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Synthesis of *bis*-Phosphate Iminoaltritol Enantiomers and Structural Characterization with Adenine Phosphoribosyltransferase

Lawrence D. Harris^{*,†,‡}, Rajesh K. Harijan^{‡,||}, Rodrigo G. Ducati[‡], Gary B. Evans^{†,§}, Brett M. Hirsch[‡], and Vern L. Schramm^{*,‡}

[†]The Ferrier Research Institute, Victoria University of Wellington, 69 Gracefield Rd, Lower Hutt, 5010, New Zealand [‡]Department of Biochemistry, Albert Einstein College of Medicine, Bronx, New York 10461, United States [§]The Maurice Wilkins Centre for Molecular Biodiscovery, The University of Auckland, Auckland, New Zealand

Abstract

Phosphoribosyl transferases (PRTs) are essential in nucleotide synthesis and salvage, amino acid, and vitamin synthesis. Transition state analysis of several PRTs has demonstrated ribocation-like transition states with a partial positive charge residing on the pentose ring. Core chemistry for synthesis of transition state analogues related to the 5-phospho- α -D-ribose 1-pyrophosphate (PRPP) reactant of these enzymes could be developed by stereospecific placement of *bis*-phosphate groups on an iminoaltritol ring. Cationic character is provided by the imino group and the *bis*-phosphates anchor both the 1- and 5-phosphate binding sites. We provide a facile synthetic path to these molecules. Cyclic-nitrone redox methodology was applied to the stereocontrolled synthesis of three stereoisomers of a selectively monoprotected diol relevant to the synthesis of transition-state analogue inhibitors. These polyhydroxylated pyrrolidine natural product analogues were *bis*-phosphorylated to generate analogues of the ribocationic form of 5-phosphoribosyl 1-phosphate. A safe, high yielding synthesis of the key intermediate represents a new route to these transition state mimics. An enantiomeric pair of iminoaltritol *bis*-phosphates (L-DIAB and D-DIAB) was prepared and shown to display inhibition of *Plasmodium falciparum* orotate

*Corresponding Authors: lawrence.harris@vuw.ac.nz., vern.schramm@einstein.yu.edu.

^{||}L.D.H and R.K.H. contributed equally to this work.

Author Contributions

L.D.H. and G.B.E. conceived the synthetic routes and conducted all chemical synthesis and characterizations. R.K.H. conducted the crystallography experiments. R.G.D. and B.M.H. purified target proteins and assayed inhibitors. V.L.S. conceived the inhibitor experiments and interpreted results. All authors contributed to writing the manuscript.

ASSOCIATED CONTENT

Supporting Information

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Enzyme preparation, crystallization, and structure determination; crystallization and crystal handling; crystallographic data collection and refinement statistics; structural folds of ScAPRTs; superimposition of ScAPRT complexes; and supplementary results and discussion; experimental section for chemical syntheses; NMR spectra for synthetic compounds (PDF)

Accession Codes

The coordinates and structure factors are deposited at the Protein Data Bank with codes 5VJN and 5VJP.

Notes

The authors declare no competing financial interest.

phosphoribosyltransferase and *Saccharomyces cerevisiae* adenine phosphoribosyltransferase (ScAPRT). Crystallographic inhibitor binding analysis of L- and D-DIAB bound to the catalytic sites of ScAPRT demonstrates accommodation of both enantiomers by altered ring geometry and *bis*-phosphate catalytic site contacts.

Graphical Abstract

Essential steps in the synthetic pathways of purine and pyrimidine nucleotides, histidine and tryptophan, NAD^+ , purine and pyrimidine base salvage, tetrahydromethanopterin synthesis, and other secondary metabolites depend on the transfer of the ribose 5-phosphate group from 5-phospho- α -D-ribose 1-pyrophosphate (PRPP) to an acceptor molecule.¹ Several phosphoribosyltransferases have been subjected to transition state analysis by kinetic isotope effects and quantum chemical analysis to reveal ribocation-like transition states.^{2–5} The few transition state analogues described for phosphor-ibosyltransferases have been focused on the ribosylated product of the reaction rather than the PRPP ribosyl donor.^{5–8} We sought to provide ribocation transition state mimics of PRPP as potential inhibitor probes for this relatively untargated class of important biosynthetic enzymes. Transition state analogues are chemically stable mimics of the transition states of their cognate enzymes. For the PRPP transferases, the challenge is to make chemically stable mimics of PRPP, which is an intrinsically chemically unstable molecule. Iminoribitols are known transition state mimics for ribosyltransferases (Figure 1). Direct iminoribitol mimics of PRPP face a problem of chemical instability with substitution of the anomeric carbon of ribose with both imino and pyrophosphate groups. Therefore, we approached the synthesis by insertion of a methylene bridge between the anomeric carbon and phosphate group, to generate *bis*-phosphorylated derivatives of 2,5-dideoxyaltritol (Scheme 1). The synthesis of D- and L-2,5-dideoxy-2,5-imino-altritol 1,6-bisphosphate (D-DIAB and L-DIAB) are described together with inhibition profiles for several phosphoribosyltransferases and the crystal structures of *Saccharomyces cerevisiae* adenine phosphoribosyltransferase (ScAPRT) in complex with both enantiomers.

