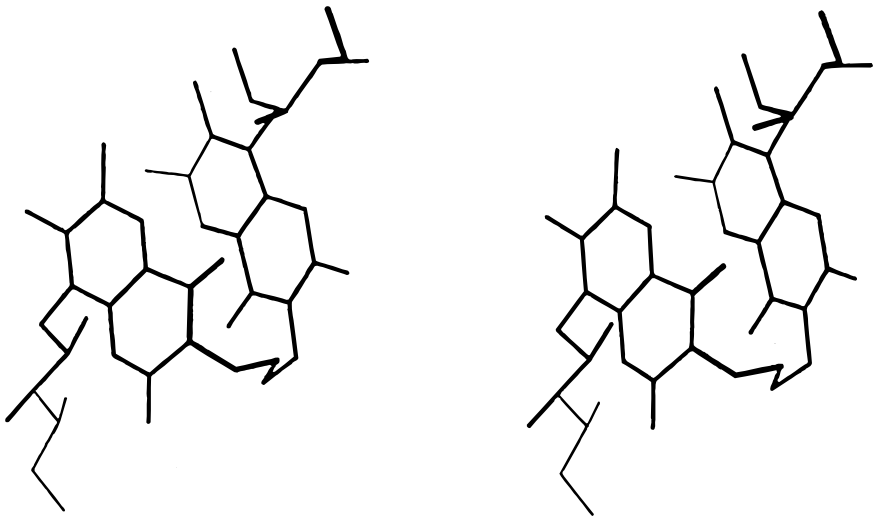


Figure 1. Molecular model of bis(lumazine) **25**, programmed for walleyed viewing. The model was generated using the Sculpt 2.5 program (Interactive Simulations, Inc.) by placing the structure in a $\pi-\pi$ stacked conformation and then minimizing the energy.

Table 1. Inhibitory Activities of Lumazines and Uracils against Light Riboflavin Synthase and Lumazine Synthase

	K_i (μM)	
compound	riboflavin synthase[a]	lumazine synthase[b]
18	280	5000
19	3000	3700
20	670	1500
24	320 ± 470 (K_i, partial inhibition) 300 ± 30 (K_{is})[c]	840 ± 490 (K_i, partial inhibition) 2200 ± 660 (K_{is})
25	37 ± 30 (K_i, mixed inhibition)[d] 57 ± 12 (K_{is})[c]	>1000
26	>1000	>1000
27	120	
28 (epimer A)	120 (pH 7.4)	
28 (epimer A)	38 (pH 6.8)	
29	55	
30	75	
31	58	
32	70	
33	17	
34	17	
35	15	
36	20	
37	540	470
38	390	330
39		200
40		360
41		430
42a	>1000	440 ± 220 (K_i, mixed inhibition) 640 ± 300 (K_{is})
42b	>1000	180 ± 88 (K_i, mixed inhibition) 350 ± 22 (K_{is})
42c	>1000	130 ± 33 (K_i, mixed inhibition) 140 ± 15 (K_{is})
43a	>1000	290 ± 120 (K_i, competitive inhibition)
43b	>1000	690 ± 290 (K_i, mixed inhibition) 1500 ± 640 (K_{is})
43c	>1000	290 ± 130 (K_i, mixed inhibition) 580 ± 170 (K_{is})
2	100	–

[a] Recombinant β_{60} capsids from *Bacillus subtilis*. [b] Recombinant riboflavin synthase from *E. coli*. [c] K_{is} is the equilibrium constant for the reaction EI + S ⇌ EIS. [d] Strictly speaking, the data observed for bis(lumazine) **25** cannot be interpreted in terms of any simple inhibition type, but it is most consistent with mixed type inhibition.

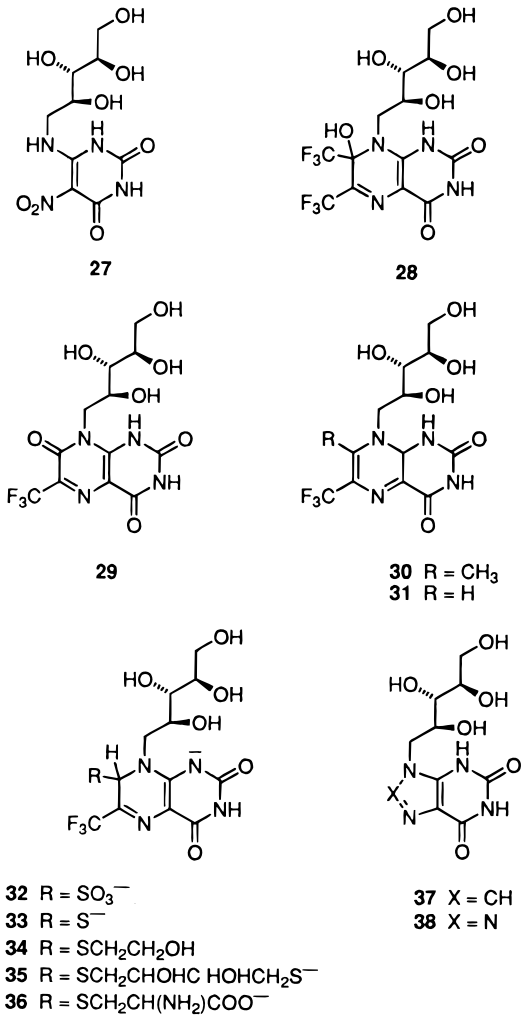
considered to be expedient from the synthetic point of view. The three bis(lumazines) **24**, **25**, and **26** (Scheme 2) were eventually adopted as targets for synthesis. Molecular modeling of the bis(lumazine) **25** having a C-4 linker (Figure 1) indicated that the desired stacking interaction of the two lumazines could indeed occur in this system. An examination of the inhibitory activities of the bis(lumazines) **24**, **25**, and **26** could possibly provide some insight into the distances between the donor and acceptor sites of the enzyme as well as the relative orientation of the lumazines during the enzymatic reaction.

