Biochemical Pharmacology, Vol. 51, pp. 1687–1700, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
PII S0006-2952(96)00213-4

Effects of Modifications in the Pentose Moiety and Conformational Changes on the Binding of Nucleoside Ligands to Uridine Phosphorylase from *Toxoplasma gondii*

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ABSTRACT. One hundred and fifty analogues of uridine, with various modifications to the uracil and pentose moieties, have been tested and compared with uridine with respect to their potency to bind to uridine phosphorylase (UrdPase, EC 2.4.2.3) from *Toxoplasma gondii*. The effects of the α- and β-anomers, the L- and D-enantiomers, as well as restricted *syn* and *anti* rotamers, on binding were examined. Pseudo-, lyxo-, 2,3′-anhydro-2′-deoxy-, 6,5′-cyclo-, 6,3′-methano-, $O^{5',6}$-methano- and carbocyclic uridines did not bind to the enzyme. Ribosides bound better than the corresponding xylosides, which were better than the deoxyribosides. The binding of deoxyribosides was in the following manner: 2′,3′-dideoxynucleosides > 2′,5′-dideoxynucleosides > 2′-deoxyribosides > 3′- and 5′-deoxyribosides. α-2′-Deoxyribosides bound to the enzyme, albeit less tightly than the corresponding β-anomers. The acyclo- and 2,2′-anhydrouridines bound strongly, with the 2,2′-anhydro-derivatives being the better ligands. 2,5′-Anhydrouridine bound to UrdPase less effectively than 2,2′-anhydrouridine and acyclouridine. Arabinosyluracil was at best a very poor ligand, but bound better if a benzyl group was present at the 5-position of the pyrimidine ring. This binding was enhanced further by adding a 5-benzyloxybenzyl group. A similar enhancement of the binding by increased hydrophobicity at the 5-position of the pyrimidine ring was observed with ribosides, α- and β-anomers of the 2′-deoxyribosides, acyclonucleosides, and 2,2′-anhydronucleosides. Among all the compounds tested, 5-(benzyloxybenzyl)-2,2′-anhydrouridine was identified as the best ligand of *T. gondii* UrdPase with an apparent K_i value of 60 ± 3 nM. It is concluded that the presence of an *N*-glycosyl bond is a prerequisite for a nucleoside ligand to bind to *T. gondii* UrdPase. On the other hand, the presence of a 2′-, 3′-, or 5′-hydroxyl group, or an *N*-glycosyl bond in the β-configuration, enhanced but was not essential for binding. Furthermore, the potency of the binding of 2,2′-anhydrouridines (fixed *high syn* isomers) in contrast to the weaker binding of the 6,1′-anhydro- or 2,5′-anhydrouridines (fixed *syn* isomers), and the complete lack of binding of the 6,5′-cyclo, $O^{5',6}$-methano- and 6,3′-methanouridines (fixed *anti* isomers) to *T. gondii* UrdPase indicate that the binding of ligands to this enzyme is in the *syn/high syn* conformation around the *N*-glycosyl bond. The results also indicate that the parasite but not the mammalian host UrdPase can participate in hydrogen bonding with *N*3 of the pyrimidine ring of nucleoside ligands. *T. gondii*

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Received 4 August 1995; accepted 6 February 1996.

UrdPase also has a larger hydrophobic pocket adjacent to the C5 of the pyrimidine moiety than the host enzyme, and can accommodate modifications in the pentose moiety which cannot be tolerated by the host enzyme. Most prominent among these modifications is the absence and/or lack of the *ribo* orientation of the 3′-hydroxyl group, which is a requirement for a ligand to bind to mammalian UrdPase. These differences between the parasite and host enzymes can be useful in designing specific inhibitors or "subversive" substrates for *T. gondii* UrdPase. BIOCHEM PHARMACOL 51;12:1687–1700