Five-membered cyclic nitrones add versatile functionality in the synthesis of cyclic amines. For example, the syntheses of pyrrolidine alkaloids, aza-sugars, and other natural products rely on the robustness of the transformations available to these nitrones.^{9–13} A general strategy for the synthesis of nitrones that allows facile access to many diastereoisomers of the iminosugar motif has been the focus of several studies.^{14–16}

We have reported several strategies to incorporate five-membered *N*-heterocycles and iminosugars into small-molecule, rationally designed, transition-state analogue inhibitors of ribosyltransferases implicated in cancer, infectious diseases, gout, and gastric ulcers.¹⁷ Here,

we expand the approach to synthesize *bis*-phosphate D-DIAB (Scheme 1), a putative transition-state analogue inhibitor of phosphoribosyltransferases. Inhibitors designed to inhibit activity of glycosidases have occasionally shown surprising levels of activity for their enantiomers.^{18–21} Thus, we also developed an efficient synthesis of enantiomer L-DIAB. We previously reported the synthesis of alcohol **2** (Scheme 1) with the 2,5-*cis*-dihydroxymethyl configuration, from iminoribitol imine **5a** by addition of a lithiated dithiane, hydrolysis, and reduction.²² This is a long and difficult synthesis. A facile route to **2** is desirable. We considered the previously synthesized nitrone **6a**²³ as a superior electrophile, but safety is a concern in the use of selenium dioxide/acetone/hydrogen peroxide²⁴ in its synthesis—especially for large scale reactions. A nitrone/ hydroxylamine/amine redox-strategy^{12,25,26} (Scheme 1) would use iminoribitol derivative **7** as a starting point for a number of stereoisomers and eliminate the need to resynthesize each stereoisomer from different sugars.¹⁴

With the exception of a few literature examples,²⁷ the 2,5-*trans*-hydroxymethyl-configuration (*cf.* target D-DIAB), resulting from stereoselective additions to cyclic-nitrones or imines of this type, has been installed in one of two ways: (i) where the configuration of the 3-position induces the favored 2,3-*trans*-relationship¹² or (ii) where the configuration of the 2-position has been inverted following addition of a suitable nucleophile. We envisioned a strategy employing reoxidation^{12,13,28} of a vinyl-adduct of hydroxylamine after addition of Grignard^{29–31} to nitrone **6a**, with subsequent oxidation/reduction (redox) steps, which could afford the desired 2,5-*trans*-configuration in compound **4** (Scheme 1). The intermediate α,β -unsaturated ketonitrone **3** in this case would double-up as a useful 2,5-*trans*-hydroxymethyl (2,3-*cis*-hydroxymethyl) synthon. An alternative synthon for this purpose has been utilized by Marradi and colleagues.²⁸ However, the harsh conditions required for deprotection of this protected-hydroxymethyl equivalent limits the synthetic utility of the synthon in subsequent transformations; hence, we felt the vinyl group would allow for a wider range of functional group manipulations. We report, herein, expedient syntheses of two enantiomeric *bis*-phosphates D-DIAB and L-DIAB and improved synthesis of alcohol **2** from a common intermediate: iminoribitol derivative **7**.

