Design, Synthesis, and Evaluation of 9-D-Ribitylamino-1,3,7,9-tetrahydro-2,6,8-purinetriones Bearing Alkyl Phosphate and α,α-Difluorophosphonate Substituents as Inhibitors of Riboflavin Synthase and Lumazine Synthase

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Received September 2, 2003

Lumazine synthase and riboflavin synthase catalyze the last two steps in the biosynthesis of riboflavin, an essential metabolite that is involved in electron transport processes. To obtain structural probes of these two enzymes, as well as inhibitors of potential value as antibiotics, a series of ribitylpurinetriones bearing alkyl phosphate and α,α-difluorophosphonate substituents were synthesized. Since the purinetrione ring system and the ribityl hydroxyl groups can be alkylated, the synthesis required the generation of these two moieties in protected form before the desired alkylation reaction could be carried out. These substances were designed as intermediate analogue inhibitors of lumazine synthase that would bind to its phosphate-binding site. All of the compounds were found to be effective inhibitors of both Bacillus subtilis lumazine synthase as well as Escherichia coli riboflavin synthase. Molecular modeling of the binding of 3-(1,3,7,9-tetrahydro-9-D-ribityl-2,6,8-trioxopurin-7-yl)propane 1-phosphate provided a structural explanation for how these compounds are able to effectively inhibit both enzymes. Interestingly, the enzyme kinetics of these new compounds in comparison with the parent purinetrione demonstrated unexpectedly that the phosphate and phosphonate substituents contributed negatively to the binding. A possible explanation for these effects on lumazine synthase would be that the inorganic phosphate in the assay buffer competes with the substituted purinetriones for binding to the enzyme. This would be consistent with the observed increase in Kₘ of the 3,4-dihydroxybutanone-4-phosphate substrate from 5.2 μM in Tris buffer or from 6.7 μM in MOPS buffer to 50 μM in phosphate buffer when tested on Bacillus subtilis lumazine synthase. However, when tested in Tris buffer vs Mycobacterium tuberculosis lumazine synthase, three of the phosphate inhibitors displayed inhibition constants in the 4–5 nM range, indicating that they are much more potent than the parent purinetrione. Under these conditions, the phosphate moieties of the inhibitors do contribute positively to their binding. The α,α-difluorophosphonate analogue, which is expected to have enhanced metabolic stability relative to the phosphates, was also found to be an inhibitor of Mycobacterium tuberculosis lumazine synthase with a Kᵢ of 60 nM.

Although the intricate details remain to be established, the lumazine synthase-catalyzed reaction most likely proceeds along the mechanistic pathway outlined in Scheme 2. 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.

Introduction

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). In a wonderful example of efficient recycling catalyzed by lumazine synthase, the product 1 of the riboflavin synthase-catalyzed reaction is condensed with the four-carbon phosphate 2 to form the riboflavin synthase substrate 3. 1–5

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10.1021/jo030278k CCC: $27.50 © 2004 American Chemical Society
Published on Web 03/03/2004

which eliminates phosphate to yield the enol 6. Tautomeration of the enol 6 to the ketone 7, ring closure, and dehydration of the covalent hydrate 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. As shown in Figure 1, the observed conformation of the phosphonate side chain of 9 implies that the phosphate of 5 would be stabilized by hydrogen bonding to the Arg136 residue and to the neighboring Thr95 (not shown). However, the details of the isomerization of the trans Schiff base to a cis Schiff base, or possible initial formation of a thermodynamically less stable cis Schiff base, have not been established. In addition, the timing of the phosphate release relative to the conformational change of the side chain to form 7 is still an open question. For example, a case has recently been made for the movement of the whole phosphate side chain of 5 toward a cyclic conformation before phosphate elimination occurs.

The purinetrione 10 was recently synthesized as part of a program to obtain lumazine synthase inhibitors that might have value as structural probes of the active site of lumazine synthase. Compound 10 acted as a partial inhibitor of Bacillus subtilis lumazine synthase and displayed a Ki of 46 μM. In addition, it was a competitive inhibitor of Escherichia coli riboflavin synthase with a Ki of 0.61 μM. These Ki values indicated that the purinetrione 10 was among the most potent inhibitors of these two enzymes. The present research project was undertaken to test the hypothesis that the attachment of alkyl phosphate side chains to the N-7 nitrogen of the purinetrione 10 would position the phosphate near the phosphate-binding site of the enzyme and thus result in an increase in potency for inhibition of lumazine synthase. Since the exact linker chain length that would be most favorable was an open question, a homologous series of four phosphates were planned in which the linker length was varied from three to six carbons. The resulting inhibitors might also be of potential therapeutic value as antibiotics because certain Gram-negative pathogenic bacteria and yeasts lack an efficient riboflavin uptake.
system and are therefore absolutely dependent on endogenous riboflavin biosynthesis.11–14

Results and Discussion

Since the ribityl hydroxyl groups and the pyrimidinedione ring of 10 have the potential to be alkylated, the distillation of alkyl phosphate side chains on N-7 of the purinetrione system 10 requires that the ribityl substituent and the pyrimidinedione ring be generated in protected forms. Consequently, commercially available 6-chloro-2,4-dimethoxypyrimidine (11) was chosen as the starting material (Scheme 3). Our plan was to carry the dimethoxypyrimidine moiety through to a relatively late stage in the synthesis and then unmask the pyrimidinedione system under acidic conditions. This strategy would also allow the distillation and removal of tert-butylidimethylsilyl protecting groups for the alcohols.

