Synthesis, Biological Activity and Molecular Modeling of 6-Benzylthioinosine Analogues as Subversive Substrates of *Toxoplasma gondii* Adenosine Kinase

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Toxoplasma gondii is the most common cause of secondary CNS infections in immunocompromised persons such as AIDS patients. The major route of adenosine metabolism in *T. gondii* is direct phosphorylation to adenosine 5′-monophosphate (AMP) catalyzed by the enzyme adenosine kinase (EC 2.7.1.20). Adenosine kinase in *T. gondii* is significantly more active than any other purine salvage enzyme in this parasite and has been established as a potential chemotherapeutic target for the treatment of toxoplasmosis. Subversive substrates of *T. gondii*, but not the human, adenosine kinase are preferentially metabolized to their monophosphorylated forms and become selectively toxic to the parasites but not their host. 6-Benzylthioinosine (BTI) was identified as an excellent subversive substrate of *T. gondii* adenosine kinase. Herein, we report the synthesis of new analogues of BTI as subversive substrates for *T. gondii* adenosine kinase. These new subversive substrates were synthesized starting from tribenzoyl protected D-ribose. To accomplish the lead optimization process, a divergent and focused combinatorial library was synthesized using a polymer-supported trityl group at the 5′-position. The combinatorial library of 20 compounds gave several compounds more active than BTI. Structure−activity relationship studies showed that substitution at the para position plays a crucial role. To investigate the reasons for this discrimination, substrates with different substituents at the para position were studied by molecular modeling using Monte Carlo Conformational Search followed by energy minimization of the enzyme−ligand complex.

Introduction

Toxoplasma gondii is an intracellular parasitic protozoan belonging to the phylum Apicomplexa. The parasite has a high incidence of infections in humans as well as in warm-blooded animals. Approximately 1 billion people worldwide are seropositive to *T. gondii*, including more than 60 million people in the United States.[1] Infection with *T. gondii* is asymptomatic unless the host immune system is seriously compromised such as in AIDS, organ transplantation, or unborn children of infected mothers.[2] Toxoplasmosis is the most common and life-threatening infection of the central nervous system,[2,3] and its therapy has not changed in the past three decades. Sulfonamides and pyrimethamine are two drugs widely used to treat toxoplasmosis in humans. Although these drugs are helpful in the acute stage of the disease, usually they do not eradicate infection. Furthermore, in over half of AIDS patients, prolonged exposure to this regimen can induce severe side-effects such as bone marrow suppression and severe skin rashes.[4−6] Hence, there is a critical need to develop new and effective drugs with significant low host toxicity.

Rational drug design is usually based on exploitation of biochemical and physiological differences between pathogen and host. One potential target for chemotherapeutic intervention against *T. gondii* is purine metabolism.[6,7] These parasites replicate rapidly and require large amounts of purines for the synthesis of nucleic acids and other vital components.[6−8] However, *T. gondii* is a purine auxotroph and hence must rely on purine salvage from the host.[7] Furthermore, since *T. gondii* can convert adenine to guanine nucleotides, but not the reverse, only salvaged adenine, adenosine, hypoxanthine, or inosine can fulfill all of its purine requirements for nucleic acid synthesis.[6,9] Adenosine is incorporated into the nucleotide pools of *T. gondii* at a 10-fold higher rate than any other purine nucleobase or nucleoside.[6,9] The major route of adenosine metabolism in *T. gondii* is direct phosphorylation to AMP, from which all other purine nucleotides can be synthesized. This reaction is catalyzed by adenosine kinase (EC 2.7.1.20), which is almost 10 times more active than any other purine salvage enzyme in these parasites.[6] In mammalian cells, adenosine is catabolized to hypoxanthine by the sequential reactions of adenosine deaminase (EC 3.5.4.4) and purine nucleoside phosphorylase (EC 2.4.2.1), respectively.[7,11] Neither of these enzymes have any appreciable activity in *T. gondii*.[6]

T. gondii adenosine kinase has been established as an excellent potential chemotherapeutic target for the treatment of toxoplasmosis.[7,10−12] Structure−activity relationships as well as biochemical, metabolic, and molecular studies established that the substrate specificity as well as other characteristics of *T. gondii* adenosine kinase differ significantly from those of the human enzyme.[7,10−12] These studies have also demon-

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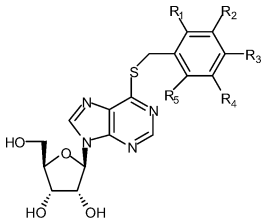
Table 1. Binding Affinity (apparent K_m) of Substituted 6-Benzylthioinosine Analogues, 6-benzylthioinosine, and Adenosine to *Toxoplasma gondii* Adenosine Kinase



compound	R_1	R_2	R_3	R_4	R_5	K_m [μM]
6a	CH_3	H	H	H	H	1.5 ± 0.6
6b	F	H	H	H	H	*a*
6c	Cl	H	H	H	H	1.4 ± 0.2
6d	H	CH_3	H	H	H	1.3 ± 0.6
6e	H	CF_3	H	H	H	2.9 ± 1.3
6f	H	NO_2	H	H	H	3.0 ± 1.6
6g	H	H	CH_3	H	H	3.3 ± 1.1
6h	H	H	OCF_3	H	H	140.7 ± 8.3
6i	H	H	Br	H	H	7.5 ± 2.6
6j	H	H	OCH_3	H	H	2.6 ± 0.4
6k	H	H	*tert*-butyl	H	H	112.8 ± 11.7
6l	H	H	$COOCH_3$	H	H	96.1 ± 1.6
6m	H	H	Cl	H	H	2.1 ± 1.0
6n	H	H	NO_2	H	H	1.1 ± 0.2
6o	H	H	CN	H	H	0.9 ± 0.1
6p	H	H	F	H	H	1.2 ± 0.3
6q	Cl	H	Cl	H	H	0.7 ± 0.2
6r	Cl	H	H	H	F	37.7 ± 2.3
6s	H	Cl	Cl	H	H	2.5 ± 1.3
6t	CH_3	H	CH_3	H	CH_3	149.6 ± 2.7
6-benzylthioinosine	H	H	H	H	H	2.4 ± 1.2
adenosine	–	–	–	–	–	2.4 ± 0.2

[a] Could not be determined.

strated that 6-benzylthioinosine (BTI) is a substrate for the parasite but not the human adenosine kinase.[7,10,11] Furthermore, BTI was shown to be preferentially metabolized to its monophosphorylated form and becomes selectively toxic to *T. gondii* but not their host, thereby acting as a subversive substrate.[7,10,11] BTI and a few other members (**6a**, **6d**, **6g**, and **6f**) of this class of compounds have been previously synthesized and tested as inhibitors of nucleoside transport systems in mammalian cells.[13] However, none of these compounds were shown to enter into or metabolized by uninfected mammalian cells.[7,11–14] In view of the fact that BTI is a substrate for the parasite adenosine kinase (but not the host) and becomes selectively toxic to *T. gondii*,[7,10,11] modification of the chemical structure of BTI could further potentiate its antitoxoplasmic efficacy. Therefore, we have focused on optimizing this lead compound by combinatorial synthesis with the aim of designing more potent subversive substrates for the *T. gondii* adenosine kinase. Herein we report the synthesis of a new class of BTI analogues that act as potent and selective subversive substrates for *T. gondii* but not the human, adenosine kinase.