We also investigated the effects on enzyme catalysis and structural properties upon binding of the *bis*-phosphate transition-state analogue inhibitors to phosphoribosyltransferases adenine phosphoribosyltransferase (APRT; EC 2.4.2.7) and orotate phosphoribosyltransferase (OPRT; EC 2.4.2.10). These are examples of key enzymes in purine salvage and pyrimidine metabolism found among many organisms. The phosphoribosyltransferases catalyze the Mg²⁺-dependent reversible transfer of the 5-phosphoribosyl moiety from α -D-5-phosphoribosyl 1-pyrophosphate (PRPP) to adenine or orotate, resulting in the formation of adenosine mono-phosphate (AMP) or orotidine monophosphate (OMP) and inorganic pyrophosphate (PP_i).^{32–34}

RESULTS AND DISCUSSION

Synthesis of nitrone **6a**²³ was substantially improved by implementing the catalytic methylrhenium trioxide (MTO)^{35–37} oxidation of secondary amines to oxidize iminoribitol **7** (Scheme 2). Nitrones **6a** (72%) and **6b** (18%) were easily separable by a combination of

recrystallization and chromatography in an excellent combined yield. We have successfully performed the MTO oxidation on a >20 g scale.

Nucleophilic addition of vinyl-Grignard to nitrone **6a** was highly efficient, yielding hydroxylamine **10** (Scheme 2) quantitatively and with complete stereoselectivity as expected on the basis of previous results.^{29,30} Reduction of the crude hydroxylamine afforded the free secondary amine **11**, which could be protected with Boc (**12a**) or Cbz (**12b**) groups. Catalytic osmium tetroxide-mediated dihydroxylation of alkenes **12a** and **12b**, diol-cleavage using NaIO₄ and reduction by NaBH₄, yielded diols **2a** and **2b** in 80% and 73% yields, respectively, over six steps from nitrone **6a**. The practical ease and enhanced safety profile of this route coupled with the high yields marks a vast improvement on the previously reported synthesis of alcohol **2a** from iminoribitol **7**.²²

Next, we turned our attention to the synthesis of *bis*-phosphate D-DIAB (Scheme 3). Conversion of hydroxylamine **10** to the vinyl-nitrone **3** was achieved in moderate yield by catalytic MTO oxidation (36%)³⁸ and was improved to 49% by utilizing stoichiometric TEMPO as the oxidant,¹³ while a standard MnO₂ oxidation protocol proved sluggish. Vinyl-nitrone **3** was a bench-stable solid.

Vinyl-nitrones similar to **3** have been synthesized by modified Stille coupling of thioimides³⁹ or by aldol-type reactions of α -methyl-substituted nitrones.⁴⁰ The α,β -unsaturated ketonitrone is an interesting and relatively unexplored functional group,⁴¹ whose chemistry appears to be limited to addition to the ketonitrone functionality,⁴² cycloadditions,⁴⁰ and unusual rearrangement reactions.⁴¹ Here, the osmium tetroxide-mediated dihydroxylation of the vinyl-nitrone **3** with subsequent cleavage by periodate and reduction by sodium borohydride yielded alcohol **13** with the desired configuration, presumably *via* the α -formyl-nitrone species.⁴³ Thus, vinyl-nitrones—in this constrained bicyclic system—can furnish the α -hydroxymethyl amine after this redox sequence. The isopropylidene unit stabilizes such systems,³⁹ and we have found that applying this type of nitrone redox chemistry to less constrained five-membered nitrogen heterocycles can be capricious. When vinyl-nitrone **3** was treated with sodium borohydride, saturated ethyl compound **19** was obtained, indicating conjugate addition of hydride to the vinyl-nitrone had occurred. Presumably rapid tautomerization of the resulting ene-hydroxylamine intermediate regenerates the nitrone, albeit in low yield.

Zinc-mediated reduction of hydroxylamine **13**, *N*-Boc-protection, and silyl-deprotection furnished diol **16** in 63% yield over three steps (Scheme 3). To prepare the *bis*-phosphate ester, we chose to employ standard phosphoramidite coupling. Initially, double installation of di-*tert*-butyl phosphate was attempted *via* di-*tert*-butyl-*N,N*-diisopropylphosphoramidite with *in situ* oxidation by *m*-CPBA, for a putative single-step, final, global deprotection. However, the product contained impurities, some of which were identified as the *H*-phosphonates.⁴⁴ These *H*-phosphonates may have arisen from facile β -elimination of the intermediate phosphite species, driven by the steric strain of the crowded environment of the 1,2,3-*cis*-setup. The installation of the *tert*-butyl phosphate by exactly this method is entirely possible in a less-crowded environment.⁵ Thus, benzyl phosphate esters were installed instead, by the same phosphoramidite methodology. With the β -elimination pathway no

longer available to the intermediate dibenzyl-phosphite, the *bis*-phosphate ester **17** could be prepared by this method with a yield of 91%. Hydrogenation of the *bis*-dibenzyl phosphate ester followed by global hydrolysis afforded *bis*-phosphate D-DIAB.