KEY WORDS. uridine; phosphorylase; toxoplasma; ligands; structure-activity

Toxoplasma gondii infection is quite common in humans but is asymptomatic in the general population. By contrast, the disease represents a major health problem for immunocompromised individuals, such as AIDS patients, and the unborn children of infected mothers [1–3]. Toxoplasmic encephalitis has become the most common cause of intracerebral mass lesions in AIDS patients and possibly the most commonly recognized opportunistic infection of the central nervous system [1–3]. Congenital toxoplasmosis is as high as 1/1000 live births and causes blindness, psychomotor or mental retardation, severe brain damage, or even death of infected children [3]. The few therapies available for the treatment of toxoplasmosis are limited by severe side-effects and development of resistance [1–4]. Therefore, the search and the need for more efficacious and less toxic therapies for the treatment of toxoplasmosis are essential. Since these parasites replicate rapidly and require large amounts of pyrimidines for the synthesis of DNA, RNA, and other macromolecules, a potential approach for therapeutic intervention against *T. gondii* is to interfere with its pyrimidine metabolism.

T. gondii can fulfill their pyrimidine requirements by either the *de novo* or the salvage pathways [5, 6]. Present information indicates that *T. gondii* differs from its mammalian host in various aspects of the pyrimidine salvage pathways [6]. Such differences offer potential targets for the chemotherapy of diseases caused by these parasites. Among those enzymatic reactions that are peculiar to the pyrimidine salvage pathways in *T. gondii* and are distinguished from those of its host are the phosphorylation and phosphorolysis of pyrimidine nucleosides. In contrast to their mammalian host, *T. gondii* are incapable of phosphorylating pyrimidine ribosides and deoxyribosides to their respective nucleoside 5′-monophosphates due to the lack of pyrimidine nucleoside kinases [6]. Pyrimidine nucleosides are ultimately converted to uracil or thymine by a single nonspecific UrdPase* (EC 2.4.2.3). Then uracil is converted by a specific uracil phosphoribosyltransferase (EC 2.4.2.9) to UMP from which all pyrimidine nucleotides can be synthesized [6]. Therefore, UrdPase provides an excellent target for chemotherapeutic intervention. Inhibitors or "subversive" substrates of *T. gondii* UrdPase could be used to impair or circumvent their pyrimidine salvage pathways.

In a systematic search for good ligands of UrdPase from *T. gondii*, a structure–activity relationship for the binding of ligands to UrdPase was previously formulated [7]. However, that study was concerned primarily with modifications in the pyrimidine nucleobase rather than in the pentose moiety. In this report, we examine the role of various modifications in the ribose moiety, as well as the effect of configuration (α- and β-anomers; D- and L-enantiomers) on the binding of nucleoside ligands to UrdPase. We also compared the binding of uridine with that of ribosyl-, arabinosyl-, xylosyl-, lyxosyl-, 2′-deoxy, 3′-deoxy, 5′-deoxy-, 2′,3′-dideoxy-, 2′,5′-dideoxy, 2′,3′-didehydro-, acyclo-, 2,2′-anhydro-, 2,3′-anhydro-2′-deoxy-, and 2,5′-anhydrouridines with various substitutions at the 5- and 6-positions of the pyrimidine ring. Such substitutions are known to enhance the binding of pyrimidine nucleobases to *T. gondii* UrdPase [7]. Furthermore, the binding of 2,2′-anhydrouridines (fixed *high syn* isomers) was compared with that of various 2,5′- or 6,1′-anhydrouridines (fixed *syn* isomers), and 6,5′-cyclo-, 6,3′-methano- and $O^{5',6}$-methanouridines (fixed *anti* isomers), as well as carbocyclic uridines (they lack the furanose ring oxygen) and pseudouridine (lacks the *N*-glycosyl bond) to determine what role the angle and modification of the glycosyl bond plays in the binding of pyrimidine nucleosides to *T. gondii* UrdPase. The data gathered from the present study, along with an extensive literature survey, allowed the formulation of structure–activity relationships for the binding of pyrimidine nucleoside ligands to UrdPase from *T. gondii*.