Nitration of the starting material was performed as described in the literature with a mixture of fuming nitric acid and sulfuric acid to afford the nitrated intermediate 15. A nucleophilic aromatic substitution reaction with ribitylamine yielded the expected product 13. The four ribityl hydroxyl groups were then protected as their tert-butylidimethylsilyl ethers to provide compound 14. The pyrimidine system was converted to the substituted purine 15 through catalytic reduction of the nitro group to the corresponding amine over palladium on carbon. Reaction of the resulting primary amine with ethyl chloroformate, followed by heating the intermediate ethyl carbamate with sodium ethoxide in refluxing ethanol, provided the protected purine system 15. Reaction of 15 with 1,4-diiodobutane, 1,5-diiodopentane, and 1,6-diiodohexane in DMF, with K₂CO₃ as the base, provided the corresponding monoalkylated products 16, 17, and 18 in good yields. Nucleophilic displacement reactions of the three iodides with silver dibenzyl phosphate resulted in the phosphates 20, 21, and 22, having linker chain lengths of four, five, and six carbons.

The dibenzyl phosphate 19, having a linker chain length of three carbons, was obtained during the exploration of a slightly different route outlined in Scheme 4. This route is simply a reversal in the sequence of steps and involves nucleophilic displacement of one of the iodides of 1,3-diiodopropane with silver dibenzyl phosphate, followed by the displacement of the second iodide with intermediate 15 as the nucleophile to afford 19. This route was not tried out of necessity because the other sequence failed. It was simply tried in order to see if the reversed sequence of steps would work.

Selective deprotection of the ribityl side chains of 19–22 with hydrogen fluoride–pyridine in THF afforded the four tetraols 23–26.16–20 Interestingly, the potential conversion of the secondary alcohols to alkyl fluorides, which is reported to occur with hydrogen fluoride–pyridine in n-hexane, was not observed in the present case.21 Cleavage of the two methyl groups and the two benzyl groups in the penultimate intermediates 23–26 with HCl in refluxing methanol afforded the four desired target compounds 27–30.

Although the four phosphates 27–30 are interesting as potential lumazine synthase and riboflavin synthase inhibitors and as structural probes of the active sites of these enzymes, their potential use as antibiotics could be limited by the fact that phosphates are in general readily hydrolyzed by phosphatases. One approach to overcome this problem would be to replace the phosphate group in these compounds with an analogue that mimics the steric and electronic character of a phosphate, but is metabolically more stable. An α,α-difluorophosphonate seems like a good choice in the present case, since it would more closely mimic the acidity of a phosphate than a simple unfluorinated phosphate would.22,23 To test whether the enzyme inhibitory activity would be retained as a result of this replacement, the α,α-difluorophosphonate analogue of the least active riboflavin synthase inhibitor 28 was synthesized. The starting material 33 (Scheme 5) was prepared as described in the literature by reaction of the anion derived from diethyl difluoromethanephosphonate with 1,4-diiodobutane.24 Reaction of the protected purinetrione 15 with the iodide 33 in the

presence of potassium carbonate in DMF afforded the alkylation product 34. The TBDMS groups were removed from 34 with hydrogen fluoride-pyridine in THF, resulting in intermediate 35. Finally, the methyl ethers and ethyl esters were cleaved in the presence of trimethylsilyl iodide to yield the desired \( R, R' \)-difluorophosphate analogue 36. This transformation proceeded in higher overall yield than the conversions of 23–26 to 27–30 with HCl–MeOH. However, when TMSI was tried for the conversions of 23–26 to 27–30, the undesired cleavage of the phosphate groups to the corresponding alkyl iodides was observed.

The phosphates 27–30, as well as the \( \alpha,\alpha' \)-difluorophosphate analogue 36, were tested as inhibitors of recombinant B. subtilis lumazine synthase \( \beta_{60} \) capsids and recombinant riboflavin synthase from E. coli. The inhibition constants and types of inhibition displayed by these compounds are listed in Table 1, along with the previously published results of the structurally related phosphonates 37–40 and 9, the phosphate 41, and the purinetrione 10. The new phosphates 27–30 and the \( \alpha,\alpha' \)-difluorophosphate 36 are reaction intermediate analogues that contain structural elements corresponding to both of the substrates 1 and 2. Consequently, these

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**Scheme 3**

(a) fuming HNO\(_3\), H\(_2\)SO\(_4\), 80–85 °C (2.5 h); (b) ribitylamine, DMF, 23 °C (48 h); (c) TBDMSCl, imidazole, 23 °C (2 days); (d) (1) H\(_2\), Pd/C, MeOH, 23 °C (3 days), (2) EtO\(_2\)COCl, Et\(_3\)N, CH\(_2\)Cl\(_2\), 0 °C (6 h), (3) NaOEt, EtOH, reflux (24 h); (e) diiodoalkane, K\(_2\)CO\(_3\), DMF, 23 °C (24 h); (f) AgOPO(OBn)\(_2\), CH\(_3\)CN, reflux (12 h); (g) HF–Py, THF, 0–23 °C (19 h); (h) HCl, MeOH, reflux (3 h).

**Scheme 4**

(a) AgOPO(OBn)\(_2\), CH\(_3\)CN, reflux (10 h); (b) 15, K\(_2\)CO\(_3\), DMF, 23 °C (24 h).

**Scheme 5**

(a) K\(_2\)CO\(_3\), DMF, 23 °C (24 h); (b) HF–Py, THF, 0–23 °C (19 h); (c) TMSI, CH\(_2\)Cl\(_2\), 23 °C (24 h).