In order to synthesize *bis*-phosphate L-DIAB, nitron **6b** was reduced with NaBH₄, and hydroxylamine **20** was isolated cleanly from the reaction mixture in 96% yield (Scheme 4). Once again, this reduction was completely stereoselective. The MTO oxidation of hydroxylamine **20** yielded a chromatographically inseparable 1:1 mixture of nitrones **8** and **6b** in 76% yield, along with 21% of recovered starting material. Vinyl magnesium bromide was added to the inseparable mixture **8/6b** to afford a separable mixture of hydroxylamine adducts **21a/b** in 82% combined yield. Hydroxylamine **21a** was reduced with zinc and *N*-Boc-protected to afford alkene **23**, in 70% yield over two steps, which was then dihydroxylated, cleaved, and reduced to give alcohol **24** in 72% yield over three steps. Silyl-group deprotection of alcohol **24** by TBAF gave diol *ent*-16 ($[\alpha]_D^{20} -51.6$ ($c = 1.55$, CHCl₃)), whose NMR spectroscopic data were identical to its enantiomer **16** ($[\alpha]_D^{20} + 48.2$ ($c = 1.25$, CHCl₃)). Elaboration of diol *ent*-16 to *bis*-phosphate L-DIAB was identical to the sequence described for *bis*-phosphate D-DIAB (*vide supra*).

Enzyme Inhibition Assays.

Recombinant *N*-terminal 6-His-tagged *Saccharomyces cerevisiae* (ScAPRT) was purified to homogeneity using a Ni-NTA His-tag affinity column, yielding approximately 60 mg of active enzyme from 20 g of cell culture.⁴⁵ Enzyme activity was assessed by use of a recently published direct and sensitive fluorimetric assay, where 2,6-diaminopurine replaced adenine, the natural substrate (Figure 2).⁴⁶

Fitting the inhibition data to the equation for competitive inhibition yielded K_i values of $8.7 \pm 1.5 \mu\text{M}$ for D-DIAB and $14.9 \pm 2.9 \mu\text{M}$ for L-DIAB (Table 1). These K_i values are lower than the previously reported $20 \mu\text{M}$ K_M for PRPP.⁴⁵ Thus, both stereoisomers are good inhibitors, binding better than the normal PRPP substrate.

The *bis*-phosphates D-DIAB and L-DIAB were also assayed for inhibition of *Plasmodium falciparum* (Pf) and human (*Homo sapiens*, Hs) orotate phosphoribosyltransferases (OPRTs). Both *bis*-phosphates inhibit PfOPRT and display 3-fold selectivity for D-DIAB (Table 1). HsOPRT showed no apparent inhibition at D-DIAB or L-DIAB concentrations of 200 μM (Table 1).

Transition state analogues for purine nucleoside phosphorylases (PNPs), 5'-methylthioadenosine phosphorylase (MTAP), and 5'-methylthioadenosine nucleosidase (MTAN) have also been synthesized in distinct enantiomeric forms to examine their differential interaction with target enzymes. In the cases of human PNP, MTAP, and *E. coli* MTAN, the relative affinities were 1200, 9600 and 2250 more tightly bound, respectively, for the correct (3*R*,4*S*) configuration than for the incorrect (3*S*,4*R*) enantiomers of tight-binding inhibitors.⁴⁷ D- and L-DIAB differ from tight-binding inhibitors by only a 2- to 3-fold binding discrimination for PfOPRT and ScAPRT. A distinguishing difference between these inhibitor classes is that the former inhibitors occupy the complete array of catalytic site

interactions related to the transition state, while the D- and L-DIAB molecules reflect binding only to the PRPP domain.

Structure Determination and Overall Structure.

ScAPRT crystallized from $(\text{NH}_4)_2\text{SO}_4$ had only sulfate at the active sites. D-DIAB and L-DIAB binding was obtained by ligand soaking (SI, Table S1). The molecular replacement using PHASER for structure determination resulted in a dimer model in the C2 space group.⁴⁸ Electron density for the N-terminal His₆ tag was not observed in either structure. Electron density was also not observed for loop II (residues Glu104 to Asp110) of chain B in the D-DIAB bound structure and the same loop of chain-A of the L-DIAB bound structure. All other amino acid residues were readily fitted into the electron density map. The densities of D-DIAB and L-DIAB are clearly defined in the structures. All resolved amino acid residues are in the most favored or are in the allowed regions of the Ramachandran plot (Table S2).

