JOC Article

Design, Synthesis, and Evaluation of Acyclic *C*-Nucleoside and *N*-Methylated Derivatives of the Ribitylaminopyrimidine Substrate of Lumazine Synthase as Potential Enzyme Inhibitors and Mechanistic Probes

Jinhua Chen,[†] Thota Sambaiah,[†] Boris Illarionov,[‡] Markus Fischer,[‡] Adelbert Bacher,[‡] and Mark Cushman*[,†]

Department of Medicinal Chemistry and Molecular Pharmacology, School of Pharmacy and Pharmacal Sciences, Purdue University, West Lafayette, Indiana 47907, and Lehrstuhl für Organische Chemie und Biochemie, Technische Universität München, D-85747 Garching, Germany

cushman@pharmacy.purdue.edu

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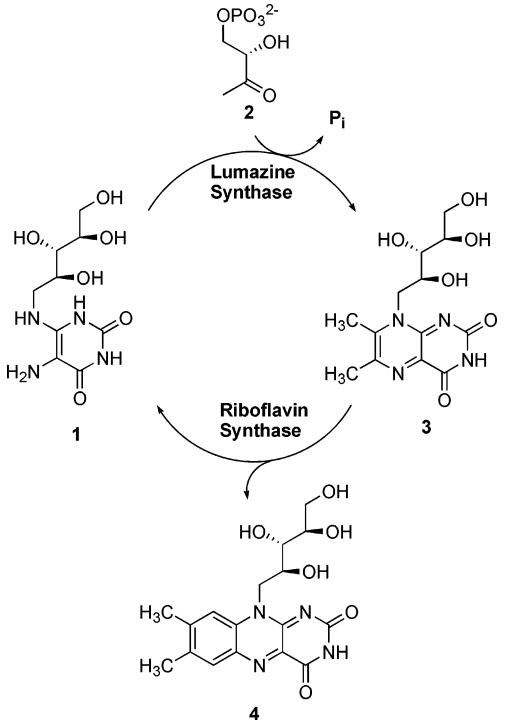
Lumazine synthase and riboflavin synthase catalyze the last two steps in the biosynthesis of riboflavin, a vitamin that is involved in many critical biochemical reactions that are essential for the maintenance of life. To obtain inhibitors and structural probes that could be useful in studying the structures of bound reaction intermediates, the ribitylamino N−H moiety of the lumazine synthase substrate was replaced by CH_2 and N−CH_3 groups. The CH_2 replacement unexpectedly and completely abolished the affinity for lumazine synthase, thus revealing a critical, yet unexplained, role of the ribitylamino N−H moiety in conferring affinity for the enzyme. In contrast, the N−CH_3 replacement resulted in an inhibitor of both lumazine synthase and riboflavin synthase. Replacement of the ribitylamino N−H moiety with epimeric C−F moieties led to inhibition of lumazine synthase and riboflavin synthase when combined with the replacement of the 5-amino group with a nitro substituent.

Introduction

Flavocoenzymes derived from riboflavin **(4)** are absolutely essential for the maintenance of life. Whereas animals obtain riboflavin **(4)** completely from dietary sources, plants and pathogenic microorganisms rely on biosynthesis to obtain it. The enzymes in the riboflavin biosynthesis pathway are therefore logical targets for the design of potential antibiotics.

The last two reactions in the biosynthesis of riboflavin are catalyzed by lumazine synthase and riboflavin synthase (Scheme 1). The riboflavin synthase-catalyzed dismutation of two molecules of 6,7-dimethyl-8-D-ribityllumazine (**3**) (Scheme 1) results in the formation of one molecule of riboflavin **(4)** and one molecule of the substituted pyrimidinedione **(1)**. Lumazine synthase catalyzes the reaction of **1** with the four-carbon phosphate **2** to form **3**, which is the substrate for riboflavin synthase.[1−4]

SCHEME 1. Biosynthesis of Riboflavin



Although the intricate details remain to be established, the lumazine synthase-catalyzed reaction has been viewed as proceeding along the mechanistic pathway outlined

* Corresponding author. Phone: 765-494-1465. Fax: 765-494-6790.
[†] Purdue University.
[‡] Technische Universität München.

(1) Plaut, G. W. E.; Smith, C. M.; Alworth, W. L. *Annu. Rev. Biochem.* **1974**, *43*, 899−922.
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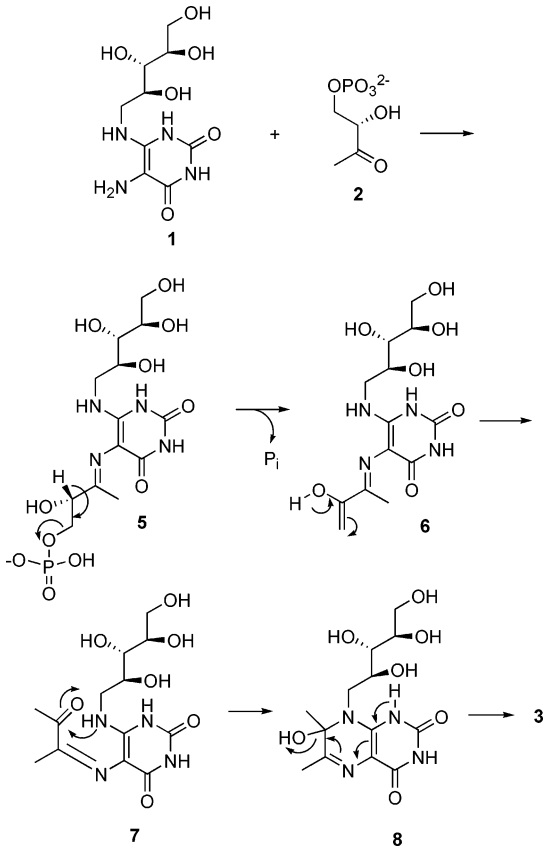
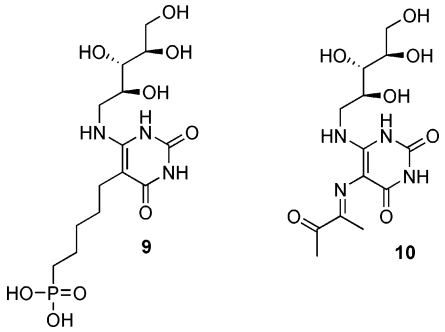
SCHEME 2. Hypothetical Lumazine Synthase Mechanism



in Scheme 2.[5] Condensation of the primary amino group of the substituted pyrimidinedione **1** with the ketone **2** is proposed to result in the formation of a Schiff base **5**, which eliminates phosphate to yield the enol **6**. Tautomerization of the enol **6** to the ketone **7**, ring closure and dehydration of **8** would provide the product **3**.