Results and Discussion

The approach that was successfully executed for the syntheses of **24**−**26** took advantage of the differences in the acidities of the 1- and 3-position protons of 6-chlorouracil (**10**). Regioselective alkylation at the N-1 and N-3 of 6-chlorouracil has been reported using LiH.[11] The regiochemistry of such alkylations has been confirmed by X-ray analysis.[12] Hence, starting with 6-chlorouracil (**10**), sequential alkylation at N-1 and then at N-3, using LiH in DMF with MOM-Cl and 1,3-dibromopropane, 1,4-dibromobutane, and 1,5-dibromopentane, provided the protected bis(uracil) derivatives **12**−**14**, respectively. Alkylation of the N-1 anion with chloromethyl methyl ether proceeded at 0 °C in 0.5 h to afford compound **11** in 99% yield. Alkylation with the dibromoalkanes, however, required a higher temperature (rt) and longer reaction time (1.5 h), producing **12**−**14** in 34−46% yield. No product was obtained when 1,2-dibromoethane was used or when the reaction conditions were varied, such as by substituting CH_3CN for DMF for easier workup and utilization of NaH instead of LiH.

Nitration of **12**−**14** with a fuming nitric acid/sulfuric acid mixture also resulted in the deprotection of the MOM groups, affording **15**−**17** in 60−95% yield. These intermediates were reacted with an excess of D-ribitylamine[13] in 50% ethanol at room temperature to afford the corresponding 5-nitro-6-D-ribitylaminouracil compounds **18**−**20**. Reduction of the two nitro groups of compounds **18**−**20** in the presence of Pd−C and HCl at 30 psi afforded the amine hydrochlorides **21**−**23**. The amine hydrochlorides **22** and **23** were initially isolated and characterized by ^{1}H NMR, which indicated the presence of the exchangeable (D_2O) N-5 protons and a 2 ppm upfield shift of the ribityl NH protons (δ 8.04 and 7.75, respectively) with respect to those of compounds **19** and **20** (δ 10.18 and 10.17, respectively). In subsequent reactions, however, the amine hydrochlorides **21**−**23** were not isolated but were instead directly condensed with 2,3-butanedione in situ to afford the bis(lumazines) **24**−**26** in a one-pot reaction from **18**−**20**. The condensation reactions, workups, and purifications of the light-sensitive compounds **24**−**26** were performed in the dark or in very dim light. The purifications of the bis(lumazines) **24**−**26** were challenging and were ultimately achieved using an acid-washed activated alumina column. The yields of the products after purification were low (9−10%). The yield during the conversion of **19** to **25** could be increased to 28%, however, by carrying out the hydrogenation and condensation reactions in distilled water instead of methanolic HCl.[14] The ^{1}H NMR spectrum (DMSO-d_6) of compounds **24**−**26** indicated the presence of the 6α and 7α methyl protons as singlets at δ 2.50−2.54 and δ 2.71−2.72, respectively. As expected, the six 7α methyl protons (δ 2.71−2.77) exchanged upon standing in CD_3OD for 24 h at 4 °C.[5]

The bis(nitrouracils) **18**−**20** and bis(lumazines) **24**−**26** were investigated as inhibitors of riboflavin synthase and lumazine synthase, and the results are listed in Table 1. For comparison, the K_i of riboflavin (**2**) (100 μM) is also listed in Table 1.[15] It was of interest to investigate the bis(nitrouracils) **18**−**20** as inhibitors of riboflavin synthase because the corresponding monomer **27** is an analogue of the riboflavin synthase product **3**, and the dissociation constant K_d of **27** was previously determined to be 650 nM when tested on the 6,7-dimethyl-8-ribityllumazine synthase of *Bacillus subtilis*.[16] The substrate analogue **27** has also been investigated as an inhibitor of recombinant riboflavin synthase from *Escherichia coli*, resulting in an inhibition constant K_i = 120 μM.[17] Although bis(nitrouracil) **18**, having a three-carbon linker chain, was the most potent (K_i = 280 μM) of the three bis(nitrouracils), it is still significantly less potent than the monomer **27** itself. This seems reasonable since after completion of the reaction the product **3** is bound to only the donor site and not the acceptor site of the enzyme, so a bis(product) analogue based on the structure of **3** would not be expected to be particularly successful as an enzyme inhibitor.