ScAPRT belongs to the type-I PRTs and is a homodimer (Figure 3 and Figure S1). Each monomer consists of five α -helices and nine β -sheets. The enzyme has two domains: domain 1 serves as a hood domain (residues 1 to 35) and a core domain (residues 36 to 178). The hood domain of ScAPRT contains α -helices (α 1, 3–13; and α 2, 31–35) and β -sheets (β 1, 14–18; and β 2, 25–30). The core domain of the enzyme consists of five parallel β -sheets (β 3, 61–67; β 4, 83–90; β 7, 123–132; β 8, 150–161; and β 9, 173–178) and three α -helices (α 3, 37–54; α 4, 69–81; and α 5, 136–148; Figure 3 and S1). The loop between two of the β -sheets (β 5, 97–104; β 6, 109–115) of the core domain is the catalytic loop.⁴⁵ In the crystal structure, the transition-state inhibitors (D-DIAB and L-DIAB) are bound at the active sites of both subunits of the ScAPRT (Figures 4 and 5). Crystal soaking experiments with 1 mM adenine together with D-DIAB and L-DIAB gave adenine bound only to chain-A and chain-B subunits, respectively (Figures 3 and 5). In the adenine-free sites, the symmetry-related Tyr107 side chain occupies the adenine binding pocket. No magnesium ion density was seen at the active sites of any of the inhibitor-bound structures, despite the presence of 5 mM MgCl_2 in the ligand soak solutions.

Binding of D-DIAB, L-DIAB and Adenine.

ScAPRT with D-DIAB has been solved to 1.78 Å resolution (Figure S1). D-DIAB is bound in the active site with low B-factors, similar to the surrounding protein residues. The O2P of D-DIAB is hydrogen bonded to water-144 (Wat144). O3 makes hydrogen bond interactions with Wat60 and (OD1)Asp130. O4 of D-DIAB makes hydrogen bond interactions with Wat29 and (OD2)Asp129. The O4P has hydrogen bond interactions with Wat88 and (OG1)Thr134. The oxygen of O5P is hydrogen bonded to Wat24. Hydrogen bond partners for O6P include (OG)Ser137 and Wat213. The nitrogen atom N1 of the iminosugar ring has hydrogen bond interactions with Wat203 (Figure 5).

L-DIAB in complex with ScAPRT was solved to 1.98 Å resolution. L-DIAB is bound to the ScAPRT active site with contacts similar to D-DIAB, but in the reverse orientation (Figure S1). The interactions of L-DIAB with the enzyme are similar to the D-DIAB bound structure, except there are fewer hydrogen bond interactions in the L-DIAB-bound structure. The oxygen of O1P is hydrogen bonded to Wat14. The O2P has hydrogen bond interactions

with Wat85 and (OG1)-Thr134. O3P is in hydrogen bond distance to (OG)Ser137 and (N)Ser137. O3 of L-DIAB is in hydrogen bond interactions with Wat77 and (OD2)Asp129. O4 is hydrogen bonded to Wat41 and (OD1)Asp130. The nitrogen atom N1 of the iminoaltritol ring has hydrogen bond interactions with Wat19 (Figure 5). In the L-DIAB structure O4P, O5P, and O6P of L-DIAB do not make favorable hydrogen bond interactions with ScAPRT (Figure 5).

The distance between the reactive N9 of adenine (or C9 in 9-deazaadenine) and the carbons of D-DIAB and L-DIAB corresponding to the reactive anomeric carbon of PRPP are an index of the inhibitor fit at the catalytic site. ScAPRT complexes with adenine and D-DIAB or L-DIAB can be compared with human (PDB ID: 1ZN7) and *Giardia lamblia* APRTase (PDB ID: 1L1R) in complexes with adenine (or 9-deazaadenine) and PRPP. The adenine binding mode is similar in all the APRTase structures. In human APRTase, the distance between N9 of adenine and reactive C1' of PRPP is 3.7 Å. This distance is within experimental uncertainty in the structure of adenine with D-DIAB in ScAPRT (3.8 Å). With 9-deazaadenine and PRPP in *G. lamblia* APRT, the distance is 3.4 Å; closer, presumably due to the transition state character of 9-deazaadenine. However, when adenine and L-DIAB are bound to ScAPRT, the ring pucker of the complex adjusts to the unfavorable configuration, causing the carbon corresponding to the anomeric carbon of bound L-DIAB structures to be 4.4 Å from the anomeric carbon position. The carbons corresponding to the anomeric carbon (C1) of PRPP correspond to C2 and C5 of D-DIAB and L-DIAB, respectively (Scheme 1; Figure 4).