X-ray crystallography of a complex of the phosphonate analogue **9** with lumazine synthase has established that the phosphate of the hypothetical Schiff base binds far away from the ribityl side chain as depicted roughly in structure **5**.[6,7] Recently, in the temperature-dependent pre-steady-state kinetic experiments of lumazine synthase from *Aquifex aeolicus*, an early optical transient absorbing at 330 nm was identified, followed by a second transient with an absorption maximum at 445 nm. The absorption at 330 nm was assigned to the Schiff base **5**, while the absorption at 445 nm could result from the enol **6** (or its cis Schiff base counterpart) or the ketone **7** (or its trans Schiff base counterpart).[8] Single turnover experiments with lumazine synthase from *Bacillus subtilis* also showed a similar initial absorption at 330 nm, followed by an absorption at 455 nm.[9] Both kinetic studies proposed that one of the subsequent steps occurring after phosphate elimination and tautomerization was the rate-determining step.[8,9]



The details of the isomerization of the trans Schiff base **10** to the cis Schiff base **7**, or possible initial formation of a thermodynamically less stable cis Schiff base, have not been established. By a semiempirical approach, the energy barrier for the **10** to **7** isomerization was calculated to be 19.6−21 kcal/mol.[10] In addition, the sequence of the phosphate release relative to the conformational change of the side chain to form **7** is still not certain. For example, it has recently been proposed that the whole phosphate side chain rotates to form a cyclic conformation with assistance from the enzyme before phosphate elimination and dehydration occur to form the cis Schiff base **7** directly.[11]

To probe the active site of lumazine synthase and gain insight into the intricate details of the reaction mechanism, we have designed metabolically stable analogues of the hypothetical reaction intermediates in Scheme 2. The crystal structure of **11** bound to *B. subtilis* lumazine synthase (Figure 1) has revealed that the ribitylamino

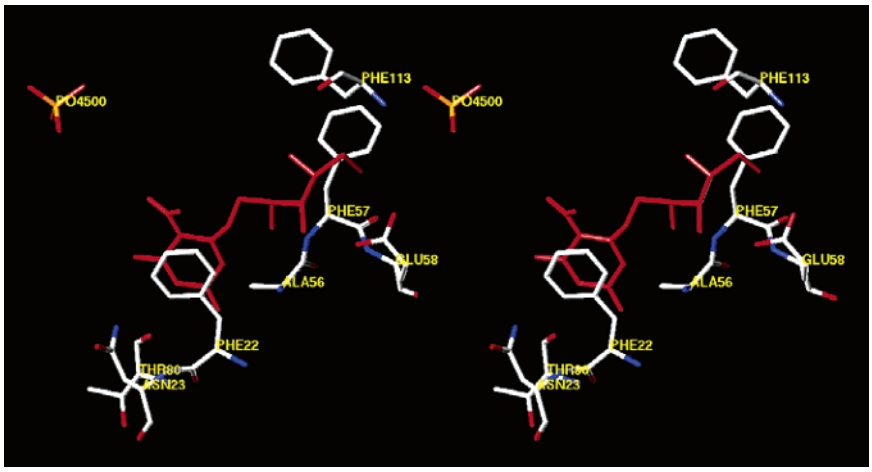


FIGURE 1. Crystal structure of the substrate analogue **11** bound in the active site of *B. subtilis* lumazine synthase. The figure is programmed for wall-eyed viewing.

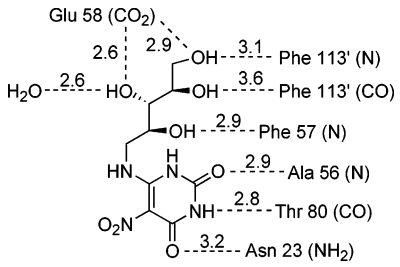
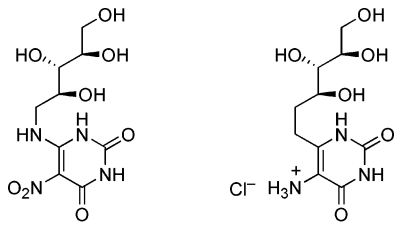
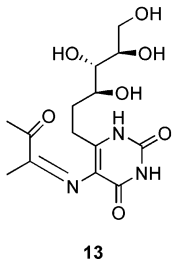


FIGURE 2. Hydrogen bonds and distances of the substrate analogue **11** bound in the active site of *B. subtilis* lumazine synthase.