Results and Discussion

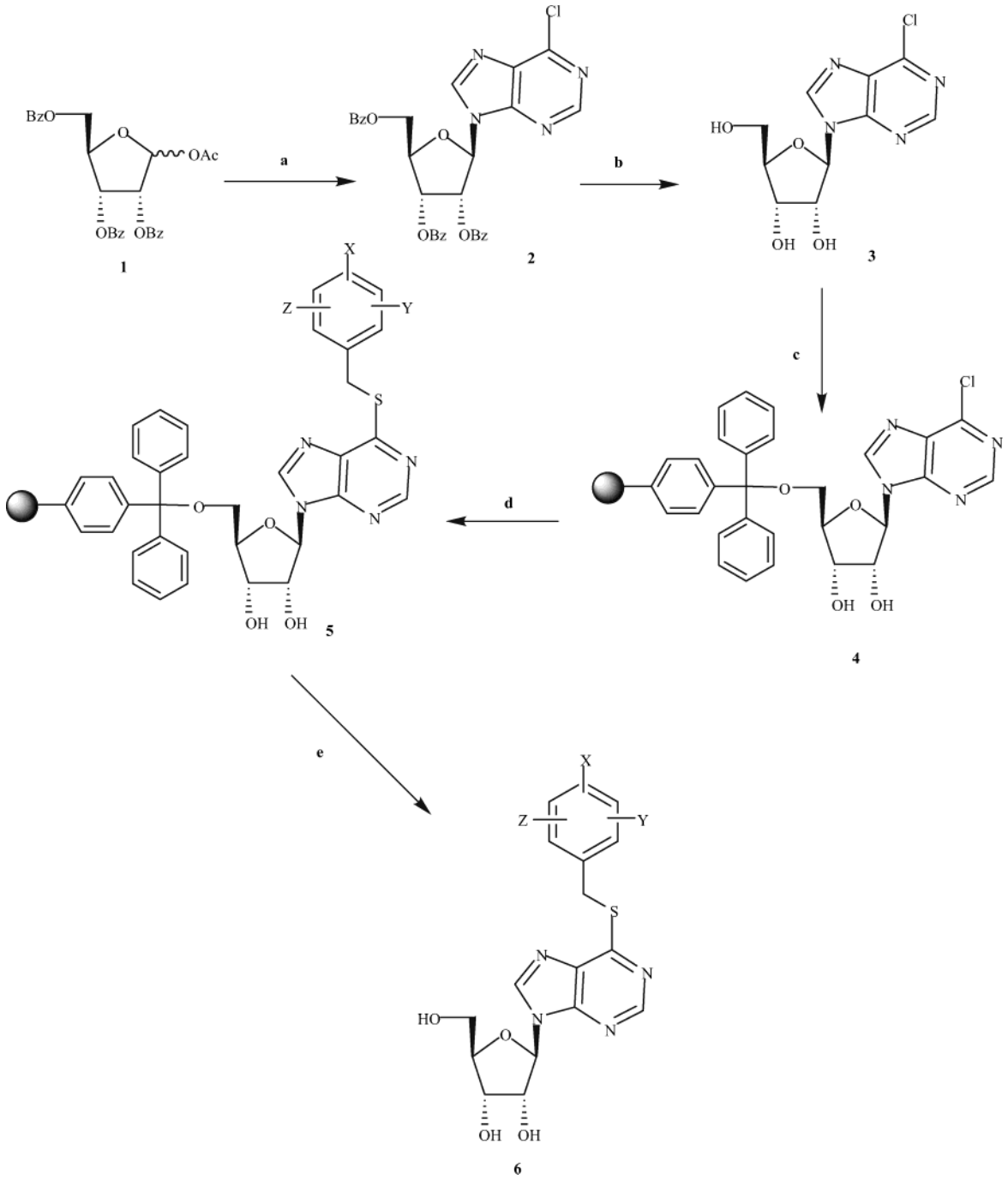
Chemistry. For the synthesis of 6-substituted 9-β-D-ribofuranosylpurine analogues, we chose a straightforward strategy amenable to combinatorial synthesis (Scheme 1), which can be adaptable for a combinatorial synthesis. The sugar 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-D-ribofuranose (**1**) was condensed[15] with 6-chloropurine to obtain 6-chloro-9-(2,3,5,-tri-*O*-benzoyl-β-D-ribofuranosyl)-9*H*-purine **(2)**. Subsequent treatment with methanolic ammonia gave 6-chloro-9-β-D-ribofuranosyl-9*H*-purine (**3**), which on coupling[16] with trityl chloride resin gave the key intermediate **4**. Nucleophilic displacement with the appropriate thiols[17] led the resin-linked 6-(substituted benzylthio)purine **5**. Cleavage from the resin with 2% trifluoroacetic acid and 5% triisopropylsilane[18] afforded the nearly pure 6-(substituted benzylthio)-purines **6** in excellent overall yield.

Structure–Activity Relationships. The binding affinities (apparent K_m values) of the newly synthesized BTI analogues to *T. gondii* and human adenosine kinase were evaluated in enzyme-based assays. The binding affinity of adenosine and BTI were also evaluated as a reference for the newly synthesized BTI analogues. BTI and its analogues did not bind to the human hepatic enzyme. On the other hand, several BTI analogues were identified as better substrates of the *T. gondii* adenosine kinase than the natural substrate (adenosine). The apparent K_m values of adenosine, BTI and the BTI analogues are reported in Table 1.

There were no differences in the biological activities of electron-rich and electron-deficient substitution at the ortho position, for example, compounds **6a** (CH_3) and **6c** (Cl). At the meta position, there seems to be a very slight preference for electron-rich substituents over electron-deficient substituents. However, these differences may be within the limits of the experimental error for the biological assay. The substitution at the para position provided more significant changes in activity compared to any other position. The para position was more amenable to modifications, which seemed to be affected by both the hydrophobicity and the electronegativity of the substituent. In the case of halogens, the increase in the electronegativity of the halogen was accompanied by increased binding to the enzyme (compounds **6i**, **6m** and **6p**). Steric bulk of the substitution at the para position appeared to play a critical role in binding. Increasing the bulkiness of substitution decreased the binding drastically (compounds **6k** and **6l**). In general, electron-deficient substituents were preferred (**6m**, **6n**, **6o**, and **6p**). In the case of multiple substitutions, it was noted that substitutions at both the ortho and para positions (**6q**) were more favorable than those on meta and para positions (**6s**). In the case of triple substitutions, the 2,4,6-trimethyl analogue (**6t**) showed drastic loss of activity, whereas a single methyl substitution at the ortho, meta, or para positions exhibited appreciable activities (compounds **6a**, **6d,** and **6g**).

Antitoxoplasmic Activity. The two best substrates, **6o** and **6q**, and the two weakest substrates, **6h** and **6k** (Table 1), were evaluated as potential antitoxoplasmic agents in cell-based assays using wild type (RH) and adenosine kinase deficient (TgAK^{-3}) strains of *T. gondii*. As a positive control, pyrimethamine and sulfadiazine, the standard chemotherapeutic agents used in the treatment of toxoplasmosis, were also evaluated. It is evident from Table 2 that all the tested compounds, **6o**, **6q**, **6h**, and **6k** tested were effective against infection with the wild-type parasites albeit to different degrees. Compounds **6o** and **6q** were more effective than **6h** and **6k**. None of these compounds, however, was effective against infection with the adenosine kinase deficient strain (TgAK^{-3}). These results demonstrate that the

Scheme 1[a]



[a] Reagents and conditions: (a) 6-chloropurine, hexamethyldisilazane, $(Me)_3SiCl$, $(Me)_3SiTfa$; (b) methanolic NH_3, overnight; (c) trityl resin, (anhydrous) pyridine, 2 days; (d) TEA, substituted benzyl thiol, 55 °C; (e) 2% TFA, 5% TIS, CH_2Cl_2 (anhydrous).

presence of *T. gondii* adenosine kinase is a requirement for these compounds to exert their antitoxoplasmic effect. Therefore, these compounds are substrates for *T. gondii* adenosine kinase in vivo as was the case with the in vitro enzyme assays. It should also be noticed that, the effectiveness of these compounds in vivo (Table 2) appears to be strongly correlated with their binding affinity (apparent K_m values) to the parasite adenosine kinase (Table 1). Furthermore, Table 2 also shows that all four compounds, **6o**, **6q**, **6h**, and **6k**, had no toxic side-effect on the survival of uninfected host cells, indicating that host toxicity is of little concern for these compounds. The lack of host toxicity is due to at least two factors. First, such 6-substituted compounds are not substrates for the human adenosine kinase.[7,10,11] Thus, no toxic nucleotides were formed in the absence of the parasite enzyme. Second, the newly synthesized compounds are analogues of *p*-nitrobenzylthioinosine (NBMPR), a known inhibitor of nucleoside transport in mammalian cells, which is not transported or metabolized by uninfected host cells.[7,11,12] Therefore, the newly synthesized compounds may act as NBMPR and not gain entry to uninfected host cells to exert toxic side-effects. Finally, Table 2 shows that the two compounds (**6o** and **6q**) were equivalent or better than sulfadiazine and/or pyrimethamine.