Crystal soaks with the DIAB isomers and adenine reveal the adenine binding pocket adjacent to D-DIAB (only in chain-A) and L-DIAB (only in chain-B). With D-DIAB, the adenine ring is sandwiched between the hydrophobic side chains of Phe27 and Ile131. The exocyclic N6 amino group is interacting with the peptide bond carbonyl of Leu26 and Wat14, while the N7 interacts with Wat191. The N7-Wat191 interaction is proposed to activate the N9 of adenine for attack of C1' on PRPP in catalysis. In the *Giardia lamblia* APRT, N7 protonation for the N9 activation role is proposed to be due to a catalytic site Glu100.⁴⁹ Similar interactions are also observed in the ScAPRT complex with L-DIAB (Figure 5). Adenine binds to only one of the two subunits, leaving the adjacent adenine-free binding pockets occupied by a symmetry-related Tyr107 side chain, suggesting subunit-sequential catalytic site activity.

Structural Basis of ScAPRT Inhibition.

Although D-DIAB and L-DIAB bind to give similar interactions at the active site of ScAPRT, L- and D-DIAB bind in reverse orientations with respect to the ScAPRT active site. The configuration of the 2', 3', 4', and 5' carbons differs for the inhibitors, causing the iminoaltritol ring to bind differently (Figure 5). Kinetic inhibition assays gave K_i values of $8.7 \pm 1.5 \mu\text{M}$ and $14.9 \pm 2.9 \mu\text{M}$, respectively, for D-DIAB and L-DIAB, despite the different binding modes. Interactions of D-DIAB to the active site of ScAPRT are slightly more favorable than L-DIAB. The N1, O4P, O5P, and O6P of L-DIAB are not in hydrogen bond interactions, a possible explanation for the weaker binding of L-DIAB.

Structural Comparisons.

The crystal structure of native unliganded (PDB ID: 1G2Q) and sulfate-bound structures (PDB ID: 1G2P) are known.⁴⁵ When compared to the unliganded ScAPRT structure (1G2Q), loop II (105–108) of the inhibitor bound structures is moved 5.8 Å toward the active site. A mutation in this loop (*e.g.*, E106L) decreased k_{cat} by 10^5 ; therefore, motion of loop II toward the active site is forming the catalytically competent structure.⁴⁵ Loop II is disordered in the sulfate-bound structure (1G2P; Figures 3 and S2). The loop between $\beta 1$ and $\beta 2$ (loop I) of the hood domain retracts 1.5 Å, and its position is distinct from the catalytic site. The loop from residues 160–168 (loop III) has an open conformation in the inhibitor bound (5VJN and 5VJP) and in the sulfate-bound structures (1G2P), whereas this loop has a closed conformation in the unliganded structure (1G2Q). Loop III is moved 7.8 Å away from the active site in D-DIAB, L-DIAB, and sulfate bound structures (Figures 3 and S2). These structural changes indicate that loops II and III are important in catalytic site organization.

3. CONCLUSIONS

We have highlighted the synthetic utility and flexibility of the nitron-redox methodology with iminosugars to form informative inhibitors for phosphoribosyltransferases. The redox methodology is particularly effective in the rigid, bicyclic system with the isopropylidene protecting group in place, which allows completely stereoselective hydride reductions of the nitron group and imparts additional stability.³⁵ As such, the commonly utilized iminoribitol 7– with a defined initial configuration—has led, with a careful choice of redox and nucleophilic addition strategies, to three different stereoisomers (**2a**, **15**, and **24**) of a selectively monoprotected diol. Two of these diastereoisomers (**15** and **24**) were elaborated to *bis*-phosphates D-DIAB and L-DIAB, which were shown to display weak inhibition of human OPRT, significant binding to PfOPRT, and substantial inhibition of ScAPRT. The crystal structure of D-DIAB and L-DIAB bound ScAPRT structures shows that both inhibitors bind to the enzyme active site with favorable enzyme–inhibitor interactions. We have also described significant improvements to the previously published synthesis of alcohol **2a**,^{22,23} an intermediate utilized to prepare numerous transition-state analogue inhibitors.¹⁷ In addition, compound **3**—an unusual vinyl-nitron species has demonstrated functional utility as a *trans*-hydroxymethyl synthon in the stereoselective addition to nitron **6a**.

METHODS

Recombinant Protein Expression and Purification.

Recombinant N-terminal 6× His-tagged ScAPRT was expressed and purified as previously described,⁴⁵ to >95% purity with modifications (see SI).