MATERIALS AND METHODS

Chemicals

The sources of the pyrimidine nucleosides and analogues used in this study are indicated in Table 1 by the following abbreviations: ALD, Aldrich Chemical Co. Inc., Milwaukee, WI; CAL, Calbiochem-Behring Corp., La Jolla, CA; HL, Hoffmann-LaRoche Inc., Nutley, NJ.; JPS, Dr. Jean-

* *Abbreviations:* acyclo, 1-[(2-hydroxyethoxy)methyl]; AM-BAU, aminomethyl-BAU or 5-benzyl-1-[(1′-aminomethyl-2′-hydroxyethoxy)-methyl]uracil; AM-BBAU, aminomethyl-BBAU or 5-benzyloxybenzyl-1-[(1′-aminomethyl-2′-hydroxyethoxy)methyl]uracil; DA-BAU, deoxyamino-BAU or 5-benzyl-1-[(2′-aminoethoxy)methyl]uracil; HM-BBAU, hydroxymethyl-BBAU or 5-(3-benzyloxybenzyl)-1-[(2′-hydroxy-1′-hydroxymethyl)methyl]uracil; *m*-methoxy-BAU, 5-(3-methoxybenzyl)-1-[(2′-hydroxyethoxy)methyl]uracil; *m*-hydroxy-BAU, 5-(3-hydroxy-benzyl)-1-[(2′-hydroxyethoxy)methyl]uracil; 2,2′-anhydro, 2,2′-anhydro-1-(β-D-arabinofuranosyl); 2,3′-anhydro-2′-deoxy, 2,3′-anhydro-1-(2′-deoxy-β-D-xylofuranosyl); 2,5′-anhydro-, 2,5′-anhydro-1-(β-D-ribofuranosyl); 6,1′-anhydro-6-hydroxypsicouridine, 6,1′-anhydro-1-(β-D-psicofuranosyl)-6-hydroxyuracil; arabinosyl, 1-(β-D-arabinofuranyosyl); 6,3′-methano, 6,3′-methano-(β-D-ribofuranosyl); 6,5′-cyclo, 6,5′-cyclo-(β-D-ribofuranosyl); β-D-lyxo, 1-(β-D-lyxofuranosyl); β-D-xylo, 1-(β-D-xylofuranosyl); and UrdPase, uridine phosphorylase.

Pierre Sommadossi, University of Alabama at Birmingham, Birmingham, AL; SIG, Sigma Chemical Co., St. Louis, MO; SYN, synthesized by the authors by the following previously published methods: the 5-benzyluridines and their acyclo-, arabinosyl-, 2,2′-anhydro-, 2,3′-anhydro-2′-deoxy-, and 5′-azido-derivatives [8–13]; the 5- or 6-(phenylselenenyl)uridines and their acyclo-derivatives [14–17]; 5-phenylthioacyclouridine [18]; 5-benzyloxybenzylbarbituric acid acyclonucleoside [19, 20]; acyclopseudouridine and its derivatives [21]; the 5-aza-6-thio-derivatives of 2,2′-anhydrouridine [22]; 1-allyloxymethyl-derivatives of uracil and thymine [23]; 3′-deoxyarabinosyluracil [24–26]; α-uridine [27]; α-thymidine [28]; α-2,2′-anhydroxylouracil [29]; L-uridine [30]; L-2′-deoxyuridine, L-thymidine, and L-2,2′-anhydrouridine [31]; L-2′,3′-dideoxyuridine [32]; L-arabinosyluracil [33]; α- and β-anomers of D-xylosides [29] and D-lyxosides [34]; L-xylosides [35]; the D- and L-enantiomers of 3′-deoxy-2,2′-anhydrouridine [36]; carbocyclic uridine [37]; carbocyclic 5-methyluridine [38]; the 2′-deoxycarbocyclic uridines [39]; the 2,2′-anhydrocarbocyclic uridines [40]; 3′-amino-3′-deoxycarbocyclic uridine [41]; 3′,4′-didehydrocarbocyclic thymidine [42]; 2′,3′-didehydro-2′,3′-dideoxyuridine (D4U) [43]; the 3′-thia derivatives of 2′,3′-dideoxyuridine [44]; 2,5′-anhydrouridine [40]; 6,3′-methanouridines [45]; 5-(phenylthiomethyl)uridine, 6,5′-cyclouridine, 6,5′-cyclo-2′-deoxyuridine, and their 4- and 5-substituted derivatives [46, 47]; dioxolanethymidine [48]; α- and β-[4-(hydroxy)tetrahydrofuran-2-yl]thymine [49]; 6,1′-anhydro-6-hydroxypsicouridine [50] and $O^{5',6}$-methanouridine [51]. L-Arabinosylthymine was synthesized from the condensation of 2,3,5-tri-O-benzyl-L-arabinofuranosyl chloride with persilated thymine in the presence of trimethylsilyl triflate. It was purified by silica gel chromatography followed by debenzylation with Pd/H_2 to give the final product. The detailed chemical synthesis of this compound will be published elsewhere. The chemical structures of the different classes of compounds studied are shown in Figs. 1–4. [2-^{14}C]Uridine (50 Ci/mol) was obtained from Moravek Biochemicals, Inc., Brea, CA; silica gel G/UV_{254} polygram TLC plates from Brinkmann Instruments, Inc., Westbury, NJ; and bovine γ-globulin and dye reagent for protein estimation from Bio-Rad Laboratories, Richmond, CA. All other chemicals were purchased from the Sigma Chemical Co.