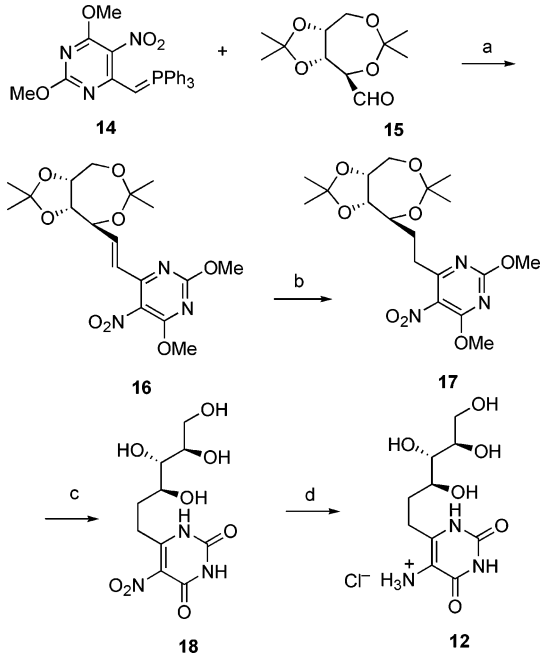
nitrogen has no obvious direct role in binding the ligand to the enzyme (Figure 2).[12] Therefore, replacement of that nitrogen with carbon, as shown in **12**, an analogue of **1**, was expected to yield new *C*-nucleoside analogues that could possibly bind to the enzyme. The *C*-nucleoside analogues of the hypothetical reaction intermediates could theoretically be observed in situ from the reaction of **12** and **2** catalyzed by the enzyme. Since these *C*-nucleoside derivatives, such as **13**, are close structural analogues of the hypothetical reaction intermediates, they should be recognized by the enzyme, or even formed by the enzyme in the presence of the *C*-nucleoside **12**, but they obviously could not complete the catalytic cycle. The interactions of these metabolically stable analogues with the enzyme could be studied by enzyme kinetics, X-ray diffraction and NMR, thus providing insight into the behavior of the enzyme-bound reaction intermediates.





It has been found that many *C*-nucleosides have potent antibacterial, anti-viral and anti-cancer activities.[13] The biological activities of *C*-nucleosides are believed to be the result of their isosterically mimicking *N*-nucleosides and their resistance to enzymatic hydrolysis.[13]

SCHEME 3. Synthesis of the *C*-Nucleoside Analogue of the Lumazine Synthase Pyrimidine Substrate[a]



[a] Reagents and conditions: (a) toluene, reflux (24 h); (b) $NaBH_4$, dioxane−ethanol (12 h); (c) Amberlite-IR-120 (H^+), MeOH−H_2O, reflux (4.5 h); (d) H_2, Pd/C, HCl−MeOH (10 h).

Results and Discussion

The Wittig reaction has been widely used in the synthesis of *C*-nucleosides.[14−17] In most cases, alkenes have not been isolated as products because a free hydroxyl group from the carbohydrate moiety adds to the alkene intermediate, resulting in the formation of cyclic carbohydrate derivatives.[14,16] Therefore, to obtain an acyclic *C*-nucleoside using the Wittig approach, a fully protected acyclic ribose derivative **15** and the ylide **14** were prepared as described in the literature.[15,18] As shown in Scheme 3, the Wittig reaction of **14** with **15** was carried out by heating at reflux in toluene and the trans alkene **16** (J = 15 Hz) was the only product obtained.[15] Deprotection of the alkene **16** with Amberlite-IR-120 (H^+) resin yielded a mixture of products, presumably due to the intramolecular addition of the nucleoside hydroxyl groups to the alkene.[15,16] To obtain the acyclic

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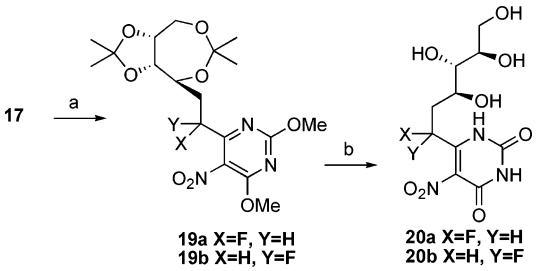
C-nucleoside, the alkene **16** was first reduced with sodium borohydride in dioxane and ethanol. Compound **17** was then deprotected with Amberlite-IR-120 (H^+) resin. The structure of the product **18** was confirmed by X-ray diffraction. The amino compound **12**, which is the *C*-nucleoside analogue of the natural substrate **1**, was prepared as the hydrochloride from **18** by catalytic hydrogenation with Pd/C in the presence of hydrochloric acid.

Surprisingly, the *C*-nucleoside analogue **12** of the substrate **1** was completely inactive against *B. subtilis* lumazine synthase, even though the X-ray structure of the crystalline complex formed from **11** and lumazine synthase indicates no direct contact of the ribitylamino group with the protein.[12] Separate binding experiments in the absence of substrate revealed that **12** has essentially no affinity for either lumazine synthase or riboflavin synthase. It is conceivable that steric factors could play a role in the unexpected inactivity of the *C*-nucleoside **12**, since the ribitylamino nitrogen of the substrate **1** is part of a "vinylogous amide" system, resulting in sp^2-character and planar geometry, which is different from that of **12**. However, this scenario is not consistent with the fact that the nitro precursor to **12**, compound **18**, did display inhibitory activity vs *B. subtilis* lumazine synthase (K_i 264 μM) and *Escherichia coli* riboflavin synthase (K_i 37 μM).

In addition to possible steric factors, the greater electron density afforded by the nonbonding electrons in the ribitylamino atom of the substrate **1** might explain the inactivity of **12**. To gain some insight into this possibility, both epimers of the fluorinated nitro-*C*-nucleosides **20a** and **20b** were desired. If electronic factors are important, the fluorinated compounds might be more active than the parent compound **18**. Homocyclic *C*-nucleosides with halogens, including iodine and chlorine on the bridge-carbon, have been reported previously.[19,20] However, *C*-nucleosides with fluorine on the proximal carbon, which is attached to the heterocycle, have not been reported. Fluorination of the proximal carbon may not only improve the biological activity by increasing the electron density, thus more closely mimicking a nitrogen atom, it will also facilitate the study of ligand−protein interaction by REDOR ^{19}F NMR.[21] Deprotonation of the benzylic carbon of **17** by potassium diisopropylamide at −90 °C, followed by electrophilic fluorination with *N*-fluorobenzenesulfonimide, provided two separable epimers **19a** and **19b**, each in 10% yield.[22] The configurations of the fluorinated carbon of **19a** and **19b** have not been determined. Both epimers were deprotected using Amberlite-IR-120 (H^+) resin to give **20a** (epimer A) and **20b (**epimer B) in excellent yield (Scheme 4).