Molecular Modeling. Prior to the current studies, preliminary structure−activity relationship studies have

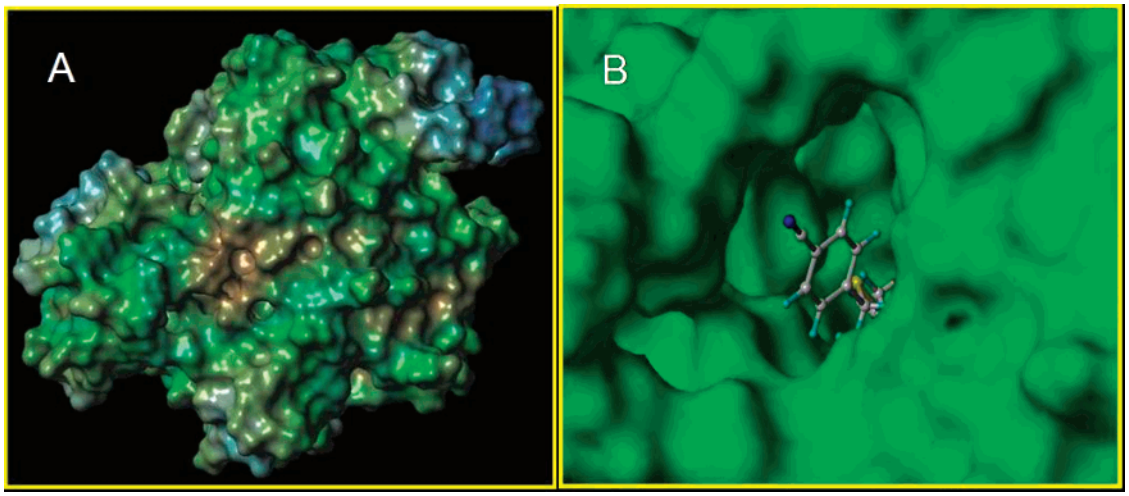


Figure 1. (a) Connolly surface representation of *Toxoplasma gondii* adenosine kinase with its hydrophobic potential mapped on it. Notice the dark brown spot representing the hydrophobic patch on the enzyme surface. This is the hydrophobic region where benzyl substituents of the ligands position themselves. (b) Docked 6-benzylthioinosine (BTI) analogue in *T. gondii* adenosine kinase.

Table 2. Effect of Different Substituted 6-(Substitutedbenzylthio)Purine Ribosides Analogues, and Known Therapeutic Compounds on Percent Survival[a] of Wild Type (RH) and Adenosine Kinase Deficient (TgAK^{-3}) Strains of *Toxoplasma gondii* Grown in Human Fibroblasts in Culture

		concentration [μM]				
compound	infection	0	5	10	25	50
6q	wild type (RH)	100	94.8	79.1	34.5	0
	TgAK^{-3}	100	100	100	100	100
	none[b]	100	98.1	99.2	94.0	99.5
6o	wild type (RH)	100	61.5	45.2	7.56	0
	TgAK^{-3}	100	100	100	100	100
	none[b]	100	97.4	96.9	100	99.7
6h	wild type (RH)	100	93.1	64.9	54.5	44.0
	TgAK^{-3}	100	100	100	97.6	95.1
	none	100	100	99.9	97.4	98.6
6k	wild type (RH)	100	83.3	71.7	47.2	12.2
	TgAK^{-3}	100	100	100	100	99.9
	none	100	99.7	100	99.1	97.1
sulfadiazine	wild type (RH)	100	75.7	64.5	59.2	46.6
	none[b]	100	98.2	99.7	99.8	100
pyrimethamine	wild type (RH)	100	53.8	36.3	11.8	6.9
	none[b]	100	100	98.8	100	100

[a] Survival of parasites was measured by incorporation of [5,6-^{3}H]uracil as previously described by el Kouni et al.[11] [b] Survival of uninfected human fibroblasts was measured by the MTT method as previously described.[11]

been performed by el Kouni and co-workers,[10] and from these preliminary studies several *T. gondii* adenosine kinase ligands have been identified, including 7-iodotubercidin[10] which is quite potent but cytotoxic and nonselective inhibitor.[19] A complex of 7-iodotubercidin bound to *T. gondii* adenosine kinase has also been reported.[20] The important findings from the detailed study of these reported crystal structures of the complexes coupled with the detailed structure−activity relationship analysis were the following: (a) 2′-OH and 3′-OH are crucial for binding. (b) Two hydrophobic pockets exist, one at the S^6 region and another at N^7 region. (c) The size of the hydrophobic pocket is larger at S^6 as compared to that at N^7.

T. gondii adenosine kinase is a 363 residue monomeric protein that catalyzes the transfer of the γ-phosphate group from ATP to adenosine to form ADP and AMP, respectively.[20] The protein consists of two domains: a large domain (α/β) and a small domain (α/β). The substrates and the products are bound at the domain interfaces or the hinge. This binding pocket is subdivided into three regions, the ribose binding region, the purine binding region, and the hydrophobic region. The ribose binding region is mostly hydrophilic and provides the anchoring of the adenosine in the proper orientation by forming a network of hydrogen bonds with residues Asp24, Gly69, and Asn73. The purine binding region is hydrophobic in its property and it also forms hydrogen bonds with the surrounding residues, with most of the binding mediated via trapped water molecules. It has been hypothesized[20] that residues Leu46, Leu142, Tyr169, and Phe201 line the putative binding pocket in the apo form of the enzyme and present the hydrophobic surface for the binding of the adenosine. Hence, we propose that this hydrophobic interaction is strengthened when the residues actually move into the pocket and results in the hinge bending. During adenosine binding, Gly68 and Gly69 side chains undergo conformational changes, causing domain movements and binding pocket formation. Because of these simultaneous movements, there are a few extra residues which add to the initial set of hydrophobic residues to ultimately define the binding pocket (Figure 1). Residues Ile22, Ala44, Thr45, Leu46, Leu138, Thr140, Leu142, Tyr169, Thr172, Ala199, and Phe201 along with Leu205 form a cavity that accommodates the purine base. These include the same set of residues, which line the putative binding pocket in the apo state and subsequently form the hydrophobic region of the adenosine binding pocket in the complexed structure.

In our studies, four compounds were selected for docking calculations with the *T. gondii* adenosine kinase. The natural substrate (adenosine), the lead compound (BTI) and the two most active compounds, 6-(4-cyanobenzylthio)-9-β-D-ribofuranosylpurine (**6o**) and 6-(2,4-dichlorobenzylthio)-9-β-D-ribofuranosylpurine (**6q**). The lowest energy conformation of various BTI analogues as obtained from the MC/EM studies is shown in Figure 2. The MC/EM method predicts the binding mode of the natural substrate adenosine with fairly high accuracy (with RMSD of 0.006 Å from the reported crystal structure). In the case of BTI, the benzyl group

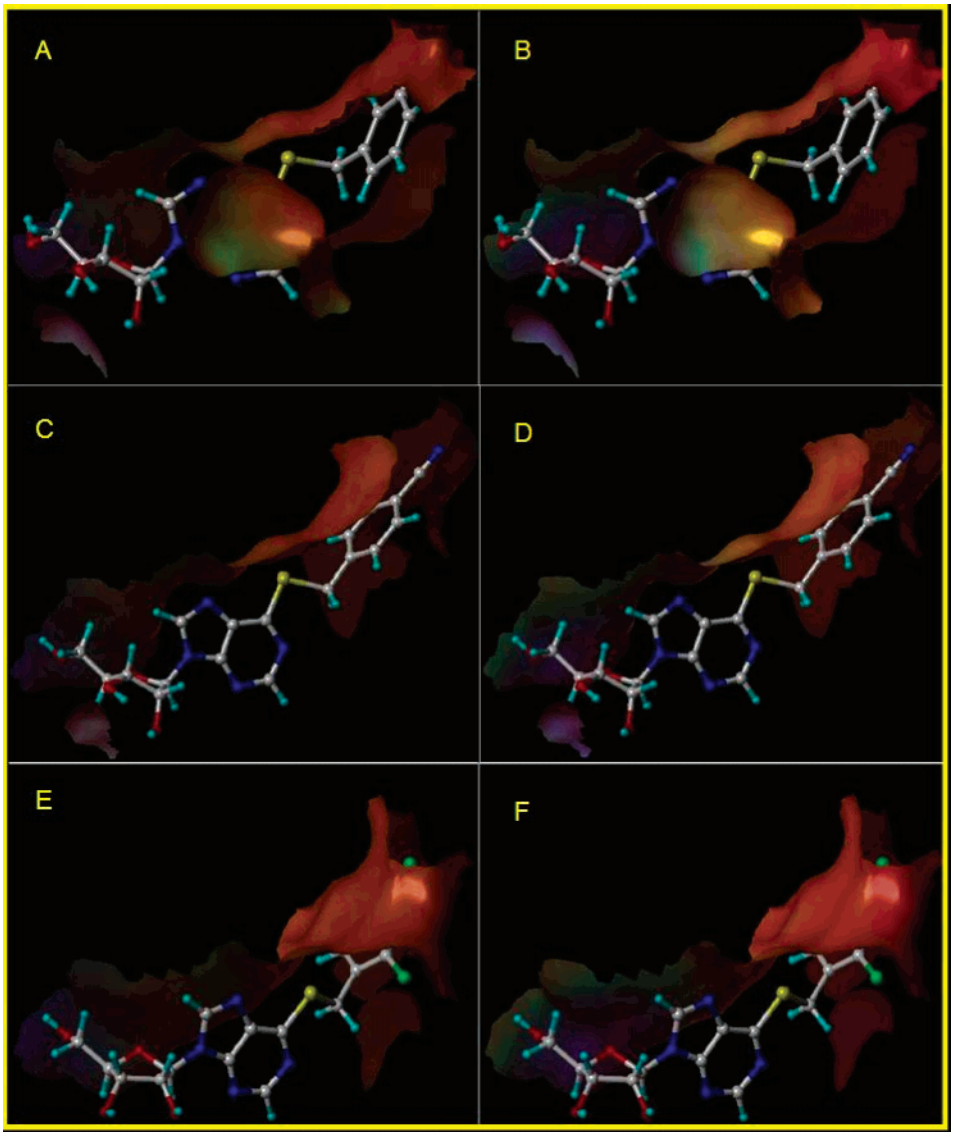


Figure 2. Connolly surface representation of the binding mode of 6-benzylthioinosine (BTI) (images A and B), 6-(4-cyanobenzylthio)-9-β-D-ribofuranosylpurine (**6o**) (images C and D) and 6-(2,4-dichlorobenzylthio)-9-β-D-ribofuranosylpurine (**6q**) (images E and F) with their hydrophobicity (left column) and electrostatic potential (right column) mapped.