Maintenance of T. gondii

Tachyzoites of the RH strain of *T. gondii* were propagated by intraperitoneal passage in female CD1 mice (Charles River Laboratories, Wellington, MA) weighing 20–25 g. A 2- or 3-day transfer period was used to provide the parasites for enzyme assays. Mice were injected intraperitoneally with an inoculum (10^6 cells) of *T. gondii* contained in 0.2 mL of sterile PBS, pH 7.2, and killed after 2–3 days by inhalation of ether. The parasites were harvested from the peritoneal cavity by injection, aspiration, and reinjection of 3–5 mL of PBS (2–3 times). The peritoneal fluid was examined microscopically to determine the concentration of *T. gondii* and to ascertain the extent of contamination by host cells. Two-day transfers generally produced parasite preparations that contained very little contamination and had a viability of >95%.

Preparation of Extracts

Extracts of *T. gondii* were prepared by homogenizing live parasites in ice-cold (3:1, v/w) 20 mM potassium phosphate buffer (pH 8) containing 1 mM EDTA, and 1 mM dithiothreitol using a polytron homogenizer (Brinkmann) followed by centrifugation at 105,000 *g* for 1 hr at 4°. The supernatant fluids (cytosol) were collected and used as the enzyme source.

UrdPase Assay

Assays were run under conditions where activity was linear with time and enzyme concentrations. Activities were determined by following the formation of [2-^{14}C]uracil from [2-^{14}C]uridine. The assay mixture contained a saturating concentration of phosphate (20 mM potassium phosphate, pH 8), 1 mM EDTA, 1 mM dithiothreitol, 125 μM [2-^{14}C]uridine (9 Ci/mol), five different concentrations of the inhibitor when present, and 40 μL of enzyme preparation (~0.05 mg protein/mL) in a final volume of 80 μL. Incubation was carried out at 37° for 30 min. The reaction was terminated by boiling for 1 min, followed by freezing for at least 20 min. Proteins were removed by centrifugation, and 5 μL of the supernatant fluid was spotted on silica gel TLC plates. The plates were then developed in a mixture of chloroform and methanol (90:10, v/v). R_f values for uridine and uracil were 0.09 and 0.39, respectively. The amounts of radioactivity in the substrate (uridine) and product (uracil) were calculated on a percentage basis using a Berthold LB-284 Automatic TLC-Linear Analyzer.

Determination of Uridine Apparent K_m

The apparent K_m value for uridine was determined using the same assay conditions for the standard assay except for the substrate concentrations used. The range of uridine concentrations was 25–900 μM. The uridine apparent K_m value was calculated using a computer program developed by Dr. Sungman Cha, Brown University, Providence, RI, and Dr. Fardos N. M. Naguib, which employs the Wilkinson–Cleland procedure [52, 53] for the estimation of V_{max} and K_m. The apparent K_m for UrdPase from *T. gondii* under these assay conditions was 65 ± 6.2 μM.

Determination and Significance of Apparent K_i *Values*

Apparent K_i values were estimated from Dixon plots ($1/v$ vs [I]) [54] of the data by a computer program with least square fitting developed by Dr. S. Cha and Dr. F. N. M. Naguib.