The compounds prepared in this study were tested as inhibitors of recombinant *B. subtilis* lumazine synthase β_{60} capsids, recombinant *Mycobacterium tuberculosis* lumazine synthase, and recombinant *E. coli* riboflavin

SCHEME 4. Synthesis of the Fluoro-*C*-nucleoside Derivatives[a]



[a] Reagents and conditions: (a) (1) KDA, THF, −90 °C (1 h), (2) *N*-fluorobenzenesulfonimide, −78 to +23 °C (3 h); (b) Amberlite-IR-120 (H^+), MeOH−H_2O, reflux (4 h).

synthase. In the case of **20a** and **20b**, the enzyme kinetics with *B. subtilis* lumazine synthase was performed both with variable **1** substrate concentration (and fixed **2** concentration) as well as with variable **2** concentration (and fixed **1** concentration), and the results are listed separately in Table 1. A comparison can be made between the inhibition constants observed with the *C*-nucleoside **18** and the corresponding epimers **20a** and **20b** when tested vs *B. subtilis* lumazine synthase using variable **1** concentration. The relevant K_i values are 264 μM for **18** and 221 μM for **20b**, with **20a** being inactive. Several conclusions can be drawn from the data. The first is that the more active epimer **20b** is not significantly more active than the corresponding unfluorinated compound **18**, so the hope that activity could be improved through fluorination of the methylene unit connected to the heterocycle ultimately turned out to be unfruitful. Second, the activity is significantly different vs different lumazine synthases, as both **20a** and **20b** were active vs *M. tuberculosis* lumazine synthase, with K_i values of 113 and 74 μM, respectively. It is interesting to note that the inactive epimer **20a** vs *B. subtilis* lumazine synthase is active vs *M. tuberculosis* lumazine synthase. Third, the fluorinated epimer **20a** is more active vs *E. coli* riboflavin synthase (K_i 21 μM) than its unfluorinated counterpart **18** (K_i 37 μM).

In addition to probing the effects of installing a potential hydrogen-bond acceptor (fluorine) on the methylene group attached to the heterocyclic system of **18**, the effects of attaching a hydroxyl group, which could act as both a hydrogen-bond donor as well as an acceptor, were investigated. Many alditol-type acyclic *C*-nucleosides and *C*-glycosides have been synthesized as potential anti-cancer and anti-viral agents.[13,23,24] Acyclic protected ribose **21**,[18] a byproduct obtained during the synthesis of **15**, was used to prepare the trans alkene **22** by a Wittig reaction with **14** (Scheme 5). Catalytic dihydroxylation of compound **22** with AD-mix-α in the presence of methanesulfonamide in a mixture of *tert*-butyl alcohol and water gave compound **23** and its isomer in a ratio of 6:1, based on the relevant signal integration in the ^{1}H NMR spectrum of crude reaction mixture.[25,26] Compound

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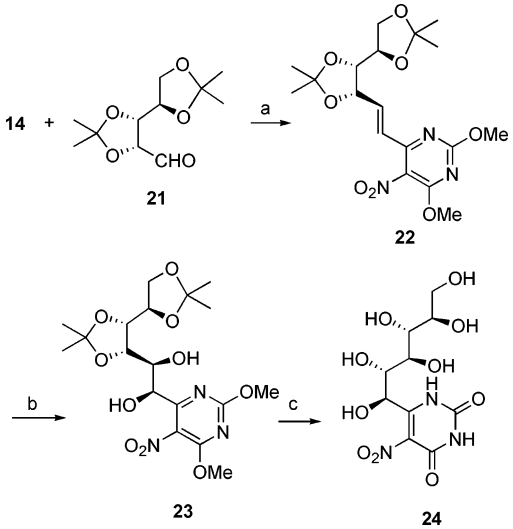
TABLE 1. Inhibition Constants vs *B. subtilis* Lumazine Synthase,[a] *M. tuberculosis* Lumazine Synthase,[b] and *E. coli* Riboflavin Synthase[c]

compd	enzyme	substrate with variable concn[d]	K_s,[e] μM	k_{cat},[f] m^{-1}	K_i,[g] μM	K_{is},[h] μM	mechanism
12	*B. subtilis* LS	1			inactive		uncompetitive
18	*B. subtilis* LS	1	2.52 ± 0.23	2.25 ± 0.04	264 ± 28		competitive
	E. coli RS		2.02 ± 0.21	5.48 ± 0.11	37.1 ± 4.5	856 ± 268	partial
20a	*B. subtilis* LS	1	6.4 ± 0.3	2.67 ± 0.04	>1000		uncompetitive
	B. subtilis LS	2	65.5 ± 3.0	2.72 ± 0.05	>3000		uncompetitive
	M. tuberculosis LS	1	215 ± 24	0.83 ± 0.04	113 ± 11		competitive
	E. coli RS		2.45 ± 0.22	12.8 ± 0.30	21.2 ± 1.8		competitive
20b	*B. subtilis* LS	1	7.03 ± 0.58	2.74 ± 0.07	221 ± 33		competitive
	B. subtilis LS	2	74.9 ± 4.2	2.84 ± 0.06		733 ± 106	uncompetitive
	M. tuberculosis LS	1	185 ± 15	0.78 ± 0.03	74 ± 6.0		competitive
	E. coli RS		3.19 ± 0.30	15.6 ± 0.5		89.7 ± 8.0	uncompetitive
24	*B. subtilis* LS	1			inactive		uncompetitive
	E. coli RS				inactive		uncompetitive
26	*B. subtilis* LS	1			inactive		uncompetitive
	E. coli RS				inactive		uncompetitive
31	*B. subtilis* LS	1	5.76 ± 0.33	2.48 ± 0.05	inactive		uncompetitive
	M. tuberculosis LS	1	72.1 ± 3.8	0.75 ± 0.01		540 ± 52	uncompetitive
	E. coli RS				inactive		uncompetitive
32	*B. subtilis* LS	1	8.42 ± 0.66	2.49 ± 0.07	180 ± 19		competitive
	B. subtilis LS	2	74.4 ± 3.8	4.41 ± 0.07	492 ± 64		competitive
	M. tuberculosis LS	1	406 ± 36	3.88 ± 0.23	341 ± 42		competitive
	E. coli RS		2.37 ± 0.28	12.8 ± 0.41	147 ± 40	738 ± 267	mixed