occupies the hydrophobic region at the S^6 region of the binding pocket in a favorable manner. It is even evident from the superimposition of the 10 low energy conformers that the change in conformation takes place only at the glycosidic bond level or sugar ring, but the overall orientation of the benzyl group was always in the same region. The orientation of the base is "anti" in all the conformations. The sugar conformation is 2′-endo in all the low energy conformations. In the case of 6-(4-cyanobenzylthio)-9-β-D-ribofuranosylpurine (**6o**), a similar trend is observed, the benzyl group positions itself in the hydrophobic pocket and also the presence of cyano group at the para position, increases the net van der Waals interaction with the surrounding residues. Here again, as in the case of BTI, the sugar conformation is 2′-endo, which is the preferred conformation of sugar for reactions catalyzed by kinases. In the case of 6-(2,4-dichlorobenzylthio)-9-β-D-ribofuranosylpurine (**6q**), the position of the benzyl group is not changed, but the net electronegative potential on the phenyl ring is increased. This slight increase is presumably responsible for the enhanced binding of **6q** compared to **6o**. Both, **6q** and **6o** are better substrates than adenosine, which can be attributed to a combination of enhanced hydrophobicity and enhanced electronegative potential. The sugar ring in all the low-energy conformers has the same envelope conformation as that of adenosine during phosphate transfer from ATP.[20] This similarity with the transition state conformation may further add to its better binding ability.

The S^6 hydrophobic region is a long tubular pocket lined with hydrophobic groups. The pocket seems to broaden near its end, where it forms a funnellike shape. The tubular shape of the pocket can accommodate the phenyl moiety of the benzyl substituent as well as single atom substituents (e.g., halogens) that are electronegative and can form favorable van der Waals interaction with the residues lining the pocket.

The conclusions from our modeling studies are as follows: (a) We confirmed that the S^6 benzyl substitution occupies the hydrophobic pocket, and this pattern seems not to be affected by the different substitution pattern on the phenyl ring of the benzyl group; (b) the hydrophobic interactions inside this binding pocket can

be increased by increasing the hydrophobicity of the substitution on the phenyl ring, although caution should be exercised in order not to introduce steric hindrance; (c) small electron-deficient substituents at the ortho and para position enhance the binding energy by van der Waals interactions and putative hydrogen bonding, the effect being more pronounced at the para position than at the ortho position; however, here again care should be exercised not to introduce any steric clashes with the side chains of the residues lining the pocket.

In summary, we have designed a novel class of potent and selective antitoxoplasmosis agents. The basic protocol for the combinatorial synthesis of this class and other related classes of compounds has been developed. We have generated several analogues which are better substrates for *T. gondii* adenosine kinase relative to the natural substrate (adenosine). This enhanced activity can be attributed to the combined effect of hydrophobic as well as van der Waals interactions, as confirmed by molecular modeling studies. The two most potent (**6q** and **6o**) as well as the two weakest (**6h** and **6k**) analogues were tested in cell-based assays and were compared to sulfadiazine and pyrimethamine, the drugs of choice for treatment of toxoplasmosis in terms of their antitoxoplasmosis activity. In addition, these compounds were devoid of host-toxicity (Table 2). To further confirm the potential usefulness of these two compounds, in vivo animal efficacy tests are warranted.

Experimental Section

Solid-phase synthesis was performed on Argonaut Quest 210 organic synthesizer. Melting points were determined on a Meltemp II laboratory device and are uncorrected. NMR spectra were recorded on a Bruker AMX400 MHz Fourier Transform spectrometer; chemical shifts are reported in parts per million-(δ), and signals are quoted as s (singlet), d (doublet), t (triplet), m (multiplet), and dd (double of doublets). UV spectra were obtained on a Beckman DU-650 spectrophotometer. Optical rotations were measured on a JASCO DIP-370 digital polarimeter. TLC was performed on Uniplates (silica gel) purchased from Analtech. Co. and Elemental analysis were performed by Atlantic Microlab Inc., Norcross, GA.

6-Chloro-9-(2, 3, 5,-tri-*O*-benzoyl-β-D-ribofuranosyl)-9*H*-purine (2). 6-Chloropurine (2.80 g, 0.018 mol) and **1** (8.38 g, 0.016 mol) were weighed together in a reaction flask, and 100 mL of anhydrous acetonitrile was added. To the mixture were added hexamethyldisilazane (3.48 mL, 0.016 mol), trimethylsilyl chloride (3.15 mL, 0.025 mol), and trimethyl silyl triflate (4.77 mL, 0.025 mol). The mixture was refluxed for 2.5 h, dichloromethane was added, and the solution was extracted with sat. $NaHCO_3$ solution. The organic phase was dried over $MgSO_4$ and evaporated. Residue was then purified by column chromatography (hexanes−EtOAc 80:20). Upon concentrating the suitable fractions, it yielded (9.3 g, 94% yield) of **2** as a white lustrous solid. mp = 114−115 °C; $[\alpha]^{25}_D$ −63.772 (*c* 0.085, $CHCl_3$); UV (CH_2Cl_2) λ_{max} 264 nm; ^{1}H NMR ($CDCl_3$): δ = 8.61 (s, 1H); 8.28 (s, 1H); 7.26−8.09 (m, 15H); 6.45 (d, 1H, *J* = 4.9 Hz); 6.42 (dd, 1H, *J* = 4.9, 5.5 Hz); 6.25 (dd, 1H, *J* = 5.2, 5.3 Hz); 4.94 (dd, 1H, *J* = 3.3, 12.4 Hz); 4.85−4.87 (m, 1H); 4.70 (dd, 1H, *J* = 4.1, 12.4 Hz). ^{13}C NMR ($CDCl_3$): δ = 65.1, 69.3, 70.2, 75.4, 81.6, 128.4, 129.7, 130.5, 132.8, 144.8, 147.9, 152.0, 154.9, and 167.0.

6-Chloro-9-β-D-ribofuranosyl-9*H*-purine (3). Compound **2** (10.3 g, 1.1 mmol) was dissolved in methanolic ammonia (100 mL) and the solution was stirred at room temperature overnight. The products were concentrated under reduced pressure and the residue was purified by short column chromatography on silica gel. The appropriate fractions, eluted with $CHCl_3$-EtOH (95:5 v/v), were combined and evaporated to give the title compound **3** as a white solid. (1.79 g, 36% yield). mp = 161−163 °C. ^{1}H NMR (DMSO-d_6) δ 8.97 (s, 1H), 8.84 (s, 1H), 6.06 (d, 1H, *J* = 5.1 Hz), 5.59 (d, 1H, *J* = 5.9 Hz), 5.28 (d, 1H, *J* = 5.1 Hz), 5.12 (t, 1H, *J* = 5.5 Hz), 4.58 (m, 1H), 4.21 (m, 1H), 4.10 (m, 1H), 3.5−3.8 (m, 2H); ^{13}C NMR (DMSO-d_6) δ = 151.8, 151.6, 149.4, 145.8, 131.4, 88.3, 85.8, 74.1, 70.1, 61.0.

Resin-Coupled 6-Chloro-9-β-D-ribofuranosyl-9*H*-purine (4). Five grams of the trityl resin and 2 g of **(3)** were combined together in a 25 mL round-bottom flask. The vessel was purged with nitrogen followed by addition of anhydrous pyridine (15 mL). Proper care should be taken to make the reaction conditions completely anhydrous. The reaction mixture was stirred gently for 2 days. Then resin beads were washed with 4 portions (4 × 15 mL) of pyridine, followed by 3 portions (3 × 15 mL) of ether, completely dried, and weighed. The difference in weight, i.e., 1.9 g corresponds to the amount of **3** coupled with the resin. This corresponds to 1.33 mmol of **3** being loaded per gram of the resin **4**.