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TABLE 1. Inhibition Constants vs Bacillus subtilis Lumazine Synthase and Escherichia coli Riboflavin Synthase

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<tr>
<th>compd</th>
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<th>variable 2 concn</th>
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<td>37</td>
<td>$K_i$</td>
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<td>190 $\pm$ 39</td>
<td>$8200 \pm 3200$</td>
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<td></td>
<td>$K_{is}$</td>
<td>2000 $\pm$ 360</td>
<td>$&gt;1000$</td>
<td>$&gt;1000$</td>
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</table>

$^a$ Recombinant riboK capsids from B. subtilis. $^b$ Recombinant riboflavin synthase from E. coli. $^c$ The concentration of the dihydroxybutanone phosphate substrate was held constant at 100 $\mu$M during the assay, while the concentration of the pyrimidinedione substrate was varied. $^d$ The concentration of the pyrimidinedione substrate was held constant at 170 $\mu$M during the assay, while the concentration of the dihydroxybutanone phosphate was varied. $^e$ $K_i$ is the substrate dissociation constant for the equilibrium $E + S \rightarrow P$. $^f$ $K_{es}$ is the rate constant for the process $E + S \rightarrow P$. $^g$ $K_i$ is the inhibitor dissociation constant for the process $E + I \rightarrow EI$. $^h$ $K_{is}$ is the inhibitor dissociation constant for the process $E + S + I \rightarrow EI$.

Compounds can be classified as "bisubstrate analogue inhibitors" of lumazine synthase that could potentially achieve increased potency through an entropic advantage resulting from the linking of structural moieties that mimic each substrate. Since these compounds can potentially inhibit the binding of both substrates to lumazine synthase, it is possible that apparent differences in the inhibition mechanism and the inhibition constants could result from which substrate concentration is varied and which is held constant during the course of the enzyme inhibition experiments. To explore this question in more detail, two sets of inhibition experiments were conducted with lumazine synthase, one with variable pyrimidinedione concentration and one with variable dihydroxybutanone phosphate concentration. The results listed in Table 1 document an apparent change in inhibitory mechanism from partial inhibition with 27, or from competitive inhibition with 28, to uncompetitive inhibition as the substrate with the variable substrate concentration is switched from 1 to 2. Moreover, the
change in the substrate whose concentration is varied was found to have significant effects on the apparent $K_i$ values. For example, for the inhibition of lumazine synthase by compound 29, the $K_i$ value changed from 271 to 852 $\mu$M as the substrate with variable concentration changed from 1 to 2.

The $\alpha,\alpha$-difluorophosphonate analogue 36 ($K_{i}$ 132 $\mu$M) is similar to the corresponding phosphate 28 ($K_{i}$ 168 $\mu$M) in potency as an inhibitor of lumazine synthase. The phosphate 28 is an uncompetitive inhibitor of lumazine synthase, while the $\alpha,\alpha$-difluorophosphonate 36 is a partial inhibitor of lumazine synthase. In addition, it should be noted that the $\alpha,\alpha$-difluorophosphonate 36 was a significantly more potent competitive inhibitor of riboflavin synthase ($K_{i}$ 39 $\mu$M) than the corresponding phosphate 28 ($K_{i}$ 332 $\mu$M). The $\alpha,\alpha$-difluorophosphonate modification to increase metabolic stability therefore appears to offer the additional advantage of conferring greater activity in some of the present testing systems.

When the potencies of the phosphates 27–30 are compared using variable pyrimidinedione substrate 1 concentration, the most potent compound proved to be 27 ($K_{i}$ 41 $\mu$M), in which the phosphate is separated from the purinetrione ring system by a three-carbon linker. That was followed by 30 ($K_{i}$ 78 $\mu$M), 28 ($K_{i}$ 168 $\mu$M), and 29 ($K_{i}$ 271 $\mu$M). In comparison with the other phosphates, the three-carbon linker of 27 makes it the closest structural analogue of the reaction intermediate 5, and this may explain why it is the most potent lumazine synthase inhibitor.

With variable dihydroxybutanone phosphate substrate 2 concentration, the potencies of 29 ($K_{i}$ 852 $\mu$M) and 30 ($K_{i}$ 175 $\mu$M) can be compared with that of the parent purinetrione 10 ($K_{i}$ 46 $\mu$M), whose enzyme inhibition studies were performed previously with variable substrate 2 concentration. It is interesting to note that the potency of the parent compound 10 ($K_{i}$ 46 $\mu$M) is greater than that of either of the phosphates 29 or 30, indicating an unexpected opposite of the expected increase in potency anticipated through the incorporation of the phosphate side chains that could bind to the phosphate-binding site of lumazine synthase. One possible explanation for this effect would be that the enzyme kinetics experiments were carried out, as usual, in phosphate buffer, and the inorganic phosphate could conceivably bind to the phosphate-binding site of the enzyme and thus inhibit the binding of 29 and 30. To examine this hypothesis in more detail, the $K_m$ values for the binding of the dihydroxybutanone phosphate substrate were determined in 50 mM Tris [tris-(C-hydroxymethyl)aminomethane] buffer, 50 mM MOPS [3-(N-morpholino)propanesulfonic acid] buffer, and 50 mM phosphate buffer, all at pH 7. The $K_m$ values for the substrate 2 were $5.2 \pm 0.5$ $\mu$M in Tris buffer, $6.7 \pm 0.4$ $\mu$M in MOPS buffer, and $50 \pm 10$ $\mu$M in phosphate buffer. Inorganic phosphate therefore does appear to inhibit the binding of the substrate 2, and most likely the inhibitors 29 and 30, to lumazine synthase. This could possibly explain why the purinetrione 10 appears to be a better inhibitor than 29 or 30 in phosphate buffer, since the purinetrione does not bind to the phosphate-binding site of the enzyme.

Like the purinetrione 10, all of the new compounds 27 ($K_{i}$ 3.63 $\mu$M), 28 ($K_{i}$ 332 $\mu$M), 29 ($K_{i}$ 2.44 $\mu$M), 30 ($K_{i}$ 17.3 $\mu$M), and 36 ($K_{i}$ 39.4 $\mu$M) were competitive inhibitors of recombinant E. coli riboflavin synthase. However, they are all less potent inhibitors than the purinetrione 10 ($K_{i}$ 0.61 $\mu$M). On the other hand, the previously reported phosphonates 37, 9, and 38 were all inactive as riboflavin synthase inhibitors, so the present phosphate and $\alpha,\alpha$-difluorophosphonate replacements for the phosphonates of these compounds, when combined with attachment to a purinetrione system, do make a positive contribution to the enzyme inhibitory activity.