ScAPRT Inhibition Assays.

Enzyme activity assays for ScAPRT were performed at 25 °C in 50 mM HEPES at pH 7.5 in 250 μL total reaction volumes, and each individual initial rate was the average of triplicate measurements. Reaction mixtures contained 2 mM MgCl_2 , 250 μM 2,6-diaminopurine (2,6-

DAP, a fluorescent and easily quantitated product),⁴⁶ 250 μ M PRPP, and 1 mM DTT and were initiated by the addition of ScAPRT (100 nM). DAP consumption was monitored by a change in fluorescence (Ex: 280 nm/Em: 345 nm) over the course of 1 h using a SpectraMax M5 plate reader (Molecular Devices), at variable concentrations of either D-DIAB or L-DIAB (0–200 μ M). Inhibition data for *bis*-phosphates D-DIAB and L-DIAB with OPRTs contained 500 μ M orotidine 5'-monophosphate, 50 mM Tris-HCl, at pH 8.0, 5 mM MgCl₂, 1 mM pyrophosphate, and 25 nM PfOPRT or 200 nM HsOPRT at 25 °C. Reaction rates were monitored at 266 nm. Initial reaction rates (triplicate) were used to calculate the K_i values.

Crystallographic Inhibitor Binding and Structure Determination.

ScAPRT was used for the crystallographic inhibitor binding studies with D-DIAB and L-DIAB. Protein (10 mg mL⁻¹) was incubated with 2 equiv of D-DIAB and L-DIAB separately for 2 h on ice. The cocrystallization of the incubated protein–ligand mixtures was then screened by the sitting drop vapor diffusion crystallization method at 22 °C using the Microlytic (MCSG1–4) crystallization conditions as described in the SI, Table S1. The X-ray diffraction data collection, data processing, structure determination, refinement, and structure analysis are detailed in the SI.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Ribocationic transition states for human purine nucleoside phosphorylase (PNP), *Plasmodium falciparum* hypoxanthine-guanine-xanthine phosphoribosyltransferase, their transition state analogues, and the rationale for synthesis of the bis-phosphate iminoaltritol for other phosphoribosyltransferases (PRTases).

Figure 2.

Reaction catalyzed by ScAPRT monitored by the direct and sensitive decrease in fluorescence signal as 2,6-diaminopurine is converted to 2-amino-AMP in the absence or presence of D-DIAB or L-DIAB.

Figure 3.

Subunit structures of ScAPRT complexes with D-DIAB and L-DIAB. (A) Structural fold and superposition of the ScAPRT-D-DIAB (brick red) and ScAPRT-L-DIAB complexes (light blue). Loop I is from Asn19 to Gly24. Loop II is from Lys105 to Gly108, and loop III is from Asp163 to Lys165. B and C are the structural superposition of ScAPRT-D-DIAB (brick red) and ScAPRT-L-DIAB (light blue), respectively with (B) unliganded ScAPRT (cyan) and (C) ScAPRT-sulfate bound (pink). (D) Superimposition of ScAPRT-D-DIAB (brick red) and ScAPRT-L-DIAB (light blue) with ScAPRT-sulfate bound (pink), showing no significant loop movement in these structures.

Figure 4.

Stereoview omit maps of D- and L-DIAB at the active sites of ScAPRT (A, B) and atomic numbering of the enantiomers in the crystal structures (C, D). (A) The omit (Fo–Fc) difference electron density map of the D-DIAB structure from subunit A. D-DIAB is added to the map as a stick model. (B) The omit (Fo–Fc) difference electron density map of the L-DIAB structure with L-DIAB added as a stick model. The (Fo–Fc) difference maps were calculated after 15 cycles of omit refinement by REFMAC5, leaving out the active site ligand. The contour levels are at 2.5σ for the D-DIAB (PDB code 5VJN) and the L-DIAB complexes (PDB code 5VJP). Atomic numbering of the D-DIAB (C) and L-DIAB (D) used in the crystal structure contacts.

Figure 5. Stereoviews of the active site of ScAPRT complexed with D-DIAB (subunit A) and L-DIAB (subunit B). Panels A and B are the active site residue interactions with D-DIAB and L-DIAB, respectively. Hydrogen bond interactions are shown by dotted lines. The red spheres are water molecules interacting with D-DIAB and L-DIAB. (C) Superimposition of ScAPRT catalytic sites with bound D-DIAB and L-DIAB. The displacement of the imino-altritol rings is apparent. The superimposed sulfate from the ScAPRT-sulfate structure shows its position in the active site corresponding to the β -1-phosphate of the PRPP pyrophosphate.