[a] Recombinant β_{60} capsids from *B. subtilis*. [b] Recombinant lumazine synthase from *M. tuberculosis*. [c] Recombinant riboflavin synthase from *E. coli*. [d] The assays with lumazine synthase were performed with one variable substrate concentration and one fixed substrate concentration. [e] K_s is the substrate dissociation constant for the equilibrium E + S ⇌ ES. [f] k_{cat} is the rate constant for the process ES → E + P. [g] K_i is the inhibitor dissociation constant for the process E + I ⇌ EI. [h] K_{is} is the inhibitor dissociation constant for the process ES + I ⇌ ESI.

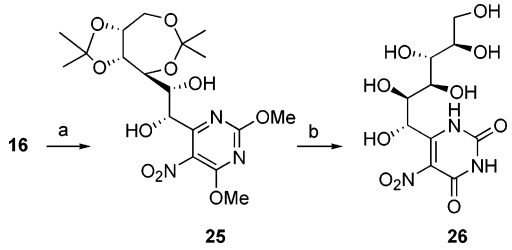
SCHEME 5. Synthesis of Pyrimidinealditol 24[a]



[a] Reagents and conditions: (a) toluene, reflux; (b) AD-mix-α, methanesulfonamide, 0 °C (12 h), 15 °C (48 h); (c) Amberlite-IR-120 (H^+), MeOH−H_2O, reflux (4.5 h).

23 was isolated by column chromatography in 61% yield. The structure of **23** was determined by X-ray diffraction, as shown in the Supporting Information. Similarly, dihydroxylation of **16** with AD-mix-β gave **25** and its isomer in a ratio of 9:1, based on the ^{1}H NMR spectrum of crude reaction mixture (Scheme 6). Compound **25** was isolated by column chromatography in 52% yield. Finally, **24** and **26** were prepared from **23** and **25**, respectively, by heating with Amberlite-IR-120 (H^+) resin in aqueous

SCHEME 6. Synthesis of Pyrimidinealditol 26[a]



[a] Reagents and conditions: (a) AD-mix-β, methanesulfonamide, 0 °C (12 h), 15 °C (72 h); (b) Amberlite-IR-120 (H^+), MeOH−H_2O, reflux (4.5 h).

methanol at reflux. Both **24** and **26** were inactive as inhibitors of *B. subtilis* lumazine synthase and *E. coli* riboflavin synthase. Comparison of the results with these two compounds with the *C*-nucleoside **18** shows that the two additional hydroxyl groups prevent binding to both lumazine synthase and riboflavin synthase.

In view of the disappointing results obtained with the *C*-nucleoside **12** and the two alditols **24** and **26**, more conservative approaches to "trapping" the intermediates in the lumazine synthase pathway were investigated. Assuming the resulting compound were an enzyme substrate, *N*-methylation of the ribitylamino group of the natural substrate **1** (Scheme 2) would block, at some stage, the final conversion to the product **3**, thus allowing us to possibly "see" the bound intermediate analogue forms. This could also result in enzyme inhibition. To investigate this hypothesis, the *N*-methylated analogue **32** was synthesized (Scheme 7). Substitution of 6-chloro-5-nitro-2,4-dimethoxypyrimidine (**29**)[27] with methyl-

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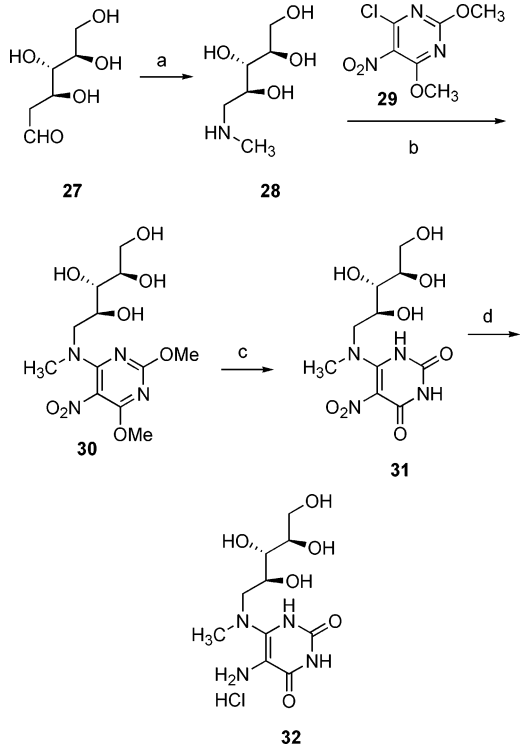
SCHEME 7. Synthesis of the *N*-Methylated Substrate Analogue 31[a]



[a] Reagents and conditions: (a) (1) methylamine, MeOH, room temperature (4 h), (2) H_2, 40 psi, PtO_2, room temperature (12 h); (b), K_2CO_3, DMF, room temperature (12 h); (c) concd HCl, MeOH, reflux (2 h); (d) H_2, Pd/C, concd HCl, MeOH (12 h).

ribitylamine (**28**),[28] followed by deprotection of intermediate **30**, gave nitro compound **31**. Compound **31** was reduced by catalytic hydrogenation in the presence of hydrochloric acid to give amino compound **32**. The K_i value of **32** vs *B. subtilis* lumazine synthase were found to be 180 μM in the presence of variable substrate **1** concentration, and 492 μM in the presence of variable substrate **2** concentration. When tested vs *M. tuberculosis* lumazine synthase, the amino compound **32** displayed a K_i of 341 μM with variable **1** concentration, and the K_i detected vs *E. coli* riboflavin synthase was 147 μM. Interestingly, the nitro precursor **31** was less active than **32** vs *M. tuberculosis* lumazine synthase, and it was inactive vs *B. subtilis* lumazine synthase and *E. coli* riboflavin synthase. This is the reverse of the situation with the *C*-nucleosides, in which the nitro compound **18** was active and the amino compound **12** was inactive.