General Procedure for the Solid-Phase Parallel Synthesis of 6-(Substituted benzylthio)-9-β-D-ribofuranosylpurine Analogues. Step I. Compound **4** [363 mg (∼100 mg of **3**)] was added to each microfrit-equipped reaction vessel (RV), followed by 10 mL of methanol. The appropriate thiols[16] (0.1 mL) were then added, followed by 1 mL of triethylamine (TEA). The reaction mixture was stirred gently (upward stroke = 50%, time = 4 s) at 55 °C for 5 h. After 5 h, the RV's were drained and the beads were washed successively with 3 × 10 mL of methanol, methylene chloride, and ether, then dried by purging with nitrogen. No analysis was done at this stage.

Step II. Cleavage of 6-(Substituted benzylthio)-9-β-D-ribofuranosylpurine Analogues from the Resin. The 10 RV's containing the dry resin from step I were filled with 10 mL of methylene chloride followed by the addition of 0.5 mL of triisopropylsilane (TIS) and 0.2 mL of trifluoroacetic acid (TFA). The reaction mixture was agitated (upward stroke = 50%, time = 2 s) for the next 10 min after which contents of the RV's were transferred to side B of the synthesizer in the corresponding RV's using a transfer cannula. Then 3 mL of ether was added along with satd $NaHCO_3$ (2 mL) to neutralize the excess TFA, and the organic layer containing the final product was transferred to a vial for further workup. It is important that the extraction has to be performed immediately in order to prevent difficult purification and loss of yield due to cleavage of glycoside bond. The organic layer was concentrated, and a short flash column (5% MeOH: 95% CH_2Cl_2) was used to remove traces of impurities. The average yield was ∼94%. The analogues were recrystalized from methanol.

6-(2-Methylbenzylthio)-9-β-D-ribofuranosylpurine (6a). Crystalline white solid. Yield = 95%; mp 190−191 °C; $[\alpha]^{25}_D$ −57.544 (*c* 0.17, MeOH); UV (H_2O) λ_{max} 292 nm (ϵ 38600 pH 2), 292 nm (ϵ 40800 pH 7), 292 nm (ϵ 39600 pH 11); ^{1}H NMR (CD_3OD): δ = 8.73 (s, 1H); 8.58 (s, 1H); 7.42 (d, 1H, *J* = 7.14 Hz); 7.14 (m, 3H); 6.08 (d, 1H, *J* = 5.76 Hz); 4.72 (t, 1H, *J* = 5.6 Hz); 4.70 (s, 2H); 4.33−4.35 (m, 1H); 4.14−4.16 (m, 1H); 3.88 (dd, 1H, *J* = 2.74, 12.37 Hz); 3.76 (dd, 1H, *J* = 3.0, 12.46 Hz); 2.42 (s, 3H). Anal. ($C_{18}H_{20}N_4O_4S$): C, H, N, S.

6-(2-Fluorobenzylthio)-9-β-D-ribofuranosylpurine (6b). Crystalline white solid. Yield = 94%; mp 62−64 °C; $[\alpha]^{25}_D$ −51.761 (*c* 0.29, MeOH); UV (H_2O) λ_{max} 290.5 nm (ϵ 36000 pH 2), 290 nm (ϵ 39700 pH 7), 290.5 nm (ϵ 38600 pH 11); ^{1}H NMR (CD_3OD): δ = 8.73 (s, 1H); 8.59 (s, 1H); 7.56 (td, 1H, *J* = 1.57, 7.68 Hz); 7.24−7.30 (m, 1H); 7.06−7.11 (m, 2H); 6.07 (d, 1H, *J* = 5.7 Hz); 4.7−4.72 (m, 3H); 4.33−4.35 (m, 1H); 4.14−4.16 (m, 1H); 3.88 (dd, 1H, *J* = 2.82, 12.36 Hz); 3.76 (dd, 1H, *J* = 3.09, 12.37 Hz). Anal. ($C_{17}H_{17}FN_4O_4S$): C, H, N, S.

6-(2-Chlorobenzylthio)-9-β-D-ribofuranosylpurine (6c). Crystalline white solid. Yield = 97%; mp 208−210 °C; $[\alpha]^{25}_D$ −44.883 (*c* 0.10, MeOH); UV (H_2O) λ_{max} 290.5 nm (ϵ 35900 pH 2), 291 nm (ϵ 34400 pH 7), 290.5 nm (ϵ 40900 pH 11); ^{1}H NMR (CD_3OD): δ = 8.74 (s, 1H); 8.59 (s, 1H); 7.64−7.66 (m, 1H); 7.40−7.42 (m, 1H); 7.21−7.27 (m, 2H); 6.07 (d, 1H, *J* = 5.65 Hz); 4.80 (s, 2H); 4.71 (t, 1H, *J* = 5.36); 4.33−4.35 (m, 1H); 4.13−4.15 (m, 1H); 3.88 (dd, 1H, *J* = 2.90, 12.51 Hz); 3.75 (dd, 1H, *J* = 3.0, 12.19 Hz). Anal. ($C_{17}H_{17}ClN_4O_4S$): C, H, N, S.

6-(3-Methylbenzylthio)-9-β-D-ribofuranosylpurine (6d). Crystalline white solid. Yield = 93%; mp 177−179 °C; $[\alpha]^{25}_D$ −58.507 (*c* 0.13, MeOH); UV (H_2O) λ_{max} 292 nm (ϵ 32800 pH 2), 292 nm (ϵ 31600 pH 7), 291.5 nm (ϵ 32900 pH 11); ^{1}H NMR (CD_3OD): δ = 8.71 (s, 1H); 8.59 (s, 1H); 7.22−7.26 (m, 2H); 7.16 (t, 1H, *J* = 7.46 Hz); 7.05 (d, 2H, *J* = 6.38 Hz); 6.07 (d, 1H, *J* = 5.7 Hz); 4.72 (t, 1H, *J* = 5.65 Hz); 4.63 (s, 2H,); 4.33−4.35 (m, 1H); 4.13−4.16 (m, 1H); 3.88 (dd, 1H, *J* = 2.89, 12.53 Hz); 3.75 (dd, 1H, *J* = 2.69, 12.42 Hz); 2.29 (s, 3H). Anal. ($C_{18}H_{20}N_4O_4S$): C, H, N, S.

6-(3-Trifluoromethylbenzylthio)-9-β-D-ribofuranosylpurine (6e). Crystalline white solid. Yield = 91%; mp 178−180 °C; $[\alpha]^{25}_D$ −42.611 (*c* 0.16, MeOH); UV (H_2O) λ_{max} 290.5 nm (ϵ 34900 pH 2), 290.5 nm (ϵ 33600 pH 7), 290.5 nm (ϵ 37000 pH 11); ^{1}H NMR (CD_3OD): δ = 8.72 (s, 1H); 8.60 (s, 1H); 7.80 (s, 1H); 7.75 (d, 1H, *J* = 7.23 Hz); 7.46−7.53 (m, 2H); 6.07 (d, 1H, *J* = 5.7 Hz); 4.75 (s, 2H); 4.71 (t, 1H, *J* = 5.39 Hz); 4.33−4.35 (m, 1H); 4.13−4.16 (m, 1H); 3.88 (dd, 1H, *J* = 2.85, 12.40 Hz); 3.76 (dd, 1H, *J* = 3.13, 12.38 Hz). Anal. ($C_{18}H_{17}F_3N_4O_4S$): C, H, N, S.

6-(3-Nitrobenzylthio)-9-β-D-ribofuranosylpurine (6f). Crystalline white solid. Yield = 95%; mp 164−166 °C; $[\alpha]^{25}_D$ −42.713 (*c* 0.14, MeOH); UV (H_2O) λ_{max} 286 nm (ϵ 44100 pH 2), 286 nm (ϵ 41800 pH 7), 286 nm (ϵ 39900 pH 11); ^{1}H NMR (CD_3OD): δ = 8.73 (s, 1H); 8.61 (s, 1H); 8.40 (s, 1H); 8.09 (d, 1H, *J* = 8.0 Hz); 7.9 (d, 1H, *J* = 7.32 Hz); 7.53 (t, 1H, *J* = 7.94 Hz); 6.07 (d, 1H, *J* = 5.6 Hz); 4.78 (s, 2H); 4.70 (t, 1H, *J* = 5.37 Hz); 4.34 (t, 1H, *J* = 3.59 Hz); 4.15 (d, 1H, *J* = 2.97 Hz); 3.88 (dd, 1H, *J* = 2.75, 12.48 Hz); 3.75 (dd, 1H, *J* = 2.98, 12.32 Hz). Anal. ($C_{17}H_{17}N_5O_6S$): C, H, N, S.