Significant differences were found in the potencies of the three bis(lumazines) **24**−**26** as riboflavin synthase inhibitors. The most potent of the three proved to be the compound **25** (K_i = 37 μM) with a C-4 linker chain, followed by inhibitor **24** (K_i = 320 μM), with a C-3 linker, and **26** (K_i > 1000 μM), with a C-5 linker. Obviously, the

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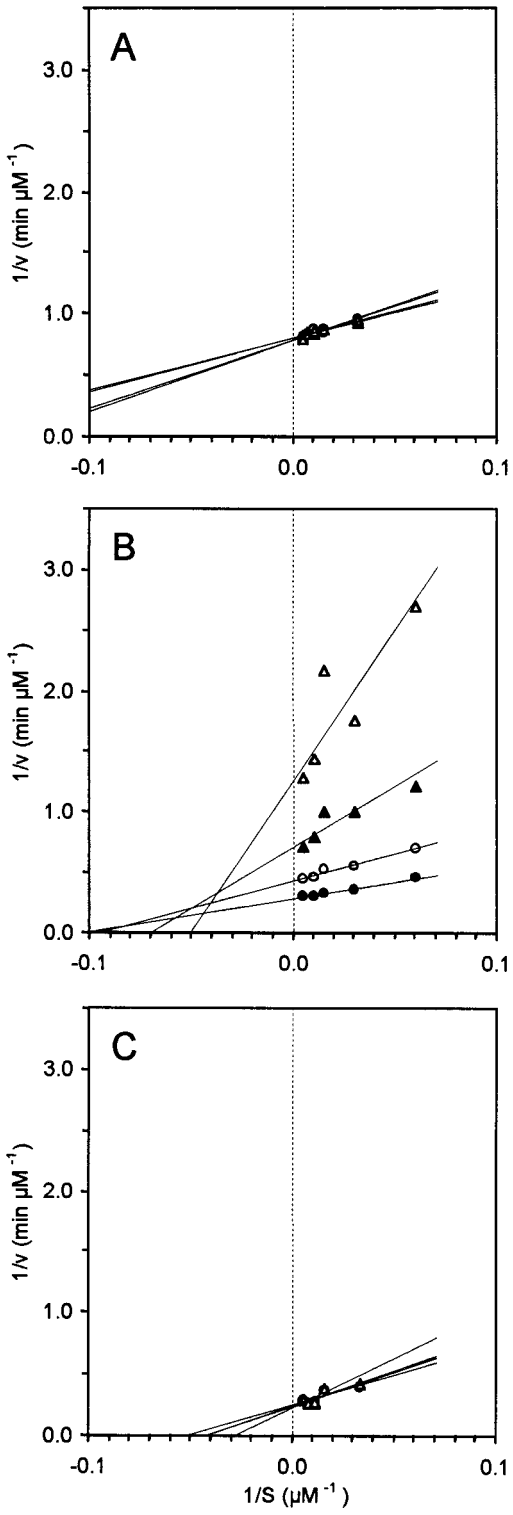
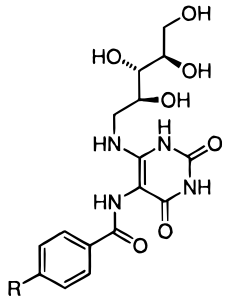


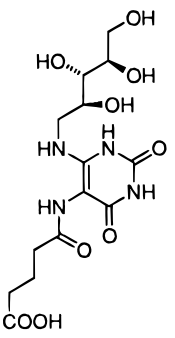
Figure 2. Inhibition of riboflavin synthase from *E. coli* by the bis(lumazines): A, **24**; B, **25**; C, **26**. The concentration of the substrate, 6,7-D-ribityllumazine (**1**), ranged from 30 μM to 190 μM. Inhibitor concentrations: ●, 0 μM; ○, 50 μM; ▲, 150 μM; △, 250 μM. The initial velocity of riboflavin formation, *v*, was determined at steady-state conditions. The kinetic data were fitted with a nonlinear regression method using the program Dynafit from P. Kuzmic.[29] In the case of compound **25** (B), each velocity curve was fitted separately according to the mechanism of Michaelis and Menten. Lineweaver−Burk plots were obtained from the best fits.

length of the linker chain plays a critical role in the effectiveness of the bis(lumazines) as riboflavin synthase inhibitors. The significantly greater potency of **25** relative to the other two compounds **24** and **26** suggests that it may in fact be functioning as a bisubstrate inhibitor. On the other hand, the potency of **25** relative to **33**−**36** argues against **25** acting as a bisubstrate inhibitor, since one would normally expect a bisubstrate inhibition to be more potent. In addition, the kinetic data for **25** was complicated and did not fit any simple type of inhibition pattern, although it was most consistent with mixed type inhibition. As shown in Figure 2, the Lineweaver−Burk lines did not intersect at one point, and the secondary plot (not shown) was nonlinear. The mechanism of the reaction catalyzed by riboflavin synthase is complex (Scheme 1) and involves two different binding sites for the substrate and perhaps also for the inhibitor. It is also possible that the reaction could involve two molecules of the substrate **1** bound to different subunits of the trimeric enzyme, or, alternatively, that they could be bound to the same subunit during the catalytic event. When tested as a possible substrate for riboflavin synthase, no evidence was observed for the transfer of a four-carbon unit from one lumazine fragment of **25** to the other one, which would have resulted in the formation of riboflavin and ribitylpyrimidine moieties.