Scheme 1.
Targets and Retrosynthetic Analysis

Scheme 2.

Synthesis of Nitrones *via* MTO and Improved Synthesis of Alcohol 2^a

^a*Reagents and conditions:* (i) MTO (0.2 mol %), H₂O₂ (30%) in CH₂Cl₂, r.t. then MeOH, 15 min, r.t. then dropwise addition of 7 in CH₂Cl₂, 0 °C, 0.5 h, 72% for **6a** and 18% for **6b**. (ii) Vinyl magnesium bromide, THF, −70 to 0 °C. (iii) Zn, AcOH, r.t., 4–16 h. (iv) Boc₂O, MeOH, r.t., 1 h, 85% over 3 steps for **12a** or CbzCl, K₂CO₃, PhMe, H₂O, r.t., 1–3 h, 84% over 3 steps for **12b**. (v) OsO₄ (4 mol %), NMO, MeCN, H₂O, r.t., 6 h. (vi) (1) NaIO₄, H₂O, EtOH, r.t., then (2) NaBH₄, r.t., 5 min to 1 h, 93% over 3 steps for **2a** and 87% over 3 steps for **2b**.

Scheme 3.

Synthesis of *bis*-phosphate D-DIAB^a

^a*Reagents and conditions:* (i) MTO (0.2 mol %), H₂O₂ (30%) in CH₂Cl₂, r.t. then MeOH, 15 min, r.t. then dropwise addition of **10** in CH₂Cl₂, 0 °C, 0.5 h, 36%. (ii) TEMPO, CH₂Cl₂, r.t., 10 min, 49%. (iii) OsO₄ (4 mol %), NMO, MeCN, H₂O, r.t., 6 h. (iv) (1) NaIO₄, H₂O, EtOH, r.t., then (2) NaBH₄, r.t., 5 min to 1 h, 47% over 3 steps. (v) Zn, AcOH, r.t., 4–16 h, quant. (vi) Boc₂O, MeOH, r.t., 1 h, 74%. (vii) TBAF, THF, r.t., 85%. (viii) (1) *N,N*-diisopropylidibenzylphosphoramidite, CH₂Cl₂, r.t., 3 h, Ar(g) then (2) *m*-CPBA, −40 to 0 °C, 1 h, 91%. (ix) H₂, 10% Pd/C, MeOH, r.t., 16 h; (x) MeOH, conc. HCl, r.t., 20 min, 76% over 3 steps. (xi) NaBH₄, MeOH, r.t., 30 min, 27%.

Scheme 4. Synthesis of *bis*-Phosphate L-DIAB^a

^a*Reagents and conditions:* (i) NaBH₄, MeOH, r.t., 30 min, 96%. (ii) MTO (0.2 mol %), H₂O₂ (30%) in CH₂Cl₂, r.t. then MeOH, 15 min, r.t., then dropwise addition of **20** in CH₂Cl₂, 0 °C, 0.5 h, 76% (recovered 21% of **20**). (iii) vinyl magnesium bromide, THF, −70 to 0 °C, 82% (combined yield; isolated yields 46% for **21a**, 31% for **21b**). (iv) Zn, AcOH, r.t., 4–16 h. (v) Boc₂O, MeOH, r.t., 1 h, 70% over 2 steps. (vi) OsO₄ (4 mol %), NMO, MeCN, H₂O, r.t., 6 h. (vii) (1) NaIO₄, H₂O, EtOH, r.t., then (2) NaBH₄, r.t., 5 min to 1 h, 72% over 3 steps. (viii) TBAF, THF, 16 h, 72%. (ix) (1) *N,N*-diisopropylidenedibenzylphosphoramidite, CH₂Cl₂, r.t., 3 h, Ar(g), then (2) *m*-CPBA, −40 to 0 °C, 1 h, 58%. (x) H₂, 10% Pd/C, MeOH, r.t., 16 h. (xi) MeOH, conc. HCl, r.t., 20 min.

Table 1.Inhibition Data for *bis*-Phosphates D-DIAB and L-DIAB^a

compound	K_i (ScAPRT/ μ M)	K_i (PfOPRT/ μ M)	K_i (HsOPRT/ μ M)
D-DIAB	8.7 ± 1.5	39 ± 4	>200
L-DIAB	14.9 ± 2.9	103 ± 17	>200

^a Initial reaction rates were used to calculate the K_i values for both compounds in triplicate.