6-(4-Methylbenzylthio)-9-β-D-ribofuranosylpurine (6g). Crystalline white solid. Yield = 94%; mp 110−112 °C; $[\alpha]^{25}_D$ −56.432 (*c* 0.37, MeOH); UV (H_2O) λ_{max} 292.5 nm (ϵ 32300 pH 2), 292.5 nm (ϵ 31100 pH 7), 292 nm (ϵ 30200 pH 11); ^{1}H NMR (CD_3OD): δ = 8.70 (s, 1H); 8.58 (s, 1H); 7.32 (d, 2H, *J* = 6.98 Hz); 7.1 (d, 2H, *J* = 6.92 Hz); 6.07 (d, 1H, *J* = 5.59 Hz); 4.71 (t, 1H, *J* = 5.36 Hz); 4.62 (s, 2H,); 4.33−4.35 (m, 1H); 4.13−4.16 (m, 1H); 3.88 (dd, 1H, *J* = 2.8, 12.3 Hz); 3.76 (dd, 1H, *J* = 3.0, 12.36 Hz); 2.29 (s, 3H). Anal. ($C_{18}H_{20}N_4O_4S$): C, H, N, S.

6-(4-Trifluoromethoxybenzylthio)-9-β-D-ribofuranosylpurine (6h). Crystalline white solid. Yield = 91%; mp 130−132 °C; $[\alpha]^{25}_D$ −54.081 (*c* 0.19, MeOH); UV (H_2O) λ_{max} 290.5 nm (ϵ 14300 pH 2), 290.5 nm (ϵ 16000 pH 7), 291 nm (ϵ 16400 pH 11); ^{1}H NMR (CD_3OD): δ = 8.71 (s, 1H); 8.59 (s, 1H); 7.57 (d, 2H, *J* = 8.27 Hz); 7.19 (d, 2H, *J* = 7.64 Hz); 6.07 (d, 1H, *J* = 5.36 Hz); 4.69−4.72 (m, 3H); 4.33−4.35 (m, 1H); 4.13−4.16 (m, 1H); 3.88 (dd, 1H, *J* = 2.6, 12.15 Hz); 3.76 (dd, 1H, *J* = 2.47, 12.27 Hz). Anal. ($C_{18}H_{17}F_3N_4O_5S$): C, H, N, S.

6-(4-Bromobenzylthio)-9-β-D-ribofuranosylpurine (6i). Crystalline white solid. Yield = 97%; mp 156−158 °C; $[\alpha]^{25}_D$ −48.688 (*c* 0.23, MeOH); UV (H_2O) λ_{max} 291.5 nm (ϵ 28700 pH 2), 291.5 nm (ϵ 11800 pH 7), 291.5 nm (ϵ 17900 pH 11);^{1}H NMR (CD_3OD): δ = 8.71 (s, 1H); 8.59 (s, 1H); 7.38−7.44 (m, 4H); 6.07 (d, 1H, *J* = 5.75 Hz); 4.71 (t, 1H, *J* = 5.38 Hz); 4.64 (s, 2H); 4.33−4.35 (m, 1H); 4.13−4.16 (m,1H); 3.88 (dd, 1H, *J* = 2.8, 12.39 Hz); 3.75 (dd, 1H, *J* = 3.08, 12.37 Hz). Anal. ($C_{17}H_{17}BrN_4O_4S$): C, H, N, S.

6-(4-Methoxybenzylthio)-9-β-D-ribofuranosylpurine (6j). Crystalline white solid. Yield = 93%; mp 102−104 °C; $[\alpha]^{25}_D$ −50.844 (*c* 0.43, MeOH); UV (H_2O) λ_{max} 292.5 nm (ϵ 32800 pH 2), 292.5 nm (ϵ 31600 pH 7), 292 nm (ϵ 31100 pH 11); ^{1}H NMR (CD_3OD): δ = 8.71 (s, 1H); 8.58 (s, 1H); 7.36 (d, 2H, *J* = 8.6 Hz); 6.84 (d, 2H, *J* = 8.65 Hz); 6.07 (d, 1H, *J* = 5.77 Hz); 4.71 (t, 1H, *J* = 5.40 Hz); 4.62 (s, 2H); 4.33−4.35 (m, 1H); 4.13−4.16 (m,1H); 3.88 (dd, 1H, *J* = 2.73, 12.43 Hz); 3.74−3.77 (m, 4H). Anal. ($C_{18}H_{20}N_4O_5S$): C, H, N, S.

6-(4-*tert*-Butylbenzylthio)-9-β-D-ribofuranosylpurine (6k). Crystalline white solid. Yield = 92%; mp 74−76 °C; $[\alpha]^{25}_D$ −43.770 (*c* 0.16, MeOH); UV (H_2O) λ_{max} 292.5 nm (ϵ 29400 pH 2), 292.5 nm (ϵ 29800 pH 7), 292.5 nm (ϵ 31700 pH 11); ^{1}H NMR (CD_3OD): δ = 8.71 (s, 1H); 8.58 (s, 1H); 7.31−7.38 (m, 4H); 6.07 (d, 1H, *J* = 5.76 Hz); 4.72 (t, 1H, *J* = 5.41 Hz); 4.64 (s, 2H); 4.33−4.35 (m, 1H); 4.14−4.16 (m,1H); 3.88 (dd, 1H, *J* = 2.77, 12.35 Hz); 3.76 (dd, 1H, *J* = 2.77, 12.40 Hz); 1.28 (s, 9H). Anal. ($C_{21}H_{26}N_4O_4S$): C, H, N, S.

6-(4-Acetoxybenzylthio)-9-β-D-ribofuranosylpurine (6l). Crystalline white solid. Yield = 93%; mp 178−180 °C; $[\alpha]^{25}_D$ −61.260 (*c* 0.18, MeOH); UV (H_2O) λ_{max} 291 nm (ϵ 8000 pH 2), 290.5 nm (ϵ 11500 pH 7), 285 nm (ϵ 16600pH 11); ^{1}H NMR (CD_3OD): δ = 8.72 (s, 1H); 8.60 (s, 1H); 7.94 (d, 2H, *J* = 8.07 Hz); 7.59 (d, 2H, *J* = 8.17 Hz); 6.07 (d, 1H, *J* = 5.68 Hz); 4.74 (s, 2H); 4.71 (t, 1H, *J* = 5.18 Hz); 4.33−4.35 (m, 1H); 4.13−4.16 (m,1H); 3.86−3.88 (m, 4H); 3.76 (dd, 1H, *J* = 3.0, 12.59 Hz). Anal. ($C_{19}H_{20}N_4O_6S$): C, H, N, S.

6-(4-Chlorobenzylthio)-9-β-D-ribofuranosylpurine (6m). Crystalline white solid. Yield = 96%; mp 134−136 °C; $[\alpha]^{25}_D$ −52.053 (*c* 0.26, MeOH); UV (H_2O) λ_{max} 291 nm (ϵ 24700 pH 2), 291 nm (ϵ 16900 pH 7), 291 nm (ϵ 16700 pH 11); ^{1}H NMR (CD_3OD): δ = 8.72 (s, 1H); 8.60 (s, 1H); 7.44−7.47 (m, 2H); 7.27−7.29 (m, 2H); 6.07 (d, 1H, *J* = 5.75 Hz); 4.71 (t, 1H, *J* = 5.41 Hz); 4.66 (s, 2H); 4.33−4.35 (m, 1H); 4.13−4.16 (m,1H); 3.88 (dd, 1H, *J* = 2.85, 12.40 Hz); 3.76 (dd, 1H, *J* = 3.13, 12.38 Hz). Anal. ($C_{17}H_{17}ClN_4O_4S$): C, H, N, S.

6-(4-Nitrobenzylthio)-9-β-D-ribofuranosylpurine (6n). Crystalline white solid. Yield = 94%; mp 197−198 °C; $[\alpha]^{25}_D$ −58.152 (*c* 0.21, MeOH); UV (H_2O) λ_{max} 290 nm (ϵ 38500 pH 2), 289 nm (ϵ 40300 pH 7), 289 nm (ϵ 42700 pH 11); ^{1}H NMR (CD_3OD): δ = 8.72 (s, 1H); 8.61 (s, 1H); 8.16 (d, 2H, *J* = 8.76 Hz); 7.73 (d, 2H, *J* = 8.64); 6.07 (d, 1H, *J* = 5.64 Hz); 4.79 (s, 2H); 4.70 (t, 1H, *J* = 5.38 Hz); 4.33−4.35 (m, 1H); 4.13−4.16 (m,1H); 3.88 (dd, 1H, *J* = 2.72, 12.53 Hz); 3.75 (dd, 1H, *J* = 3.12, 12.33 Hz). Anal. ($C_{17}H_{17}N_5O_6S$): C, H, N, S.