In conclusion, the results described here demonstrate a critical, although still undefined, role for the ribitylamino N−H group in substrate recognition by both lumazine synthase and riboflavin synthase, since N−H replacement with a methylene moiety results in an unexpected and complete abrogation of affinity for both enzymes. Partial recovery of affinity can be realized by replacement of the remaining primary amino group with a nitro group, but no further increase in activity can be realized through replacement of the CH_2 group with a CHF moiety. The different inhibitory properties discovered for the two epimers **20a** and **20b** vs *B. subtilis* lumazine synthase and *E. coli* riboflavin synthase are intriguing, and may result from steric or stereoelectronic effects, but the difference obviously cannot be explained with certainty at the present time because the absolute configurations of the fluorinated carbons have not been determined. Interestingly, there is not much difference in activity when the two epimers are tested on *M. tuberculosis* lumazine synthase. *N*-Methylation of the lumazine synthase substrate ribitylamino nitrogen provides moderately potent inhibitors of both lumazine synthase and riboflavin synthase. The utilization of the methylamino compound **32** to generate metabolically stable reaction intermediates in the presence of lumazine synthase could possibly provide information about the structures of the enzyme-bound intermediates.

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Experimental Section

5-Amino-6-*C*-[(D-ribityl)methyl]pyrimidine-2,4-dione Hydrochloride (12). Concentrated HCl (0.2 mL) and 10% Pd/C (10 mg) were added to a solution of nitro compound **18** (15 mg, 0.049 mmol) in MeOH (5 mL). After the mixture was degassed, a hydrogen balloon was attached and the mixture was stirred at room temperature for 10 h. The reaction mixture was filtered through Celite, which was then washed with water (1 mL). The solvent was evaporated from the filtrate, and the residue was dried over P_2O_5 to give **12** as a pale yellow amorphous solid (10 mg, 65%): ^{1}H NMR (300 MHz, CD_3OD) δ 3.62 (m, 2 H), 3.47 (m, 2 H), 3.41 (m, 1 H), 2.57 (t, J = 7.05 Hz, 2 H), 1.85 (m, 1 H), 1.72 (m, 1 H); ^{13}C NMR (75 MHz, D_2O) 162.3, 153.1, 152.5, 104.8, 74.6, 73.1, 71.3, 63.6, 29.1, 26.1. Anal. Calcd for $C_{10}H_{18}ClN_3O_6 \cdot H_2O$: C, 36.43; H, 6.11. Found: C, 36.27; H, 6.08.

6-*E*-(2,5:3,4-Di-*O*-isopropylidene-D-ribitylidenemethylene)-2,4-dimethoxy-5-nitropyrimidine (16). A mixture of ylide **14**[15] (0.72 g, 1.57 mmol) and aldehyde **15**[18] (0.36 g, 1.57 mmol) in dry toluene (15 mL) was heated at reflux for 24 h. After the reaction mixture was cooled to room temperature, the solvent was removed and the residue was purified by flash chromatography (2 × 20 cm column of SiO_2, EtOAc−hexanes 1:4) to give **16** as colorless glass (0.60 g, 93%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.48 (dd, J = 3.91, 15.11 Hz, 1 H), 6.72 (dd, J = 1.94, 15.11 Hz, 1 H), 4.48 (m, 1 H), 4.12 (d, J = 5.35 Hz, 1 H), 4.06 (s, 6 H), 3.99 (s, 2 H), 3.79 (dd, J = 4.35, 9.71 Hz, 1 H), 1.54 (s, 3 H), 1.40 (s, 3 H), 1.35 (s, 3 H), 1.33 (s, 3 H); EIMS (MH^+) m/z 412. Anal. Calcd for $C_{18}H_{25}N_3O_8 \cdot H_2O$: C, 50.36; H, 5.91. Found: C, 50.34; H, 6.29.

6-*C*-[(2,5:3,4-Di-*O*-isopropylidene-D-ribityl)methyl]-2,4-dimethoxy-5-nitropyrimidine (17). A solution of alkene **16** (0.167 g, 0.41 mmol) in dry dioxane (2.0 mL) was added dropwise to a suspension of $NaBH_4$ (34 mg, 0.88 mmol) in a mixture of dioxane (7.0 mL) and ethanol (2.0 mL) over a period of 15 min. After being stirred at room temperature overnight, the reaction mixture was poured into a mixture of ice (10 g) and saturated NH_4Cl solution (5 mL). The solvent was removed under reduced pressure, and the residue was extracted with $CHCl_3$ (3 × 10 mL), washed with water (10 mL) and brine (5 mL), and dried over Na_2SO_4. The solvent was removed, and the residue was purified by flash chromatography (1 × 20 cm column of SiO_2, EtOAc−hexanes 1:3) to give **17** as a colorless glass (0.072 g, 43%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.10 (m, 1 H), 4.07 (s, 3 H), 4.04 (s, 3 H), 3.95 (m, 2 H), 3.82−3.69 (m, 2 H), 2.98−2.88 (m, 1 H), 2.78−2.65 (m, 1 H), 2.33−2.20 (m, 1 H), 1.84−1.70 (m, 1 H), 1.47 (s, 3 H), 1.34 (s, 3 H), 1.33 (s, 3 H), 1.31 (s, 3 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 166.3, 163.2, 129.6, 108.1, 101.3, 79.3, 76.5, 69.3, 58.2, 55.6, 55.2, 29.8, 29.7, 28.2, 25.57, 24.9, 23.7; EIMS (MH^+) m/z 414. Anal. Calcd for $C_{18}H_{27}N_3O_8$: C, 52.30; H, 6.54; N, 10.17. Found: C, 52.63; H, 6.47; N, 9.52.