Apparent K_i values are related to K_i values by the following equation:

$$\text{apparent } K_i = \frac{K_{is}(1 + [S]/K_m)}{1 + ([S](K_m)(K_{is}/K_{ii})}$$

where K_{is} and K_{ii} are inhibition constants that would have been estimated from the replots of slope and intercept, respectively, of a Lineweaver–Burk plot vs [I]. If a compound is a competitive inhibitor with respect to uridine, $K_{ii} = \infty$ and $K_{is} = K_i$. Therefore,

$$\text{apparent } K_i = K_i(1 + [S])/K_m$$

Thus, for *T. gondii* UrdPase which has an apparent K_m = 65 μM uridine, the apparent K_i of a competitive inhibitor, measured at a uridine concentration of 125 μM, is approximately 3-fold higher than its respective K_i value. It should be noted, however, that we did not characterize the compounds used in this study with regard to the type of inhibition (competitive, non-competitive or uncompetitive) or whether or not they are substrates for the enzyme. Nevertheless, the riboside-, deoxyriboside-, acyclo- and 2,2′-anhydro-derivatives of uridine are known substrates or competitive inhibitors of UrdPase from other sources [9, 55–64], and all of the 5-position substituents included in the present study do not alter the competitive inhibition of any of the compounds studied thus far [9, 55–64]. Therefore, it is assumed that the compounds that bind to UrdPase in the present investigation are competitive inhibitors of the enzyme, i.e. bind to the same site as uridine. Under the present method of screening, if a compound is an alternative substrate for the enzyme (e.g. β-2′-deoxyuridine), the apparent K_i would be equal to its apparent K_m [65].

Protein Estimation

Protein concentrations were determined by the method of Bradford [66] using bovine γ-globulin as a standard.

RESULTS AND DISCUSSION

One hundred and fifty analogues of uridine with various modifications in the pyrimidine and pentose moieties, including α- and β-anomers, D- and L-enantiomers, and conformationally restricted (*syn* and *anti* rotamers) derivatives, were evaluated as ligands of UrdPase from *T. gondii*. Figures 1–4 show the chemical structures of uridine and the various types of derivatives studied in the present investigation. The degree of binding of these compounds to UrdPase was determined by measuring their ability to inhibit the enzyme. Inhibition was quantified by estimating the apparent K_i values for compounds that inhibited *T. gondii* UrdPase by more than 10% at a concentration of 0.9 mM. The apparent K_i values of these compounds for *T. gondii* UrdPase are shown in Table 1. Compounds are referred to in the text by their trivial name and compound number (bold type in parentheses). Also shown in Table 1 is the ratio between the apparent K_i for the reference compound uridine (**1**) and the apparent K_i for each of the compounds screened. This ratio indicates the fold-increase (values >1) or decrease (values <1) in binding of compounds, relative to uridine (**1**).

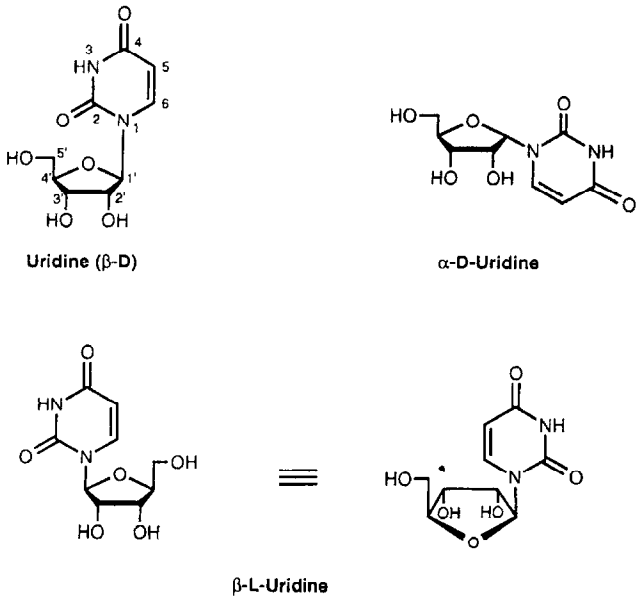


FIG. 1. Chemical structures of β-D-uridine, α-D-uridine and β-L-uridine.