6-(4-Cyanobenzylthio)-9-β-D-ribofuranosylpurine (6o). Crystalline white solid. Yield = 93%; mp 72−74 °C; $[\alpha]^{25}_D$ −49.651 (*c* 0.22, MeOH); UV (H_2O) λ_{max} 290.5 nm (ϵ 48400 pH 2), 290.5 nm (ϵ 32100 pH 7), 290.5 nm (ϵ 47400 pH 11); ^{1}H NMR (CD_3OD): δ = 8.71 (d, 1H, *J* = 1.52 Hz); 8.60 (d, 1H, *J* = 1.38 Hz); 7.63−7.68 (m, 4H); 6.07 (d, 1H, *J* = 5.67 Hz); 4.73 (s, 2H); 4.70 (t, 1H, *J* = 5.08 Hz); 4.33−4.35 (m, 1H); 4.13−4.15 (m,1H); 3.88 (dd, 1H, *J* = 1.82, 12.32 Hz); 3.75 (dd, 1H, *J* = 1.62, 12.28 Hz). Anal. ($C_{18}H_{17}N_5O_4S$): C, H, N, S.

6-(4-Fluorobenzylthio)-9-β-D-ribofuranosylpurine (6p). Crystalline white solid. Yield = 96%; mp 70−72 °C; $[\alpha]^{25}_D$ −52.178 (*c* 0.18, MeOH); UV (H_2O) λ_{max} 291.5 nm (ϵ 38100 pH 2), 291.5 nm (ϵ 37700 pH 7), 291.5 nm (ϵ 39000 pH 11); ^{1}H NMR (CD_3OD): δ = 8.72 (s, 1H); 8.59 (s, 1H); 7.48 (m, 2H); 7.01(m, 2H); 6.07 (d, 1H, *J* = 5.73 Hz); 4.71 (t, 1H, *J* = 5.37 Hz); 4.66 (s, 2H); 4.33−4.35 (m, 1H); 4.14−4.15 (m,1H); 3.88 (dd, 1H, *J* = 2.84, 12.36 Hz); 3.76 (dd, 1H, *J* = 3.06, 12.41 Hz). Anal. ($C_{17}H_{17}FN_4O_4S$): C, H, N, S.

6-(2,4-Dichlorobenzylthio)-9-β-D-ribofuranosylpurine (6q). Crystalline white solid. Yield = 97%; mp 190 °C; $[\alpha]^{25}_D$ −47.024 (*c* 0.14, MeOH); UV (H_2O) λ_{max} 284.5 nm (ϵ 10100 pH 2), 284.5 nm (ϵ 9200 pH 7), 284.5 nm (ϵ 11500 pH 11); ^{1}H NMR (CD_3OD): δ = 8.74 (s, 1H); 8.60 (s, 1H); 7.67 (d, 1H, *J* = 8.31 Hz); 7.48 (d, 1H, *J* = 2.04 Hz), 7.26 (dd, 1H, *J* = 2.08, 8.31 Hz); 6.07 (d, 1H, *J* = 5.68 Hz); 4.77 (s, 2H); 4.71 (t, 1H, *J* = 5.26 Hz); 4.33−4.35 (m, 1H); 4.14−4.16 (m,1H); 3.88 (dd, 1H, *J* = 2.76, 12.35 Hz); 3.76 (dd, 1H, *J* = 3.06, 12.40 Hz). Anal. ($C_{17}H_{16}Cl_2N_4O_4S$): C, H, N, S.

6-(2-Chloro-6-fluorobenzylthio)-9-β-D-ribofuranosylpurine (6r). Crystalline white solid. Yield = 94%; mp 102−104 °C; $[\alpha]^{25}_D$ −55.583 (*c* 0.18, MeOH); UV (H_2O) λ_{max} 290.5 nm (ϵ 38500 pH 2), 290.5 nm (ϵ 37400 pH 7), 290.5 nm (ϵ 38800 pH 11); ^{1}H NMR (CD_3OD): δ = 8.76 (s, 1H); 8.60 (s, 1H); 7.28−7.35 (m, 2H); 7.9−7.14 (m, 1H); 6.09 (d, 1H, *J* = 5.64 Hz); 4.87 (s, 2H); 4.72 (t, 1H, *J* = 5.43 Hz); 4.33−4.35 (m, 1H); 4.13−4.16 (m,1H); 3.89 (dd, 1H, *J* = 2.50, 12.15 Hz); 3.76 (dd, 1H, *J* = 2.91, 12.42 Hz). Anal. ($C_{17}H_{16}FClN_4O_4S$): C, H, N, S.

6-(3,4-Dichlorobenzylthio)-9-β-D-ribofuranosylpurine (6s). Crystalline white solid. Yield = 95%;mp 144−146 °C; $[\alpha]^{25}_D$ −53.048 (*c* 0.25, MeOH); UV (H_2O) λ_{max} 290.5 nm (ϵ 38300 pH 2), 290.5 nm (ϵ 35500 pH 7), 290.5 nm (ϵ 32200 pH 11); ^{1}H NMR (CD_3OD): δ = 8.72 (s, 1H); 8.60 (s, 1H); 7.65 (s, 1H); 7.42 (s, 2H); 6.07 (d, 1H, *J* = 5.64 Hz); 4.71 (t, 1H, *J* = 5.28 Hz); 4.64 (s, 2H); 4.34 (t, 1H, *J* = 4.01 Hz); 4.15 (d,1H, *J*

= 2.97 Hz); 3.88 (dd, 1H, J = 2.74, 12.44 Hz); 3.76 (dd, 1H, J = 2.98, 12.27 Hz). Anal. ($C_{17}H_{16}Cl_2N_4O_4S$): C, H, N, S.

6-(2,4,6-Trimethylbenzylthio)-9-β-D-ribofuranosylpurine (6t). Crystalline white solid. Yield = 93%; mp 184−186 °C; $[\alpha]^{25}_D$ −67.906 (*c* 0.20, MeOH); UV (H_2O) λ_{max} 293 nm (ϵ 41300 pH 2), 293 nm (ϵ 39600 pH 7), 293 nm (ϵ 42000 pH 11); ^{1}H NMR (CD_3OD): δ = 8.74 (s, 1H); 8.59 (s, 1H); 6.87 (s, 2H), 6.08 (d, 1h, J = 5.73 Hz); 4.73 (t, 1H, J = 5.41 Hz); 4.70 (s, 2H); 4.33−4.35 (m, 1H); 4.13−4.16 (m,1H); 3.89 (dd, 1H, J = 2.75, 12.41 Hz); 3.76 (dd, 1H, J = 2.99, 12.44 Hz); 2.36 (s, 6H); 2.24 (s, 3H). Anal. ($C_{20}H_{24}N_4O_4S$): C, H, N, S.

Molecular Modeling Study. *T. gondii* adenosine kinase functions by induced fit mechanism and involves hinge bending motion of two domains. This domain movement is complex and apparently involves further tuning of the protein structure when ATP binds to its region in this pocket. As a result, our initial modeling attempts to use the apo form (1LIO.pdb)[20] of the enzyme were far less successful than subsequent analysis using the full *T. gondii* adenosine kinase-adenosine complex[20] (1LII.pdb). The crystal coordinates of the reported enzyme−inhibitor complex were missing the residues 1−10, 239−240, 255−269, and 359−363. Residues 239−240 and 255−269 were added using the SYBYL Biopolymer module (Tripos Associates, St. Louis, MO). The terminal residues were not added because it has been shown in mutational and sequence deletion studies that their effect on the overall binding affinity was negligible.[20b] We included in our calculations the magnesium and chlorine ions as observed in the crystal structure. The determination of the binding mode of BTI analogues was complicated by the fact that the enzyme functions via an induced-fit mechanism and that only two substrate−enzyme complexes are known, one with the natural substrate adenosine and the other with 7-iodotubericidin. Neither of these enzyme complexes was ideal for these studies, because our analogues are almost ∼16 Å in length diagonally, whereas the diagonal length of the adenosine and 7-iodotubericidin is ∼8 Å. To determine the binding mode of the BTI analogues, we tested several modeling techniques that had been previously used for enzymes that function by an induced-fit mechanism. We tried Low Mode Conformational Search (LMOD), Monte Carlo Multiple Minimum/Energy Minimization (MCMM/EM), and Monte Carlo/Energy Minimization (MC/EM). Of these methods, only MC/EM was able to reproduce the crystal structure to a fairly good accuracy and has already been used for enzymes of the induced-fit type.[21−23]

All the calculations were performed on MACROMODEL 7.0 (Schrödinger Inc.) with the GB/SA continuum water solvation model. MMFF94s force field, as implemented in MACROMODEL 7.0 was used. In the case of BTI and BTI analogues, the starting conformation was obtained after performing a 50 000 step Monte Carlo conformational search in the GB/SA continuum water solvation model. Ligands were introduced into the binding pocket in a random orientation (in some trial runs they were in entirely opposite orientations). The MOLS command in BATCHMIN was used to translate the molecule along with torsional variation and the Monte Carlo conformational search with search option set to "Usage Directed" was initiated, with Polak-Ribiere first derivative Conjugate Gradient (PRCG) as a method for the subsequent energy minimization. The whole enzyme was used in the Energy minimization step. From our experience, no major computational advantage was achieved when only the residues within the 7 Å radii of the ligand were used. EM calculations were performed for 500 steps or until the energy difference between subsequent structures was 0.05 kJ/mol. All the torsional angles were allowed to be varied during the search. A total of 1000 conformations were generated during the MC/EM search. The calculations were very computationally intensive. For example, it required 29 days on R10000 Octane 2 SGI running IRIX 6.5 to perform a 1000 conformations search for a single ligand.