The bis(nitrouracils) **18**, **19**, and **20** as well as the bis(lumazines) **24**, **25**, and **26** were also investigated as inhibitors of recombinant lumazine synthase β_{60} capsids from *E. coli*, and the results are listed in Table 1. The three bis(nitrouracils) **18**, **19**, and **20** were all very weak inhibitors of the enzyme, ranging from a K_i of 1500 μM for **20** to a K_i of 5000 μM for **18**. Likewise, the three bis(lumazines) **24**, **25**, and **26** proved to be very weak or inactive as inhibitors of lumazine synthase. They are all less active than the previously published lumazine synthase inhibitors, including the ribitylpurine **37** (K_i = 470 μM), the ribitylazapurine **38** (K_i = 330 μM), and the three ribityluracils **39** (K_i = 200 μM), **40** (K_i = 360 μM), and



39 R = SO_2F
40 R = SO_3Na



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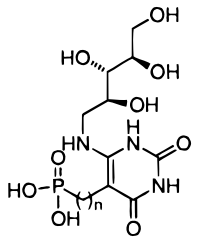
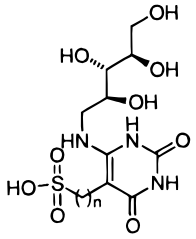


42a n = 4
42b n = 5
42c n = 6



43a n = 4
43b n = 5
43c n = 6

41 (K_i = 430 μM).[17,18] They are also less active than the three phosphonates **42a**−**c** and the three sulfonates **43a**−**c**.[19] This seems reasonable since the active site of lumazine synthase is not large enough to accommodate a bis(lumazine).[20,21]

Prior work has demonstrated that the monomeric lumazine derivatives **28**−**36** are also capable of producing significant inhibition of riboflavin synthase (Table 1).[22−24] The present investigation suggests that the incorporation of these lumazines into bisubstrate analogues of riboflavin synthase could be of interest.

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 an internal standard: s = singlet, d = doublet, m = multiplet, bs = broad singlet. The relative integrals of peak areas agreed with those expected for the assigned structures. The mass spectrum was determined on a plasma desorption mass spectrometer (PDMS), which utilizes 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.[25] The compounds were applied to a nitrocellulose-coated Mylar target and allowed to dry prior to being put into the mass spectrometer. The acceleration potential was set at 17 kV, with data being collected for 15 min. Proportions of solvents used for TLC are by volume. Analytical samples were dried in vacuo (0.2 mmHg) in an Abderhalden drying apparatus over P_2O_5 and refluxing acetone. Elemental analyses were performed by the Purdue Microanalytical Laboratory. Fractional moles of water and solvents frequently found in some analytical samples were not removed despite 24−48 h of drying in vacuo and were confirmed by their presence in the ^{1}H NMR spectrum. All solvents and chemicals were purchased from Aldrich Chemical Co. and were used as received.

1-Methoxymethyl-6-chlorouracil (11). A mixture of 6-chlorouracil (**10**) (4.00 g, 27.39 mmol) and LiH (0.33 g, 41.08 mmol) in DMF (100 mL) was stirred at 0 °C for 5 min under an atmosphere of argon, and then chloromethyl methyl ether (2.4 mL, 31.60 mmol) was added. The mixture was stirred for 0.5 h at 0 °C and then treated with water (200 mL). The pH of the solution was adjusted to 4 with 2 N HCl, and this aqueous solution was extracted with CH_2Cl_2 (3 × 100 mL). The organic layer was dried ($MgSO_4$) and evaporated under reduced pressure. The residue was treated with hexanes, and the white precipitate obtained was filtered and dried to yield **11** (5.15 g, 98.8%): mp 126−127 °C; TLC R_f 0.79 ($CHCl_3$/MeOH 15:2); ^{1}H NMR (DMSO-d_6) δ 3.31 (s, 3 H), 5.27 (s, 2 H), 6.09 (s, 1 H), 11.68 (s, 1 H).

1,3-Bis[3-(1-methoxymethyl-6-chlorouracilyl)]propane (12). A mixture of **11** (1.0 g, 5.26 mmol) and LiH (0.063 g, 7.89 mmol) in DMF (10 mL) was stirred at 0 °C for 5 min under an atmosphere of argon, and then 1,3-dibromopropane (0.531 g, 2.63 mmol) was added. The mixture was stirred for 1.5 h at room temperature and then treated with water (100 mL). The white precipitate was filtered, washed with water, and dried to afford **12** (0.41 g, 46%): mp 147−148 °C; TLC R_f 0.86 ($CHCl_3$/MeOH 15:1); ^{1}H NMR (DMSO-d_6) δ 1.82 (m, 2 H), 3.31 (s, 6 H), 3.80 (t, 4 H), 5.33 (s, 4 H), 6.16 (s, 2 H).