5-Nitro-6-*C*-[(D-ribityl)methyl]pyrimidine-2,4-dione (18). After Amberlite-IR-120 (H^+) resin (1.60 g) was washed with distilled water (3 × 5 mL), it was mixed with MeOH (3 mL), water (3 mL), and **17** (60 mg, 0.14 mmol). The mixture was heated at reflux for 4.5 h. After being cooled to room temperature, the mixture was filtered and washed with 50% MeOH (3 × 2 mL). The solvent was removed, and the residue was reprecipitated from MeOH and ether. The precipitate was dried over P_2O_5 under vacuum to give **18** as a pale yellow amorphous solid (37 mg, 85%): ^{1}H NMR (300 MHz, CD_3OD) δ 3.73−3.64 (m, 2 H), 3.61−3.52 (m, 2 H), 3.46−3.42 (m, 1 H), 2.82−2.72 (m, 1 H), 2.67−2.57 (m, 1 H), 2.04−1.94 (m, 1 H), 1.84−1.73 (m, 1 H); ^{13}C NMR (75 MHz, CD_3OD) 158.8, 158.0, 151.0, 129.0, 75.5, 74.3, 72.9, 64.6, 31.5, 28.0; EIMS (MH^+) *m/z* 306. Anal. Calcd for $C_{10}H_{15}N_3O_8 \cdot 0.13\ Et_2O$: C, 40.82; H, 5.56; N, 12.78. Found: C, 41.06; H, 5.24; N, 12.41.

6-*C*-[(2,5:3,4-Di-*O*-isopropylidene-D-ribityl)fluoromethyl]-2,4-dimethoxy-5-nitropyrimidine (19a and 19b). Diisopropylamine (0.15 mL, 1.0 mmol) was added to a solution of KO-*t*-Bu (112 mg, 1.0 mmol) in THF (2.0 mL), and the mixture was cooled to −78 °C under Ar. BuLi (0.53 mL, 1.6 M, 0.85 mmol) was slowly added and the mixture stirred at −78 °C for 10 min. After the mixture was cooled to −90 °C, a solution of **17** (0.200 g, 0.5 mmol) in THF (1.0 mL) was added slowly. After the mixture was stirred at −90 °C for 1 h, a solution of *N*-fluorobenzenesulfonimide (0.300 g, 0.85 mmol) in THF (2 mL) was added slowly. The temperature was increased to −78 °C and then to room temperature over 3 h. Saturated NH_4Cl solution (5 mL) was added, and the solvent was removed under reduced pressure. The residue was extracted with ether (3 × 20 mL) and dried over Na_2SO_4. After solvent was removed, the residue was purified by flash chromatography (2 × 15 cm column of SiO_2, hexanes−EtOAc 8:1−6:1) to give two separable isomers. The less polar epimer (**19a**) was isolated as a pale yellow glass (20 mg, 10%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.88 (ddd, J = 4.3, 8.34, 47.51 Hz, 1 H), 4.09−4.02 (m, 10 H), 3.84−3.82 (m, 1 H), 2.51−2.21 (m, 2 H), 1.40−1.22 (m, 3 H); ESIMS *m/z* (rel intensity) 432 (MH^+, 54). Anal. Calcd for $C_{18}H_{26}FN_3O_8 \cdot 0.5$ EtOAc: C, 50.52; H, 6.36. Found: C, 50.73; H, 6.20. The more polar epimer (**19b**) was also isolated as pale yellow glass (20 mg, 10%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.57 (ddd, J = 1.8, 11.1, 48.3 Hz, 1 H), 4.15−3.92 (m, 6 H), 3.74−3.69 (m, 1 H), 2.64−2.50 (m, 1 H), 2.00−1.76 (m, 1 H), 1.50 (s, 3 H), 1.38 (s, 6 H), 1.34 (s, 3 H). Anal. Calcd for $C_{18}H_{26}FN_3O_8 \cdot 0.5$ EtOAc: C, 50.52; H, 6.36. Found: C, 50.70; H, 6.10.

(1*R*,2*S*)-1-(2,6-Dimethoxy-5-nitropyrimidin-4-yl)-2-(2,2,2′,2′-tetramethyl[4,4′]bi[1,3]dioxolanyl]-5-yl)ethane-1,2-diol (23). After a mixture of water (5 mL), *tert*-butyl alcohol (5 mL), and AD-mix-α (1.54 g) was stirred at room temperature for 15 min, it appeared as two clear phases. Methanesulfonamide (0.104 g) was added, and the mixture was cooled to 0 °C. Compound **22** (0.382, 0.924 mmol) was added at once. The reaction mixture was vigorously stirred at 0 °C for 12 h and then at 15 °C for 48 h. The reaction mixture was cooled to 0 °C, and solid sodium sulfite (1.5 g) was added. The mixture was warmed to room temperature and stirred at room temperature for 1 h. It was extracted with CH_2Cl_2 (5 × 10 mL). The organic layer was washed with 1 N NaOH solution (3 × 10 mL) and water (10 mL) and dried over Na_2SO_4. Solvent was removed under reduced pressure, and the residue was purified by flash chromatography (30 g, 3 × 30 cm column of SiO_2, hexanes−EtOAc 3:2) to give **23** as a glass (0.266 g, 61%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.25 (d, J = 9.64 Hz, 1 H), 4.42 (dd, J = 5.41, 9.47 Hz, 1 H), 4.15 (m, 2 H), 4.12 (m, 1 H), 4.09 (s, 3 H), 4.08 (m, 1 H), 4.07 (s, 3 H), 3.97 (m, 1 H), 3.85 (d, J = 9.64 Hz, 1 H), 3.79 (d, J = 3.33 Hz, 1 H), 1.39 (s, 3 H), 1.36 (s, 3 H), 1.31 (s, 3 H), 1.29 (s, 3 H); EIMS (MH^+) *m/z* 446. Anal. Calcd for $C_{18}H_{27}N_3O_{10}$: C, 48.54; H, 6.11; N, 9.43. Found: C, 48.22; H, 5.91; N, 9.38.