Effect of the Conformation around the N-Glycosyl Bond

The uridine derivatives shown in Figs. 1–4 can be divided into five general categories according to the conformation of the torsion angle (χ) around the *N*-glycosyl bond (O4′-C1′-*N*1-C2): derivatives that are fixed in the *high syn* range (2,2′-anhydro-, χ ≅ 110°), derivatives that are fixed in the *syn* range (2,3′-anhydro-2′-deoxy-, χ = 78°; 2,5′-anhydro-, χ = 65°; 6,1′-anhydro-, χ = 50–80°), derivatives that are fixed in the *anti* range (6,5′-cyclo-, χ = 210°; 6,3′-methano-, χ = 265°; $O^{5',6}$-methano-, χ = 200°), derivatives that are not fixed in either position (ribosides, 2′-deoxyribosides, 3′-deoxyribosides, 5′-deoxyribosides, 2′,3′-dideoxyribosides, 2′,5′-dideoxyribosides, 2′,3′-didehydroribosides, arabinosides, xylosides, lyxosides, and acyclonucleosides), and derivatives that either lack a true *N*-glycosyl bond (carbocyclic nucleosides) or do not have one (C-nucleosides). The results of the present investigation demonstrated that the 2,2′-anhydrouridines (e.g. 2,2′-anhydrouridine, **120**) strongly bind to *T. gondii* UrdPase followed by the acyclic nucleosides (e.g. acyclouridine, **78**) > ribosides (e.g. uridine, **1**) > 2,5′-anhydronucleosides (e.g. 2,5′-anhydrouridine, **135**) > xylosides (e.g. xylouracil, **69**) > 2′,3′-dideoxynucleosides (e.g. 2′,3′-dideoxyuridine, **45**) > 6,1′-anhydronucleosides (e.g. 6,1′-anhydro-6-hydroxypsicouridine, **136**) > 2′,5′-dideoxynucleosides (e.g. 2′,5′-dideoxyridine, **58**) > 2′-deoxyribosides (e.g. 2′-deoxyuridine, **23**) > 3′-deoxyribosides (e.g. 3′-

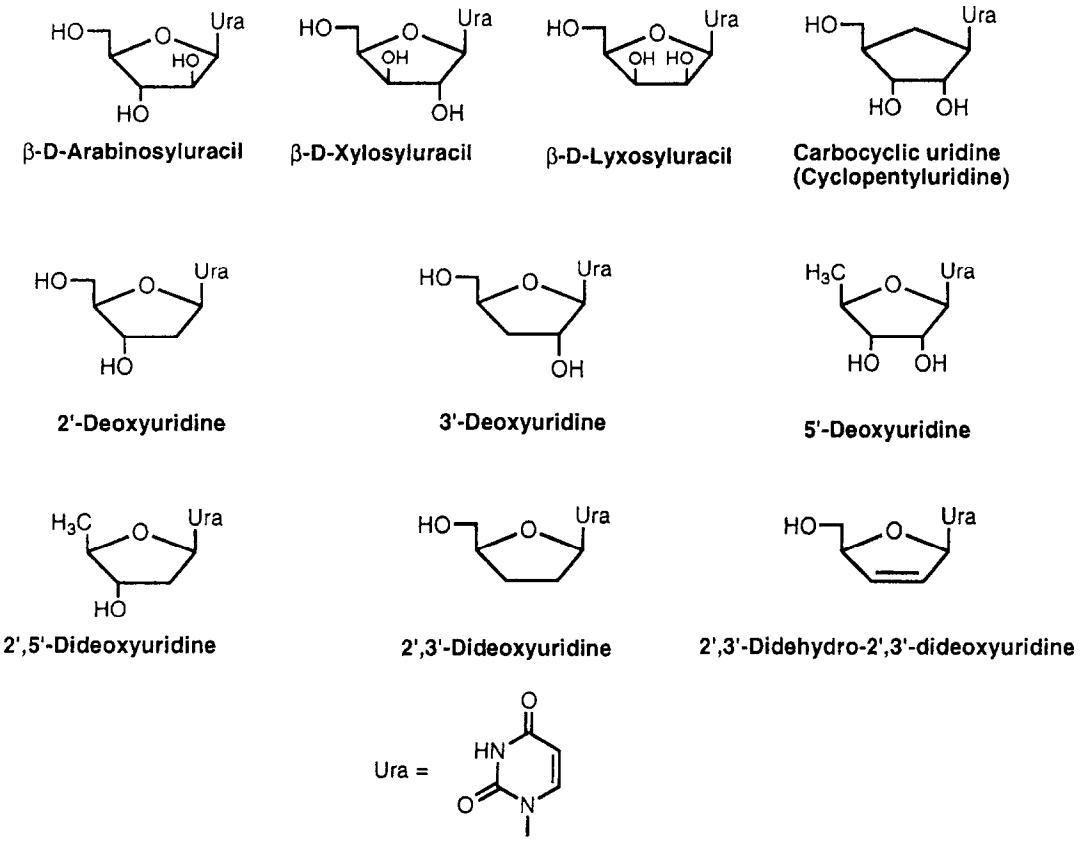
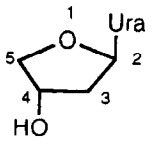