1,4-Bis[3-(1-methoxymethyl-6-chlorouracilyl)]butane (13). A mixture of **11** (1.00 g, 5.26 mmol) and LiH (0.063 g, 7.89 mmol) in DMF (25 mL) was stirred at 0 °C for 5 min under an atmosphere of argon, and then 1,4-dibromobutane (0.568, 2.63 mmol) was added. The mixture was stirred for 1.5 h at room temperature and then treated with water (100 mL). The white precipitate was filtered, washed with water, and dried to afford **13** (0.43 g, 34%): mp 138−139 °C; TLC R_f 0.95 ($CHCl_3$/MeOH 15:2); ^{1}H NMR (DMSO-d_6) δ 1.49 (m, 4 H), 3.29 (s, 6 H), 3.76 (m, 4 H), 5.31 (s, 4 H), 6.05 (s, 2 H); CI m/z 435 (MH^+). Anal. Calcd for ($C_{16}H_{20}N_4O_6Cl_2$) C, H, N, Cl. Calcd: C, 44.15; H, 4.63; N, 12.87; Cl, 16.29. Found C, 43.95; H, 4.68; N, 12.74; Cl, 16.07.

1,5-Bis-[3-(1-methoxymethyl-6-chlorouracilyl)]pentane (14). A mixture of **11** (1.5 g, 7.89 mmol) and LiH (0.076 g, 9.48 mmol) in DMF (25 mL) was stirred at 0 °C for 5 min under an atmosphere of argon, and then 1,5-dibromopentane (0.853 g, 3.95 mmol) was added. The mixture was stirred for 1.5 h at room temperature and then treated with water (15 mL). The white precipitate was filtered, washed with water, and dried to afford **14** (0.72 g, 41%): mp 110−115 °C; TLC R_f 0.95 ($CHCl_3$/MeOH 15:2); ^{1}H NMR (DMSO-d_6) δ 1.19 (m, 2 H), 1.54 (m, 4 H), 3.33 (s, 6 H), 3.77 (t, 4 H), 5.33 (s, 4 H), 6.14 (s, 2 H).

1,3-Bis[3-(6-chloro-5-nitrouracilyl)]propane (15). Fuming HNO_3 (1.5 mL) was added to concentrated H_2SO_4 (3 mL) at 0 °C, and the nitrating mixture was stirred for 5 min. Compound **12** (0.6 g, 1.40 mmol) was added to the nitrating mixture, which was stirred for 15 min at 0 °C and then at room temperature for another 15 min. Ice was added to the reaction mixture, and the resulting suspension was filtered, washed with cold water, and dried to yield **15** (0.54 g, 91%): ^{1}H NMR (DMSO-d_6) δ 1.63 (m, 1 H), 1.73 (m, 1 H), 3.64 (m, 4 H), 9.97 (bs, 2 H); FAB (negative ion) m/z 421 ($M - 1$)$^-$.

1,4-Bis[3-(6-chloro-5-nitrouracilyl)]butane (16). Fuming HNO_3 (0.5 mL) was added to concentrated H_2SO_4 (1 mL) at 0 °C, and the nitrating mixture was stirred for 5 min. Compound **13** (0.2 g, 0.46 mmol) was added to the nitrating mixture, which was stirred for 15 min at 0 °C and then at room temperature for another 15 min. Ice was added to the reaction mixture, and the resulting suspension was filtered, washed with cold water, and dried to yield **16** (0.12 g, 60%): ^{1}H NMR (DMSO-d_6) δ 1.43 (m, 4 H), 3.69 (m, 4 H), 9.87 (bs, 2 H); FAB (negative ion) m/z 435 ($M - 1$)$^-$.

1,5-Bis[3-(6-chloro-5-nitrouracilyl)]pentane (17). Fuming HNO_3 (1.0 mL) was added to concentrated H_2SO_4 (2 mL) at 0 °C, and the nitrating mixture was stirred for 5 min. Compound **14** (0.37 g, 0.81 mmol) was added to the nitrating mixture, which was stirred for 15 min at 0 °C and then at room temperature for another 15 min. Ice was added to the reaction mixture, and the resulting suspension was filtered, washed with cold water, and dried to yield **17** (0.35 g, 95%): ^{1}H NMR (DMSO-d_6) δ 1.23 (m, 2 H), 1.45 (m, 4 H), 3.62 (m, 2 H), 3.73 (m, 2 H), 9.95 (bs, 2 H); FAB (negative ion) m/z 449 ($M - 1$)$^-$.