5-Nitro-6-(*N*-methyl)ribityl-2,4-dimethoxypyrimidine (30). Methylribitylamine **(28)** (1.4 g, 8.48 mmol) and K_2CO_3 (1.17 g, 8.48 mmol) were added to a solution of 6-chloro-2,4-dimethoxy-5-nitropyrimidine **(29)**[27] (0.93 g, 4.24 mmol) in DMF (20 mL). After the reaction mixture was stirred at room temperature for 12 h, the solvent was removed under reduced pressure. The residue was dissolved in water (10 mL), extracted with $CHCl_3$ (3 × 10 mL), washed with brine, and dried over Na_2SO_4. The solvent was removed, and the residue was purified by flash chromatography (2 × 20 cm column of SiO_2, 230−400 mesh, CH_2Cl_2−MeOH 97:3−95:5) to give **30** as a pale yellow glass (1.03 g, 70%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.08−3.80 (m, 10 H), 3.73−3.69 (m, 1 H), 3.55−3.43 (m, 2 H), 2.99 (s, 3 H). Anal. Calcd for $C_{12}H_{20}N_4O_8$: C, 41.38; H, 5.79. Found: C, 41.40; H, 5.87.

5-Nitro-6-(*N*-methyl)ribitylpyrimidine-2,4-dione (31). Concentrated HCl (2.0 mL) was added slowly to a solution of **30** (180 mg, 0.517 mmol) in MeOH (2 mL). The mixture was heated at reflux for 2 h. After the reaction mixture was cooled to room temperature, ether (5 mL) was added, and the solid was collected by filtration, washed with ether (2 × 5 mL), and dried under vacuum to give **31** as pale yellow amorphous solid (110 mg, 50%): ^{1}H NMR (300 MHz, CD_3OD) δ 4.19−4.06 (m, 2 H), 3.97−3.82 (m, 1 H), 3.78−3.60 (m, 2 H), 3.27−3.03 (m, 2 H), 2.70 and 2,71 (2 s, 3 H). Anal. Calcd for $C_{10}H_{16}N_4O_8 \cdot 1.5\ H_2O$: C, 34.59; H, 5.51. Found: C, 34.41; H, 5.31.

***B. subtilis* Lumazine Synthase Assay.**[29] Reaction mixtures contained 100 mM potassium phosphate, pH 7.0, 5 mM EDTA, 5 mM dithiothreitol, inhibitor (0−500 μM), 170 μM 5-amino-6-ribitylamino-2,4-(1*H*,3*H*)-pyrimidinedione (**1**), and *B. subtilis* lumazine synthase (16 μg, specific activity 8.5 μmol mg^{-1} h^{-1}) in a total volume of 1000 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 **(2)** to a final concentration of 50−400 μM. The formation of 6,7-dimethyl-8-ribityllumazine **(3)** was monitored photometrically at 408 nm ($\epsilon_{lumazine}$ = 10 200 M^{-1} cm^{-1}). The velocity−substrate data were fitted for all inhibitor concentrations by nonlinear regression using the program DynaFit.[30] 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. In a separate set of experiments, the L-3,4-dihydroxy-2-butanone 4-phosphate concentration **(2)** was kept constant at 100 μM and the concentration of the second substrate, 5-amino-6-ribitylamino-2,4-(1*H*,3*H*)-pyrimidinedione (**1**), was varied.

***E. coli* Riboflavin Synthase Assay.**[31] Reaction mixtures contained 100 mM potassium phosphate, 10 mM EDTA, 10 mM sodium sulfite, inhibitor (0 to 300 μM), and riboflavin synthase (4.6 μg, specific activity 45 μmol mg^{-1} h^{-1}). After preincubation, the reactions were started by the addition of various amounts of 6,7-dimethyl-8-ribityllumazine **(3)** (2.5−200 μM) to a total volume of 1000 μL. The formation of riboflavin **(4)** was measured online with a computer-controlled photometer at 470 nm ($\epsilon_{riboflavin}$ = 9600 M^{-1} cm^{-1}). The K_i evaluation was performed in the same manner as described above.

***M. tuberculosis* Lumazine Synthase Assay.** Reaction mixtures contained 50 mM Tris·HCl, pH 7.0, 100 mM NaCl, 5 mM DTT, inhibitor (0−500 μM), L-3,4-dihydroxy-2-butanone 4-phosphate **(2)** (100 μM), and lumazine synthase (64 μg, specific activity 0.041 μmol mg^{-1} h^{-1}) in a total volume of 1000 μL. The solution was incubated at 37 °C for 10 min, and the reaction was started by the addition of a small volume (up to 20 μL) of 5-amino-6-ribitylamino-2,4-(1*H*,3*H*)-pyrimidinedione (**1**) to a final concentration of 40−300 μM. The formation of 6,7-dimethyl-8-ribityllumazine (**3**) was monitored photometrically at 408 nm ($\epsilon_{lumazine}$ = 10 200 M^{-1} cm^{-1}). The velocity−substrate data were fitted for all inhibitor concentrations by

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nonlinear regression using the program DynaFit[30] as is described for the assay with *B. subtilis* enzyme.

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Supporting Information Available: X-ray crystallographic data for compounds **18** and **23**, experimental procedures for the syntheses of compounds **20a**,**b**, **22**, **24**−**26**, **28**, and **32**, and NMR spectra for compounds **17**, **18**, **20a**, **31**, and **32**. This material is available free of charge via the Internet at http://pubs.acs.org.

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