Finally, a comparison can be made between the present compounds and the previously reported pyrimidinedione inhibitors 39, 40, and 41 having an amide chain connected to phosphonate or phosphate moieties. In contrast to the present series, these amides were inactive or displayed very weak activity vs lumazine synthase, but were riboflavin synthase inhibitors in the K 160–190 $\mu$M range. All of these compounds are less potent than 27, 29, 30, and 36 vs both lumazine synthase and riboflavin synthase, indicating that the extension of the phosphate or phosphonate side chains from a purinetrione ring system is generally more favorable than connecting them to a pyrimidinedione system through an amide linkage. The one exception is the low activity ($K_{i}$ 332 $\mu$M) displayed by 28 vs riboflavin synthase.

Crystal structures are available of the complexes formed between the substrate analogue 42 and the lumazine synthases of B. subtilis and Schizosaccharomyces pombe. X-ray structures have also been determined of complexes of the intermediate analogue 9 bound

Saccharomyces cerevisiae lumazine synthase (Figure 1) and the product analogue 43 bound to S. pombe lumazine synthase.8,27 These structures allow the rational docking and energy minimization of additional lumazine synthase inhibitors. In the present case, a hypothetical model was constructed of the binding of the lumazine synthase/riboflavin synthase inhibitor 27 to B. subtilis lumazine synthase. This model was produced by overlapping the structure of 27 with that of 42 in a 15 Å radius spherical fragment surrounding the ligand in one of the sixty equivalent active sites of B. subtilis lumazine synthase.26 The structure of 42 was then removed and the energy of the complex minimized using the MMFF94 force field while allowing the ligand and the protein structure contained within a 6 Å sphere surrounding the ligand to remain flexible with the remainder of the protein structure frozen. The resulting Figure 2 was constructed by displaying the amino acid residues calculated to be involved in bonding of the protein with ligand 27, using a maximum distance of 3.5 Å between the donor and acceptor atoms to be considered a hydrogen bond. As expected, the calculated structure shows the purinetrione ring of the ligand 27 stacked with the phenyl ring of Phe22. The phosphate of the ligand is extensively hydrogen bonded with the side chain nitrogens of Arg127, as well as the backbone nitrogens of Ala85 and Thr86 and the side-chain hydroxyl of Thr86. The ribityl hydroxyl groups are hydrogen bonded to a water molecule, the side chain amino group of Lys135, the backbone carbonyl of Phe113, and the side chain carboxylate of Glu58. The purinetrione ring of the ligand is hydrogen bonded to the side-chain amino group of Lys135, the backbone nitrogen of Ala56, the backbone carbonyl of Thr80, and the side-chain nitrogen of Asn23. These contacts are summarized in Figure 3. It should be noted that these contacts are either similar or identical to those seen in the crystal structure of 42 bound to B. subtilis lumazine synthase.26

An X-ray structure of E. coli riboflavin synthase, which contains three identical subunits, has been published along with a hypothetical model of the bound substrate 28. According to these studies, as well as NMR investigations of the structure of the N-terminal domain of riboflavin synthase,29 the active site of the enzyme is derived from the intermolecular juxtaposition of residues belonging to both of the barrels in the C-terminal and N-terminal domains of the two monomer units, with one substrate...
molecule bound to each barrel. These structures allow molecular modeling of the complex formed between 27 and E. coli riboflavin synthase to proceed in a rational way. The structure displayed in Figure 4 was arrived at after docking two molecules of the ligand 27 in the active site and energy minimization using a frozen protein structure and two mobile ligand molecules. The N-barrel residues in Figure 4 are colored orange, while the C-barrel residues are green. As predicted from the known regiochemistry of the riboflavin synthase-catalyzed reaction and the available crystal structures, two molecules of the ligand are stacked in the active site with their ribityl chains pointing in opposite directions.3 The two phosphate side chains are therefore also pointing in opposite directions, and the model suggests the possibility of hydrogen bonding of each phosphate with some of the ribityl hydroxyl groups of the neighboring ligand molecule.

Human infections with drug-resistant Mycobacterium tuberculosis have been an ever-increasing worldwide health problem. New antibiotics that are effective against these resistant strains are therefore needed urgently. Consequently, the phosphates prepared in the present study were also tested vs M. tuberculosis lumazine synthase. Also, for the sake of comparison, the parent purinetrione 10 was examined, along with the three-carbon alcohol 46 corresponding to the most potent phosphate 27. The synthesis of 46 is described in Scheme 6. Alkylation of the starting material 15 with 1,3-diiodopropane in DMF in the presence of potassium carbonate as the base afforded the iodide 44, which was hydrolyzed to the alcohol 45 with sodium hydroxide in refluxing methanol. Removal of the tert-butyldimethylsilyl protecting groups and cleavage of the two methyl ethers present in 45 with methanolic hydrochloric acid yielded the desired product 46.