1,3-Bis[3-(5-nitro-6-D-ribitylaminouracilyl)]propane (18). A mixture of **15** (0.5 g, 1.18 mmol) and D-ribitylamine[13] (1.00 g, 6.62 mmol) in 50% ethanol (10 mL) was stirred at room temperature for 16 h. The ethanol was evaporated, and the solution was basified with 1 N NaOH (pH 9). This was loaded onto an anion-exchange column (2.5 cm × 58.4 cm, Dowex-1 × 2−400, 10 g) and eluted, sequentially, with water (200 mL) and 1% HCOOH. Fractions corresponding to the product (TLC) were pooled, concentrated, and loaded onto a cation-exchange column (2.5 cm × 58.4 cm, Dowex-50W × 2−400, 10 g) and eluted with water (200 mL). The product fractions were pooled and lyophilized to yield **18** (0.50 g, 65%): ^{1}H NMR (DMSO-d_6) δ 1.76 (m, 2 H), 3.43−3.72 (m, 14 H), 3.86 (m, 4 H), 4.3−5.43 (bm, 8 H), 10.18 (s, 2 H), 11.25 (bs, 2 H); FAB (negative ion) m/z 651 ($M - 1$)$^-$. Anal. Calcd for ($C_{21}H_{32}N_8O_{16}$·0.5

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HCOOH): C, 38.23; H, 4.92; N, 16.59. Found: C, 38.23; H, 5.10; N, 16.35.

1,4-Bis[3-(5-nitro-6-D-ribitylaminouracilyl)]butane (19). A mixture of **16** (0.92 g, 2.11 mmol) and D-ribitylamine[13] (1.00 g, 6.62 mmol) in 50% ethanol (25 mL) was stirred at room temperature for 16 h. The ethanol was evaporated, and the solution was basified with 1 N NaOH (pH 9). This was loaded onto an anion-exchange column (2.5 cm × 58.4 cm, Dowex-1 × 2−400, 10 g) and eluted, sequentially, with water (200 mL) and 1% HCOOH. Fractions corresponding to the product (TLC) were pooled, concentrated, and loaded onto a cation-exchange column (2.5 cm × 58.4 cm, Dowex-50W × 2−400, 10 g) and eluted with water (200 mL). The product fractions were pooled and lyophilized to yield of **19** (1.10 g, 78%): ^{1}H NMR (DMSO-d_6) δ 1.47 (m, 4 H), 3.37, 3.54, 3.70, 3.86 (m, 18 H), 4.0−5.0 (bm, 8 H), 10.18 (s, 2 H), 11.35 (bs, 2 H); FAB (negative ion) m/z 665 (M − 1)$^-$. Anal. Calcd for ($C_{22}H_{34}N_8O_{16}$·1.0 HCOOH): C, 38.77; H, 5.09; N, 15.72. Found: C, 38.60; H, 5.17; N, 15.45.

1,5-Bis[3-(5-nitro-6-D-ribitylaminouracilyl)]pentane (20). A mixture of **17** (0.30 g, 0.66 mmol) and D-ribitylamine[13] (0.6 g, 3.96 mmol) in 50% ethanol (10 mL) was stirred at room temperature for 16 h. The ethanol was evaporated, and the solution was basified with 1 N NaOH (pH 9). This was loaded onto an anion-exchange column (2.5 cm × 58.4 cm, Dowex-1 × 2−400, 10 g) and eluted, sequentially, with water (200 mL) and 1% HCOOH. Fractions corresponding to the product (TLC) were pooled, concentrated, and loaded onto a cation-exchange column (2.5 cm × 58.4 cm, Dowex-50W × 2−400, 10 g) and eluted with water (200 mL). The product fractions were pooled and lyophilized to yield **20** (0.31 g, 69%): ^{1}H NMR (DMSO-d_6) δ 1.27 (m, 2 H), 1.52 (m, 4 H), 3.37−3.72 (m, 14 H), 3.87 (m, 4 H), 4.0−5.0 (bm, 8 H), 10.17 (s, 2 H), 11.33 (bs, 2 H); FAB (negative ion) m/z 679 (M − 1)$^-$. Anal. Calcd for ($C_{23}H_{36}N_8O_{16}$·0.7 HCOOH): C, 39.94; H, 5.29; N, 15.72. Found: C, 40.24; H, 5.48; N, 15.42.