FIG. 2. Chemical structures of uridine analogues modified in the pentose moiety.

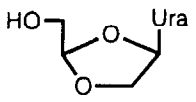
deoxyuridine, **42**) = 5'-deoxyribosides (e.g. 5'-deoxyuridine, **43**) = arabinosides (e.g. arabinosyluracil, **61**). All other compounds tested did not bind. The lack of binding of the carbocyclic uridines (e.g. carbocyclic uridine, **109**) or C-nucleosides (e.g. pseudouridine, **20**) is likely due to the presence of a cycloalkyl-heterocycle or a C–C bond, respectively, in these compounds instead of an *N*-glycosyl bond as in true nucleosides. The lack of binding of the 6,5'-cyclo- **(137–143)**, 6,3'-methano- **(144)** and $O^{5',6}$-methano- **(146)** nucleosides, on the other hand, can be attributed to the *anti*-orientation of their aglycons around the *N*-glycosyl bond. It should be pointed out that the lack of binding of the 6,5'-cyclonucleosides **(137–143)** is not entirely due to the absence of the 5'-hydroxyl group. As will be discussed below, the 5'-hydroxyl group enhances but is not essential for binding to *T. gondii* UrdPase.

In contrast to the lack of binding of the 6,5'-cyclo- **(137–143)**, 6,3'-methano- **(144)** and $O^{5',6}$-methano- **(146)** nucleosides, which are fixed in the *anti* conformation, the 2,2'-anhydrouridines **(120, 122–124)** were the best ligands of *T. gondii* UrdPase with 5-(benzyloxybenzyl)-2,2'-anhydrouridine **(124)** being the strongest binding ligand with an apparent K_i value of 60 ± 3 nM. Since the orientation of the pyrimidine moiety around the *N*-glycosyl bond in the 2,2'-anhydrouridines is fixed in the *high syn* range ($\chi \cong 110°$), we conclude that pyrimidine nucleosides bind to *T. gondii* UrdPase as *syn* rotamers and bind best at the *high syn* range. This conclusion is corroborated by the finding that compounds which do not bind to *T. gondii* UrdPase (i.e. carbocyclic uridine, **109**; 5-methyl-carbocyclic uridine, **110**) become ligands, albeit weak, when constrained in the *high syn* range (i.e. 2,2'-anhydro-carbocyclic uridine, **115**; 2,2'-anhydro-5-methyl-carbocyclic uridine, **116**).

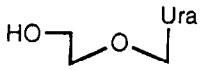
The differences between the binding capacities of 2,2'- (e.g. 2,2'-anhydrouridine, **120**), 2,5'- (e.g. 2,5'-anhydrouridine, **135**), and 6,1'-anhydro- (e.g. 6,1'-anhydro-6-hydroxypsicouridine, **136**) nucleosides to *T. gondii* UrdPase could be due to the differences in the furanose conformations and/or glycosyl torsion angles of these compounds. This presumably affects their proper fit in the active site of the enzyme. Furthermore, the *N*3 in the pyrimidine ring of the 2,2'-anhydro- and 2,5'-anhydrouridines is a hydrogen acceptor, while in the 6,1'-anhydro-nucleosides it is a hydrogen donor. Therefore, if the torsion angle around the *N*-glycosyl bond is in the favored *high syn* range as in the 2,2'-anhydrouridines ($\chi \cong 110°$), the *N*3 of the pyrimidine ring can participate in hydrogen bonding within the enzyme active site. Such hydrogen bonding may not occur if the torsion angle around the *N*-glycosyl bond is in a less than the favored position (i.e. *syn*) as in the 2,5'-anhydrouridines ($\chi = 65°$). This ability of the pyrimidine ring *N*3 to participate in hydrogen bonding could be the major factor underlying the large difference in binding between the 2,2'-anhydro-derivatives (e.g. 2,2'-anhydrouridine, **120**) and their corresponding 2,5'-anhydro-