As a first step to the determination of the activities of the phosphates 27–30 vs M. tuberculosis lumazine synthase, an attempt was made to determine the $K_m$ of the phosphate substrate 2. This proved to be impossible in 100 mM phosphate buffer, because even at a substrate concentration of 1000 $\mu$M, the initial reaction velocity still increased linearly with concentration, indicating that the apparent $K_m$ for the substrate in 100 mM phosphate buffer is much greater than 1000 $\mu$M. In contrast, in MOPS buffer (50 mM MOPS pH 7.0, 100 mM NaCl, 5 mM EDTA, 5 mM DTT), maximal reaction velocity was reached by 400 $\mu$M phosphate substrate 2 concentration, and in Tris buffer (50 mM Tris–HCl pH 7.0, 100 mM NaCl, 5 mM EDTA, 5 mM DTT), maximal reaction velocity was reached by 250 $\mu$M phosphate substrate 2 concentration. Evidently, the M. tuberculosis lumazine synthase reaction rate is much more sensitive to inorganic phosphate than the B. subtilis enzyme is. For this reason, the subsequent studies on enzyme inhibition by the phosphates 27–30 were performed in Tris buffer, using a variable pyrimidinedione 1 concentration and a constant phosphate substrate 2 concentration. As shown

![Figure 4](image-url)

**Figure 4.** Hypothetical model of the binding of two molecules of inhibitor 27 to Escherichia coli riboflavin synthase. The C-barrel is orange and the N-barrel is green. Hydrogen atoms have been omitted for clarity. The model is programmed for walleyed viewing.

**Scheme 6**

\[
\begin{align*}
\text{Reagents and conditions:} & \quad \text{(a) 1,3-diiodopropane, } K_2 CO_3, \text{ DMF, } 23^\circ C (24 h); \quad \text{(b) NaOH, MeOH, reflux (12 h); \quad (c) HCl, MeOH, reflux (3 h).}
\end{align*}
\]

in Table 2, the resulting $K_i$ values of the phosphates 27–29 were all between 4 and 5 nM! The phosphate 30, having the longest phosphate side chain, was less active, displaying a $K_i$ of 0.17 µM. In contrast, the parent purinetrione 10 displayed a $K_i$ of 9.1 µM, the three-carbon alcohol had a $K_i$ of 2.6 µM. It is obvious that the phosphate moieties of the inhibitors 27–30 play an important role in increasing the affinity of these enzyme inhibitors for the $M$. tuberculosis lumazine synthase. It is also gratifying to see inhibition constants for the phosphates 27–29 in the low nanomolar range vs $M$. tuberculosis lumazine synthase. The crystal structure of $M$. tuberculosis lumazine synthase has not yet been determined, so the structural basis for the differences seen with this enzyme vs the $B$. subtilis enzyme remain to be elucidated.

As mentioned above, a potential limitation of the phosphates 27–30 as therapeutically useful antibiotics results from their expected metabolic instability toward phosphatases. Accordingly, the α,α-difluorophosphonate 36 was also tested as an inhibitor of $M$. tuberculosis lumazine synthase. As shown in Table 2, compound 36 displayed a $K_i$ of 60 nM, which is clearly in the range of interest for possible therapeutic use.

In conclusion, the results presented here demonstrate that certain purinetriones bearing phosphate side chains can inhibit both lumazine synthase as well as riboflavin synthase, and molecular modeling with compound 27 suggests possible binding modes to each enzyme. Antibiotics that would inhibit both lumazine synthase and riboflavin synthase would be less likely to suffer from the development of antibiotic resistance by the organisms that are supposed to treat, since pathogenic microorganisms would have to simultaneously select for mutations in both enzymes in order to escape the cytotoxic effects of the antibiotics.

### Experimental Section

**General Methods.** Melting points were determined in capillary tubes and are uncorrected. Proton nuclear magnetic resonance spectra (1H NMR) were determined at 300 or 500 MHz as noted. Phosphorus nuclear magnetic resonance (31P NMR) chemical shifts are reported relative to 85% H$_3$PO$_4$ as external standard. Silica gel used for column chromatography was 230–400 mesh.

**6-Chloro-2,4-dimethoxy-5-nitroprazinomycin (12).** Sulfuric acid (12.5 mL) was added dropwise to fuming nitric acid (7.5 mL) at 0 °C, and 6-chloro-2,4-dimethoxyprazinomycin (11) (5.00 g, 28.7 mmol) was added. The reaction mixture was stirred at 80–85 °C for 2.5 h, cooled to room temperature, poured into ice–water, and stirred for 20 min. The solid was filtered, washed three times with ice–water, and dried under reduced vacuum to give pure 12 as a yellow solid (4.00 g, 64%): mp 66–67 °C (lit.15 mp 67–69 °C); 1H NMR (300 MHz, CDCl$_3$) δ 4.07 (s, 3 H), 4.11 (s, 3 H).

**2,4-Dimethoxy-5-nitro-6-ribitylamino-2,4-dimethoxy-5-nitropyrimidinamine (13).** 6-Chloro-2,4-dimethoxy-5-nitropyrimidinamine (12) (5.84 g, 26.85 mmol) was added to a solution of ribitylamine (8.83 g, 58.55 mmol) in dry DMF (200 mL), and the mixture was stirred at room temperature for 48 h under nitrogen atmosphere. The solvent was removed using reduced pressure, and the resultant residue was purified using flash chromatography (30 g × 20–30 cm column of SiO$_2$, eluting with chloroform–methanol (10:1), to afford the desired product 13 (7.46 g, 75%) as a light yellow solid: mp 70–71 °C; IR (KBr) 3346, 1598 cm$^{-1}$; 1H NMR (300 MHz, CD$_2$OD) δ 4.02 (s, 3 H), 3.99 (s, 3 H), 4.01–3.92 (m, 2 H), 3.80–3.57 (m, 5 H); 13C NMR (75 MHz, CD$_2$OD) δ 168.4, 164.9, 160.3, 113.3, 79.4, 74.5, 74.2, 71.7, 64.5, 55.9, 55.8, 45.2; EIMS (MH$^+$) m/z 335. Anal. Calcld. for C$_{11}$H$_{18}$N$_4$O$_8$: C, 53.22; H, 5.43; N, 16.76. Found: C, 53.75; H, 5.32; N, 17.02.