1,3-Bis[3-(6,7-dimethyl-8-D-ribityllumazinyl)]propane (24). A solution of **18** (0.45 g, 0.69 mmol) in MeOH (25 mL) containing 6 N HCl (5 mL) was hydrogenated at atmospheric pressure in the presence of 10% Pd−C (0.04 g) for 4 h to afford a solution of the hydrochloride intermediate **21**. The hydrogen was then replaced with argon, and 2,3-butanedione (1 mL) was added to the mixture, which was allowed to stir overnight (18 h) in the dark. The catalyst was filtered through a Celite pad, and the filtrate was evaporated to dryness (bath temperature = 40 °C). The residue was dissolved in 80% ethanol and loaded onto a column of Brockmann I acid-washed activated alumina (50 g, 2.5 cm × 58.4 cm, prepared in absolute ethanol). The column was eluted with 70% EtOH, and the eluate was evaporated to dryness under reduced pressure. The residue was dissolved in water (20 mL) and extracted with water-saturated benzyl alcohol (8 × 25 mL). The fluorescent yellow benzyl alcohol layers were pooled and filtered through a Whatman #2 filter paper. The filtrate was diluted with ether (300 mL) and extracted with water (4 × 200 mL) until the ether layer was colorless. The aqueous fractions were pooled, washed with ether (25 mL) to remove any residual benzyl alcohol, and evaporated to dryness. The residue was suspended in ethanol (5 mL) and filtered. The residue was washed with ethanol (5 mL) and ether (20 mL) and dried to yield the product of **24** (0.05 g, 10%) as a bright yellow solid: ^{1}H NMR (DMSO-d_6) δ 2.02 (m, 2 H), 2.54 (s, 6 H), 2.72 (s, 6 H), 3.98 (bs, 4 H), 3.92 (m, 5 H), 4.23 (bs, 2 H), 4.48 (bm, 2 H), 4.77 (bs, 2 H), 5.14 (bs, 2 H), 5.60 (bm, 1 H); ^{13}C NMR (DMSO-d_6) δ 18.06, 21.86, 23.37, ∼42 (obscured by solvent peak), 50.66, 63.27, 68.41, 72.50, 73.75, 130.64, 140.27, 148.89, 149.17, 154.35, 160.17.

1,4-Bis[3-(6,7-dimethyl-8-D-ribityllumazinyl)]butane (25). Procedure A. A solution of **19** (0.86 g, 1.29 mmol) in MeOH (30 mL) containing 6 N HCl (5 mL) was hydrogenated at atmospheric pressure in the presence of 10% Pd−C (0.08 g) for 4 h to afford a solution of the hydrochloride intermediate **22**. The hydrogen was then replaced with argon, and 2,3-butanedione (1.5 mL) was added to the mixture, which was allowed to stir overnight (18 h) in the dark. The catalyst was filtered through a Celite pad, and the filtrate was evaporated to dryness (bath temperature = 40 °C). The residue was dissolved in 80% ethanol and loaded onto a column of Brockmann I acid-washed activated alumina (50 g, 2.5 cm × 58.4 cm, prepared in absolute ethanol). The column was eluted with 60% EtOH, and the eluate was evaporated to dryness under reduced pressure. The residue was dissolved in water (20 mL) and extracted with water-saturated benzyl alcohol (5 × 50 mL). The fluorescent yellow benzyl alcohol layers were pooled and filtered through a Whatman #2 filter paper. The filtrate was diluted with ether (500 mL) and extracted with water (5 × 200 mL) until the ether layer was colorless. The aqueous fractions were pooled, washed with ether (25 mL) to remove any residual benzyl alcohol, and evaporated to dryness. The residue was suspended in ethanol (5 mL) and filtered. The residue was washed with ethanol (15 mL) and ether (20 mL) and dried to yield the product **25** (0.08 g, 9%) as bright yellow solid: ^{1}H NMR (DMSO-d_6) δ 1.51 (m, 4 H), 2.50 (s, 6 H), 2.71 (s, 6 H), 3.86 (bs, 5 H), 4.23 (bs, 2 H), 4.47 (bs, 3 H), 4.77 (bs, 2 H), 5.11 (bs, 3 H); ^{13}C NMR (DMSO-d_6) δ 18.00, 21.83, 24.93, ∼42 (obscured by solvent peak), 50.58, 63.28, 68.14, 72.42, 73.77, 130.64, 140.40, 148.93, 149.19, 154.44, 160.25. Anal. Calcd for ($C_{30}H_{42}N_8O_{12}$·1.0 C_2H_5OH· 3.0HCl): C, 44.58; H, 5.96; N, 13.00. Found: C, 44.59; H, 5.60; N, 12.70.

Procedure B. Palladium on carbon (10%, 50 mg) in distilled water (10 mL) was stirred in a hydrogen atmosphere for 5 min. 1,4-Bis[3-(5-nitro-6-ribitylaminouracil)]butane (**19**) (100 mg, 0.23 mmol) was added, and the solution was hydrogenated at room temperature and atmospheric pressure for 5 h. The formation of the reduction product was monitored by analytical HPLC. 2,3-Butanedione (0.5 mL, 5.7 mmol) was added, and the suspension was stirred for 1 h at room temperature under a nitrogen atmophere. The catalyst was removed by filtration, and the filtrate was concentrated to a yellow syrup under reduced pressure. The residue was dissolved in water and applied on a column of Dowex 50W × 2−400 resin (5 g) that was washed with distilled water and then with 1 N NH_4OH. Basic fractions (10 mL) were collected and evaporated to a small volume under reduced pressure. Ethanol was added, and after 12 h in the refrigerator a yellow precipitate formed and was collected by filtration to give **25** (30 mg, 28%) as a bright yellow solid: ^{1}H NMR (D_2O) δ 1.58 (m, 4 H), 2.54 (s, 6 H), 2.76 (s, 6 H exchangeable with D_2O), 3.62−3.54 (m, 4 H), 3.85−3.73 (m, 10 H), 4.30 (m, 2 H), 4.90 (m, 2 H); ^{13}C NMR (D_2O) δ 18.80, 22.20, 24.97, 42.31, 51.77, 63.26, 69.81, 72.74, 74.11, 130.95, 145.47, 149.24, 152.66, 157.86, 163.05; PDMS: 707 (MH^+); HPLC retention time 10.7 min (Phenomenex nucleosil 5 μm C-18 column, 4.6 × 250 mm, eluting with 25% methanol and detecting at 264 nm).