Enzyme Assays. Purified recombinant *T. gondii* adenosine kinase and human liver cytosol[11] were used in these experiments as the source for the parasite and host enzymes, respectively.

Overexpression of *T. gondii* Adenosine Kinase. The coding sequence of cloned *T. gondii* adenosine kinase gene *TgAK*[24] was PCR amplified and engineered into *Escherichia coli* expression vector pET21a. The coding sequence was verified by DNA sequencing. The resulting plasmid pETgAK was transformed into strain BL21(DE3)plysS competent *E. coli* cells. For the expression of adenosine kinase, cells were grown at 37 °C in LB medium (pH 7.5) containing 50 μg/mL of ampicillin, 34 μg/mL of chloramphenicol, and 1% glucose. When the OD_{600} reached 0.6, IPTG was added to a final concentration of 1 mM, and the incubation was continued for 3 h. The cells were then harvested by centrifugation at 6500*g* for 15 min at 4 °C. The cells were resuspended in 250 mL of cold 20 mM Tris-Cl pH 8.0, followed by centrifugation. The supernatant was removed, and the pellet was stored at −70 °C. The frozen pellet was resuspended in 20 mL of lysis buffer and sonicated. The suspension was recentrifuged at 33 000*g* for 30 min at 4 °C. The total protein concentration of the supernatant was determined by the Bradford method[25] and glycerol was added to a final concentration of 20% and stored at −20 °C.

Purification of the Recombinant Enzyme. The enzyme was purified to apparent homogeneity with a specific activity of 0.9 mmol/min/mg protein and a yield of 5 mg of soluble protein/L of culture by the following method. Aliquots of supernatant collected above were loaded onto a 95 mL Q-Sepharose Fast Flow column (Pharmacia) equilibrated with Buffer A (50 mM Tris-Cl (pH 8.0), 1 mM DTT). The column was then washed with five column volumes of Buffer A and eluted with a 10 column volumes linear gradient from 0 to 1 M NaCl in Buffer A at a flow rate of 5.0 mL/min. Fractions of 17 mL were collected. Adenosine kinase eluted as a broad peak at approximately 90−100 mM NaCl. The fractions containing the activity were concentrated, resuspended in Buffer A and reloaded in a 53 mL Q-Sepharose HP26/10 column (Pharmacia) equilibrated in the same buffer. The column was washed with two column volumes of Buffer A and eluted with 20 column volumes of a linear gradient 0−300 mM NaCl, and 10 mL fractions were collected. Adenosine kinase eluted as a sharp peak at approximately 100 mM NaCl. Fractions containing the activity were pooled, concentrated, resuspended in Buffer A, and kept at −75 °C. The identity and homogeneity of the purified enzyme was established by N-terminal amino acid sequencing and SDS polyacrylamide gel electrophoresis as a single band at ∼49 kD, respectively. The enzyme is stable under these conditions for months.

Evaluation of 6a−t as Alternative Substrates for Purified *T. gondii* Adenosine Kinase. Enzyme assays were run under conditions where activity was linear with time and enzyme concentration.[10,11] Adenosine kinase activity was determined by following the formation of radiolabeled AMP from adenosine. The assay mixture contains 50 mM Tris-Cl, pH 7.4; 2.5 mM ATP, 5 mM $MgCl_2$, 5 mM creatine phosphate, creatine kinase, 5 μM [8-^{14}C]adenosine (55 Ci/mol), 50 μL of enzyme in a final volume of 100 μL, in the absence or presence of various concentrations of the compound under evaluation. Incubation was carried out at 37 °C and terminated by boiling in a water bath for 2 min followed by freezing for at least 20 min. Precipitated proteins were removed by centrifugation, and 10 μL of the supernatant was spotted on silica gel TLC plates. The TLC plates were developed in a mixture of chloroform/methanol/acetic acid (102:12:6 v/v/v). The R_f values were adenosine, 0.27; adenine, 0.36; AMP, 0.17. The amounts of radioactivity in both the substrate and product(s) were calculated on a percentage basis using a computerized Berthold LB-284 Automatic TLC−Linear Analyzers (Wallac Inc., Gaithersburg, MD). Apparent K_i values of these analogues were calculated from Dixon plots 1/*v* vs [I] by least-squares fitting by computer programs developed by Dr. Naguib as previously described.[10,11] Since these compounds are competitive alternate

substrates of *T. gondii* adenosine kinase, their apparent K_i values are equal to their apparent K_m value [26] as presented in Table 1.

Evaluation of 6-(Substituted benzylthio)purine Ribosides Analogues as Potential Antitoxoplasmosis Agents against Tachyzoites in Tissue Culture. The wild-type RH and the adenosine kinase deficient mutant TgAK^{-3} [24] strains of *T. gondii* were used in these experiments. The adenosine kinase deficient mutant TgAK^{-3} was used as a control to verify that the active drugs were metabolized by adenosine kinase in vivo. The effects of purine analogues as antitoxoplasmosis agents in tissue culture was measured by their ability to inhibit the replication of intracellular *T. gondii* in tissue culture using monolayers of human foreskin fibroblasts (grown for no more than 30 passages) in RPMI 1640 medium.[11,12] The viability of intracellular parasites was evaluated by the selective incorporation of radiolabeled uracil into nucleic acids of the parasites at least in triplicates as previously described.[7,11] Briefly, confluent cells (4−5 day incubation) were cultured for 24 h in 24-well flat bottom microtiter plates (∼−5 × 10^5/1 mL/well) and incubated at 37 °C in 5% CO_2, 95% air to allow the cells to attach. The medium was then removed, and the cells were infected with isolated *T. gondii* in medium with 3% FBS (one parasite/cell). After 1 h incubation, the cultures were washed with media with 10% FBS to remove extracellular parasites. FBS was maintained at a final concentration of 10%. Seven concentrations of the compound were then added to cultures of the parasite-infected cells to give a final concentration of 0, 5, 10, 25, and 50 μM. Drugs were dissolved in 50% ethanol to give a final concentration of 2.5% ethanol when added to the wells. After an additional 18 h incubation the medium was replaced with 1 mL drug free media containing [5,6-^{13}H]uracil (2 μCi/mL) and incubated for another 6 h after which the media was removed. The fibroblasts were released from the wells by trypsinization with addition of 200 μL trypsin/EDTA (2.5×) to each well. After 10 min incubation, 1 mL of ice cold 10% trichloroacetic acid (TCA) was added to each well. The plates were then placed on a shaker to ensure the detachment of the cells. The suspended contents of each well was filtered through GF/A 2.4 cm glass microfiber filters (Whatman, Hillsboro, OR), which were prewashed each with 1 mL double distilled H_2O and dried. After filtration, the filters were washed with 10 mL of methanol, left to dry, then placed in scintillation vials containing 5 mL of Econo-Safe scintillation fluor (Research Products International Corp., Mount Prospect, IL), and radioactivity was counted using an LS5801 Beckman scintillation counter.

Absence of Toxicity of 6-(Substituted benzylthio)-purine Ribosides Analogues to Mammalian Fibroblasts. Possible toxicity against fibroblast feeder cells by the same doses of the various analogues used in the above experiments was measured, at least in triplicates, using a modification of the Microculture Tetrazolium [MTT] assay on uninfected monolayers of human foreskin fibroblasts (grown for no more than 30 passages) in RPMI 1640 medium.[11,12] Briefly, confluent cells were incubated for at least 24 h in 96-well flat bottom microtiterplates [∼−10^5/200 μL/well] at 37 °C in 5% CO_2, 95% air to allow the cells to attach. The medium was then replaced with 200 μL of fresh medium. The appropriate concentration of the compounds was dissolved in 50 μL of medium and added to each well to give the desired final concentrations. The cultures were then incubated for 48 h after which 50 μL of sterile MTT solution [2 mg/1 mL PBS] was added to each well. MTT solution was sterilized by filtration through 0.22 μm filters [Costar, Cambridge, MA]. After 4 h incubation, the medium was removed, 100 μL of dimethyl sulfoxide [DMSO] was added to each well, and the plates were shaken gently for 2−3 min to dissolve the formed formazan crystals. The absorbance was measured at 540 nm using a computerized microtiterplate reader [Themomax, Molecular Devices].

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Supporting Information Available: ^{1}H NMR spectra of representative compounds **6h**, **6k**, **6n**, **6o**, and **6q**. This material is available free of charge via the Internet at http://pubs.acs.org.

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