**TABLE 2. Inhibition Constants vs Mycobacterium tuberculosis Lumazine Synthase**

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<th>compd</th>
<th>$K_i$ (µM)</th>
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<tr>
<td>26</td>
<td>2.6 ± 0.31</td>
<td>mixed</td>
</tr>
<tr>
<td>27</td>
<td>4.5 ± 3.3</td>
<td>competitive</td>
</tr>
<tr>
<td>28</td>
<td>4.1 ± 2.3</td>
<td>competitive</td>
</tr>
<tr>
<td>29</td>
<td>4.7 ± 1.9</td>
<td>competitive</td>
</tr>
<tr>
<td>30</td>
<td>170 ± 1.8 nM</td>
<td>competitive</td>
</tr>
<tr>
<td>36</td>
<td>60 ± 6.8 nM</td>
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*The experiments were conducted at a fixed substrate concentration of 150 µM and a variable substrate concentration of 1–400 µM.*

**Experimental Section**

**General Methods.** Melting points were determined in capillary tubes and are uncorrected. Proton nuclear magnetic resonance spectra (1H NMR) were determined at 300 or 500 MHz as noted. Phosphorus nuclear magnetic resonance (31P NMR) chemical shifts are reported relative to 85% H$_3$PO$_4$ as external standard. Silica gel used for column chromatography was 230–400 mesh.

**6-Chloro-2,4-dimethoxy-5-nitropyrimidinamine (12).** Sulfuric acid (12.5 mL) was added dropwise to fuming nitric acid (7.5 mL) at 0 °C, and 6-chloro-2,4-dimethoxyprazinomycin (11) (5.00 g, 28.7 mmol) was added. The reaction mixture was stirred at 80–85 °C for 2.5 h, cooled to room temperature, poured into ice–water, and stirred for 20 min. The solid was filtered, washed three times with ice–water, and dried under reduced vacuum to give pure 12 as a yellow solid (4.00 g, 64%): mp 66–67 °C (lit.15 mp 67–69 °C); 1H NMR (300 MHz, CDCl$_3$) δ 4.07 (s, 3 H), 4.11 (s, 3 H).

**2,4-Dimethoxy-5-nitro-6-ribitylamino-2,4-dimethoxy-5-nitropyrimidinamine (13).** 6-Chloro-2,4-dimethoxy-5-nitropyrimidinamine (12) (5.84 g, 26.85 mmol) was added to a solution of ribitylamine (8.83 g, 58.55 mmol) in dry DMF (200 mL), and the mixture was stirred at room temperature for 48 h under nitrogen atmosphere. The solvent was removed using reduced pressure, and the resultant residue was purified using flash chromatography (30 g × 20–30 cm column of SiO$_2$, eluting with chloroform–methanol (10:1), to afford the desired product 13 (7.46 g, 75%) as a light yellow solid: mp 70–71 °C; IR (KBr) 3346, 1598 cm$^{-1}$; 1H NMR (300 MHz, CD$_2$OD) δ 4.02 (s, 3 H), 3.99 (s, 3 H), 4.01–3.92 (m, 2 H), 3.80–3.57 (m, 5 H); 13C NMR (75 MHz, CD$_2$OD) δ 168.4, 164.9, 160.3, 113.3, 79.4, 74.5, 74.2, 71.7, 64.5, 55.9, 55.8, 45.2; EIMS (MH$^+$) m/z 335. Anal. Calcld. for C$_{11}$H$_{18}$N$_4$O$_8$: C, 53.22; H, 5.43; N, 16.76. Found: C, 53.75; H, 5.32; N, 17.02.

**TABLE 2. Inhibition Constants vs Mycobacterium tuberculosis Lumazine Synthase**

<table>
<thead>
<tr>
<th>compd</th>
<th>$K_i$ (µM)</th>
<th>mechanism</th>
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<tbody>
<tr>
<td>10</td>
<td>9.1 ± 0.61</td>
<td>competitive</td>
</tr>
<tr>
<td>26</td>
<td>2.6 ± 0.31</td>
<td>mixed</td>
</tr>
<tr>
<td>27</td>
<td>4.5 ± 3.3</td>
<td>competitive</td>
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</tr>
<tr>
<td>36</td>
<td>60 ± 6.8 nM</td>
<td>competitive</td>
</tr>
</tbody>
</table>

*The experiments were conducted at a fixed substrate concentration of 150 µM and a variable substrate concentration of 1–400 µM.*
diiodokane (2.25 mmol) were added, and the mixture was stirred at room temperature for 24 h. The DMF was removed under reduced pressure, and the residue was purified by flash chromatography (SiO₂), eluting with hexanes—EtOAc (95:5), to afford the pure iodides

Dibenzy1 4-[9-(2,3,4,5-Tetra-O-tert-butyldimethylsilyl-o-riboyl)-7,9-dihydro-2,6-dimethoxy-8-oxopurin-5-yl]-butane 1-Phosphate (20). Compound 20 was obtained by the procedure above in 83% yield as a colorless oil: ¹H NMR (300 MHz, CDCl₃) δ 7.31 (s, 10 H), 5.01 (m, 4 H), 4.47 (d, J = 9.9 Hz, 1 H), 4.30 (dd, J = 10.98, 13.90 Hz, 1 H), 4.06 (d, J = 1.78 Hz, 1 H), 4.02 (d, J = 2.1 Hz, 2 H), 3.99 (s, 1 H), 3.96 (s, 3 H), 3.96 (s, 3 H), 3.84 (m, 2 H), 3.77 (m, 1 H), 3.66 (dd, J = 5.60, 10.35 Hz, 1 H), 3.56 (dd, J = 5.8, 10.35 Hz, 1 H), 1.72 (q, 1 H), 0.94 (s, 9 H), 0.87 (s, 18 H), 0.63 (s, 9 H), 0.20 (s, 3 H), 0.11 (s, 6 H), 0.043 (s, 3 H), 0.041 (s, 3 H), −0.071 (s, 3 H), −0.52 (s, 3 H); EIMS (MH⁺) m/z 1119. Anal. Calcld for C₄₉H₆₃N₄O₁₄P₂S₄: C, 57.83; H, 8.55; N, 5.00. Found: C, 58.00; H, 8.65; N, 5.08.