1,5-Bis[3-(6,7-dimethyl-8-D-ribityllumazinyl)]pentane (26). A solution of **20** (0.44 g, 0.65 mmol) in MeOH (15 mL) containing 6 N HCl (2 mL) was hydrogenated at atmospheric pressure in the presence of 10% Pd−C (0.04 g) for 4 h to afford a solution of the hydrochloride intermediate **23**. The hydrogen was then replaced with argon, and 2,3-butanedione (1 mL) was added to the mixture, which was allowed to stir overnight (18 h) in the dark. The catalyst was filtered through a Celite pad, and the filtrate was evaporated to dryness (bath temperature = 40 °C). The residue was dissolved in 80% ethanol and loaded onto a column of Brockman I acid-washed activated alumina (50 g, 2.5 cm × 58.4 cm, prepared in absolute ethanol). The column was eluted with 60% EtOH, and the eluate was evaporated to dryness under reduced pressure. The residue was dissolved in water (20 mL) and extracted with water-saturated benzyl alcohol (5 × 50 mL). The fluorescent yellow benzyl alcohol layers were pooled and filtered through a Whatman #2 filter paper. The filtrate was diluted with ether (500 mL) and extracted with water (5 × 200 mL) until the ether layer was colorless. The aqueous fractions were pooled, washed with ether (25 mL) to remove any residual benzyl alcohol, and evaporated to dryness. The residue was suspended in ethanol (5 mL) and filtered. The residue was washed with ethanol (15 mL) and ether (20 mL) and dried to yield the product **26** (0.04 g, 9%) as a bright yellow solid: ^{1}H NMR (DMSO-d_6) δ 1.08 (m, 2 H), 1.38 (m, 4 H), 2.54 (s, 6 H), 2.72 (s, 6 H), 3.58 (bs, 5 H), 3.71 (bs, 2 H), 3.85 (bs, 3 H), 4.02 (bs,

2 H), 4.23 (bs, 3 H), 4.46 (bs, 3 H), 4.74 (bs, 3 H), 5.10 (bs, 3 H); ^{13}C NMR (DMSO-d_6) δ 15.17, 17.99, 21.83, 23.76, ~42 (obscured by solvent peak), 50.56, 63.28, 68.31, 72.47, 73.76, 130.64, 140.27, 148.64, 149.24, 154.38, 160.17. Anal. Calcd for ($C_{31}H_{44}N_8O_{12}$·1.6 C_2H_5OH ·0.6 HCl): C, 48.17; H, 6.52; N, 13.14. Found C, 48.53; H, 6.31; N, 12.78.

Determination of Riboflavin Synthase. A solution containing buffer (100 mM sodium/potassium phosphate, 10 mM sodium sulfite, and 10 mM EDTA; 1.0 mL) and riboflavin synthase was preincubated at 37 °C. After the addition of lumazine (6 mM; 0.1 mL), riboflavin formation was followed photometrically at 470 nm. Riboflavin synthase activity was determined from the initial velocity of riboflavin formation, using a molar absorptivity of 9100 M^{-1} cm^{-1}. The amount of riboflavin synthase was determined using a specific activity of 50 μmol h^{-1} mg^{-1}.[26,27]

Determination of Lumazine Synthase. Lumazine synthase was determined photometrically at 280 nm using an absorptivity of 0.8 mL mg^{-1} cm^{-1}.[28]

Inhibition of Riboflavin Synthase. Assay mixtures contained 100 mM sodium/potassium phosphate (pH 7.4), 10 mM sodium sulfite, 10 mM EDTA, enzyme inhibitor (0−600 μM), and riboflavin synthase (4.2 μg) in a total volume of 0.62 mL. The solution was incubated at 37 °C, and the reaction was started by the addition of a small volume (0.02 mL) of 6,7-dimethyl-8-ribityllumazine to a final concentration of 0.017−0.190 mM. The formation of riboflavin was monitored photometrically at 470 nm. The kinetic data were fitted with a nonlinear regression method using the program DynaFit from P. Kuzmic.[29] Different kinetic models were considered. K_i values and standard deviations were obtained from the most likely inhibition type. In the case of compound **25**, each velocity curve was fitted separately according to the mechanism of Michaelis and Menten. Lineweaver−Burk plots were obtained from the best fits.

Inhibition of Lumazine Synthase. 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(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-butanone 4-phosphate to a final concentration of 50−310 μM. The formation of 6,7-dimethyl-8-ribityllumazine was monitored photometrically at 410 nm. The kinetic data were fitted with a nonlinear regression method using the program DynaFit from P. Kuzmic.[29] Different kinetic models were considered. K_i values and standard deviations were obtained from the most likely inhibition type.

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