Dibenzy1 5-[9-(2,3,4,5-Tetra-O-tet-t-butyldimethylsilyl-o-riboyl)-7,9-dihydro-2,6-dimethoxy-8-oxopurin-5-yl]-pentane 1-Phosphate (21). Intermediate 21 was obtained by the procedure above in 64% yield as a colorless oil: ¹H NMR (300 MHz, CDCl₃) δ 7.31 (s, 10 H), 5.09 (m, 4 H), 4.47 (dd, J = 10.14, 13.92 Hz, 1 H), 4.07 (d, J = 2.06 Hz, 1 H), 4.02 (br s, 1 H), 3.98 (s, 3 H), 3.96 (m, 1 H), 3.94 (m, 1 H), 3.93 (s, 3 H), 3.81 (m, 3 H), 3.67 (dd, J = 5.60, 10.45 Hz, 1 H), 3.56 (dd, J = 5.8, 10.35 Hz, 1 H), 1.62 (m, 4 H), 1.33 (m, 2 H), 0.94 (s, 9 H), 0.87 (s, 18 H), 0.64 (s, 9 H), 0.20 (s, 3 H), 0.107 (s, 6 H), 0.085 (s, 3 H), 0.042 (s, 3 H), 0.036 (s, 3 H), −0.060 (s, 3 H), −0.49 (s, 3 H); EIMS (MH⁺) m/z 1132. Anal. Calcld for C₅₀H₇₄N₄O₁₄P₂S₄: C, 58.30; H, 8.57; N, 4.95. Found: C, 58.28; H, 8.40; N, 4.49.
7.96. 31P NMR (121 MHz, CDCl 3) J 3 H), 3.94 (m, 3 H), 3.87 (s, 3 H), 3.85 (m, 3 H), 3.75 (br s, 2 H), 3.49 (br s, 1 H), 1.60 (m, 4 H), 1.30 (m, 2 H); 13C NMR (75 MHz, CDCl 3) J 27, 26.9, 25.6; EIMS (MNa +) m/z 713. Anal. Calcd for C 32H43N4O11P F 2: C, 52.50; H, 8.91; N, 5.44. Found: C, 52.11; H, 8.72; N, 5.33.

Diethyl 5-(7-Dihydro-2,6-dimethoxy-8-oxo-9-ribitylpurin-7-yl)-2-fluorophenylacetate (36). To a solution of 35 (0.309 g, 0.54 mmol) in dry THF (20 mL) was added anhydrous NaH to afford yellow precipitate. The NMR spectrum of the yellow precipitate was identical to that of 35. NMR (300 MHz, CDCl 3) J 3 H), 3.94 (m, 3 H), 3.85 (m, 3 H), 3.75 (br s, 2 H), 3.49 (br s, 1 H), 1.60 (m, 4 H), 1.30 (m, 2 H); 13C NMR (75 MHz, CDCl 3) J 27, 26.9, 25.6; EIMS (MNa +) m/z 713. Anal. Calcd for C 21H35F2N4OsO2P: C, 52.50; H, 8.91; N, 5.44. Found: C, 52.11; H, 8.72; N, 5.33.

Diethyl 1-(9-(2,4',5'-Tetra-O-tart-butyldimethylsilyl-beta-D-ribityl)-7,9-dihydro-2,6-dimethoxy-8-oxopurin-7-yl)-1,1-difluorophenylacetate (37). To a solution of 36 (0.309 g, 0.54 mmol) in dry THF (20 mL) was added anhydrous NaH to afford yellow precipitate. The NMR spectrum of the yellow precipitate was identical to that of 36. NMR (300 MHz, CDCl 3) J 3 H), 3.94 (m, 3 H), 3.85 (m, 3 H), 3.75 (br s, 2 H), 3.49 (br s, 1 H), 1.60 (m, 4 H), 1.30 (m, 2 H); 13C NMR (75 MHz, CDCl 3) J 27, 26.9, 25.6; EIMS (MNa +) m/z 713. Anal. Calcd for C 21H35F2N4OsO2P: C, 52.50; H, 8.91; N, 5.44. Found: C, 52.11; H, 8.72; N, 5.33.
at room temperature for 10 h and washed with CH₂Cl₂ (3 x 10 mL). The water layer was lyophilized, resulting in 36 as a colorless solid (0.235 g, 89%); mp 92–94 °C; 1H NMR (300 MHz, D₂O) δ 3.85 (m, 2 H), 3.73 (m, 3 H), 3.65 (m, 1 H), 3.57 (d, J = 2.89 Hz, 1 H), 3.53–3.42 (m, 2 H), 1.86 (m, 2 H), 1.56 (quint, J = 6.93 Hz, 2 H), 1.35 (quint, J = 6.29 Hz, 2 H); EIMS (MH⁺) m/z 489. Anal. Calcld for C₂₅H₃₃N₄F₂O₆: C, 55.41; H, 9.54; N, 6.63. Found: C, 55.12; H, 9.38; N, 6.46.

3-(2,3,4,5-Tetra-O-tet-butylmethylisilyl-1-ribityl)-7,9-dihydro-2,6-dimethoxy-8-oxopurin-7-yl-1-propanol (45). Compound 44 (238 mg, 0.25 mmol) was dissolved in a solution of 1 N NaOH – MeOH (1:1, 10 mL), and the mixture was heated at reflux for 12 h. The solvent was distilled off under reduced pressure and the residue was purified by flash chromatography (SiO₂), eluting with hexanes – ethyl acetate (5:1), to furnish compound 45 (160 mg, 75%) as a white solid: mp 188 °C; 1H NMR (300 MHz, CDCl₃) δ 9.91, 3.09 Hz, 1 H), 4.41 (m, 4 H), 4.00 (m, 7 H), 3.80 (m, 1 H), 3.68 (m, 1 H), 3.57 (m, 3 H), 1.96 (m, 2 H), 0.86 (m, 27 H), 0.59 (s, 9 H), 0.10–0.06 (m, 21 H), –0.51 (s, 3 H). Anal. Calcld for C₁₅H₂₃N₄F₂O₁₀P: C, 32.19; H, 4.26. Found: C, 32.10; H, 4.60.

3-(2,3,4,5-Tetra-O-tet-butylmethylisilyl-1-ribityl)-5,7-dihydro-2,4-dimethoxy-8-oxopurin-5-yl-propanol (46). Compound 45 (150 mg, 0.18 mmol) was dissolved in a solution of concd HCl – MeOH (1:1, 10 mL), and the mixture was heated at reflux for 3 h. The solvent was removed under reduced pressure, and the residue was reprecipitated from propanol. The solid was dried under vacuum to afford 46 (25 mg, 35%) as a white solid: mp 187–188 °C; 1H NMR (300 MHz, CDCl₃) δ 9.92 (m, 1 H), 7.95 (m, 2 H), 4.29 (m, 4 H), 4.03 (s, 3 H), 3.91 (m, 4 H), 3.74 (m, 1 H), 3.61 (m, 1 H), 3.47 (m, 1 H), 3.13 (t, J = 6.69 Hz, 2 H), 2.13 (m, 2 H), 0.84–0.77 (m, 27 H), 0.52 (s, 9 H), 0.06–0.05 (m, 18 H), 0.14 (s, 3 H), –0.59 (s, 3 H). Anal. Calcld for C₁₅H₂₃N₄O₅: C, 55.41; H, 9.54; N, 6.63. Found: C, 55.12; H, 9.38; N, 6.44.

Molecular Modeling on Lumazine Synthase. Using Sybyl (Tripos, Inc., version 6.9, 2002), the X-ray crystal structure of the complex of 5-nitro-6-ribitylamino-2,4-(1H,3H)-pyrimidinedione (42) with the lumazine synthase of B. subtilis (1RVV) was clipped to include information within a 15 Å radius of the 60 equivalent ligand molecules. The residues that were clipped in this cut complex were capped with either neutral amino or carboxyl groups. The structure of the inhibitor 27 was overlapped with the structure of 5-nitro-6-ribitylamino-2,4-(1H,3H)-pyrimidinedione (42), which was then deleted. Hydrogen atoms were added to the complex. MMFF94 charges were loaded, and the energy of the complex was minimized using the Powell method to a termination gradient of 0.05 kcal/mol while employing the MMFF94 force field. During the minimization of the complex, inhibitor 27 and the 6 Å sphere surrounding it were allowed to remain flexible, while the remaining portion of the complex was held rigid using the aggregate function. Figure 2 was constructed by displaying the amino acid residues of the enzyme that are involved in hydrogen bonding with the inhibitor 27. The maximum distance between donor and acceptor atoms contributing to the hydrogen bonds shown in Figure 1 was set to 3.5 Å.

Molecular Modeling on Riboflavin Synthase. Using Sybyl (Tripos, Inc., version 6.9, 2002), the X-ray crystal structure of E. coli riboflavin synthase (1I8D) was downloaded and two molecules of the ligand 27 were docked and oriented as suggested by the published model of the binding of two molecules of the substrate 1 in the active site, as well as by the structure of 43 bound to S. pombe riboflavin synthase. The C- and N-terminal groups were changed to neutral carboxylic acid and amino groups, and hydrogens were added to the protein structure and to the oxygen of the water molecules. MMFF94 charges were loaded, and the energy of a 6 Å radius spherical subset including and surrounding the two ligand molecules was minimized using the Powell method to a termination gradient of 0.05 kcal/mol while employing the MMFF94 force field. During energy minimization, the remaining protein structure was held rigid using the aggregate function. Figure 4 was constructed by displaying the amino acid residues in the C- and N-barrels surrounding the two ligand molecules.

Lumazine Synthase Assay. Reaction mixtures contained 100 mM potassium phosphate, pH 7.0, 5 mM EDTA, 5 mM diithiothreitol, inhibitor (0–500 μM), 170 μM 5-amino-6-ribitylamino-2,4(1H,3H)-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-butanoate-4-phosphate (2) to a final concentration of 50–400 μM. The formation of 6,7-dimethyl-8-ribityllumazine (3) was measured online with a computer-controlled photometer at 408 nm (ε₅₄₀ = 10 200 M⁻¹ cm⁻¹). The velocity–substrate data were fitted for all inhibitor concentrations with a nonlinear regression method using the program DynaFit.

Different inhibition models were considered for the calculation. K 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-butanoate-4-phosphate concentration (2) was kept constant at 100 μM and the concentration of the second substrate, 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione (1), was varied.

Riboflavin Synthase Assay. Reaction mixtures contained buffer (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 (ε₄₇₀ = 9100 M⁻¹ cm⁻¹). The K value was performed in the same manner as described above.

Acknowledgment. This research was made possible by NIH Grant No. GM 51469 as well as by support from the Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie.

Supporting Information Available: Procedures and analytical data for the synthesis of compounds 29 and 30. This material is available free of charge via the Internet at http://pubs.acs.org.

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