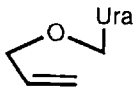
β-[4(α)-Hydroxytetrahydrofuran-2-yl]uracil



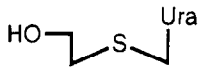
β-Dioxolaneuracil



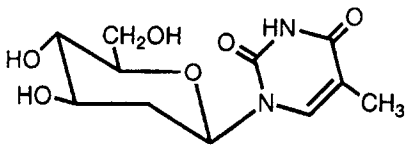
Acyclouridine



1-Allyloxymethyluracil



Thioacyclouridine



2'-Deoxyglucopyranosylthymine

FIG. 3. Chemical structures of uridine analogues that lack the pentose moiety.

(e.g. 2,5′-anhydrouridine, **135**) or 6,1′-anhydro- (e.g. 6,1′-anhydro-6-hydroxypsicouridine, **136**) nucleosides (320- and 590-fold, respectively) to *T. gondii* UrdPase.

Similarly, the better binding (75-fold) of 2,2′-anhydrouridines (**120, 122–124**) when compared with their corresponding acyclouridines (**78, 81, 84, 90**) could be explained by the involvement of the pyrimidine ring *N*3 in hydrogen bonding with *T. gondii* UrdPase. Although the acyclouridines have torsion angles around the *N*-glycosyl bond of ≅ 90° [67], they are flexible and can rotate to the favored *high syn* position [67] as that in the 2,2′-anhydrouridines. However, unlike the 2,2′-anhydrouridines, the *N*3 of the pyrimidine ring of the acyclouridines is a hydrogen donor and cannot participate in the proposed hydrogen bonding with the enzyme. In mammals, this differential binding between compounds that can participate in hydrogen bonding between *N*3 and UrdPase (i.e. 2,2′-anhydrouridine, **120**) and those that cannot (e.g. acyclouridine, **78**; 2,5′-anhydrouridine, **135**) does not exceed 6-fold [40]. Therefore, it appears that the involvement of *N*3 in hydrogen bonding with UrdPase is of more significant importance in *T. gondii* and constitutes a major difference between UrdPase from this parasite and its mammalian hosts.

Acyclouridine (**78**) and acyclothymidine (**80**) were better ligands for *T. gondii* UrdPase than their corresponding ribosides (**1, 10**). This better binding of the acyclonucleosides over their corresponding nucleosides could be attributed to the freedom of the acyclouridines to rotate around the *N*-glycosyl bond to achieve the favored *high syn* position [67]. Unlike the acyclouridines, uracil nucleosides are predominantly present as *anti* rotamers [68] and do not have the same freedom as the acyclouridines to rotate to the preferred *high syn* conformation.

The requirement for a ligand to be in the *high syn/syn* conformation to bind to *T. gondii* UrdPase could also explain the 2.3-fold better binding of barbituric acid riboside (**19**). The oxo group at the 6-position of **19** may favor the *high syn/syn* conformation. Indeed, it was shown that 6-methyluridine, which has a 6-position substitution and favors the *syn* conformation, is a substrate for UrdPase from *Salmonella typhimurium* [69]. These results demonstrated the requirement for the presence of a true *N*-glycosyl bond conformed in the *syn* position in order for a nucleoside to bind to *T. gondii* UrdPase. A similar conclusion was reached for the binding of ligands to UrdPase from various species [40, 60, 63, 69].

The poor binding of 1-allyloxymethyluracil (**107**) and 5-methyl-1-allyloxymethyluracil (**108**), when compared to acyclouridine (**78**) and 5-methylacyclouridine (**80**), respectively, could be attributed to the longer acyclo chain which may interfere with the freedom of rotation to the preferred *syn* configuration. It is also possible that the hydrophobic acyclo chain may be attracted to the proposed

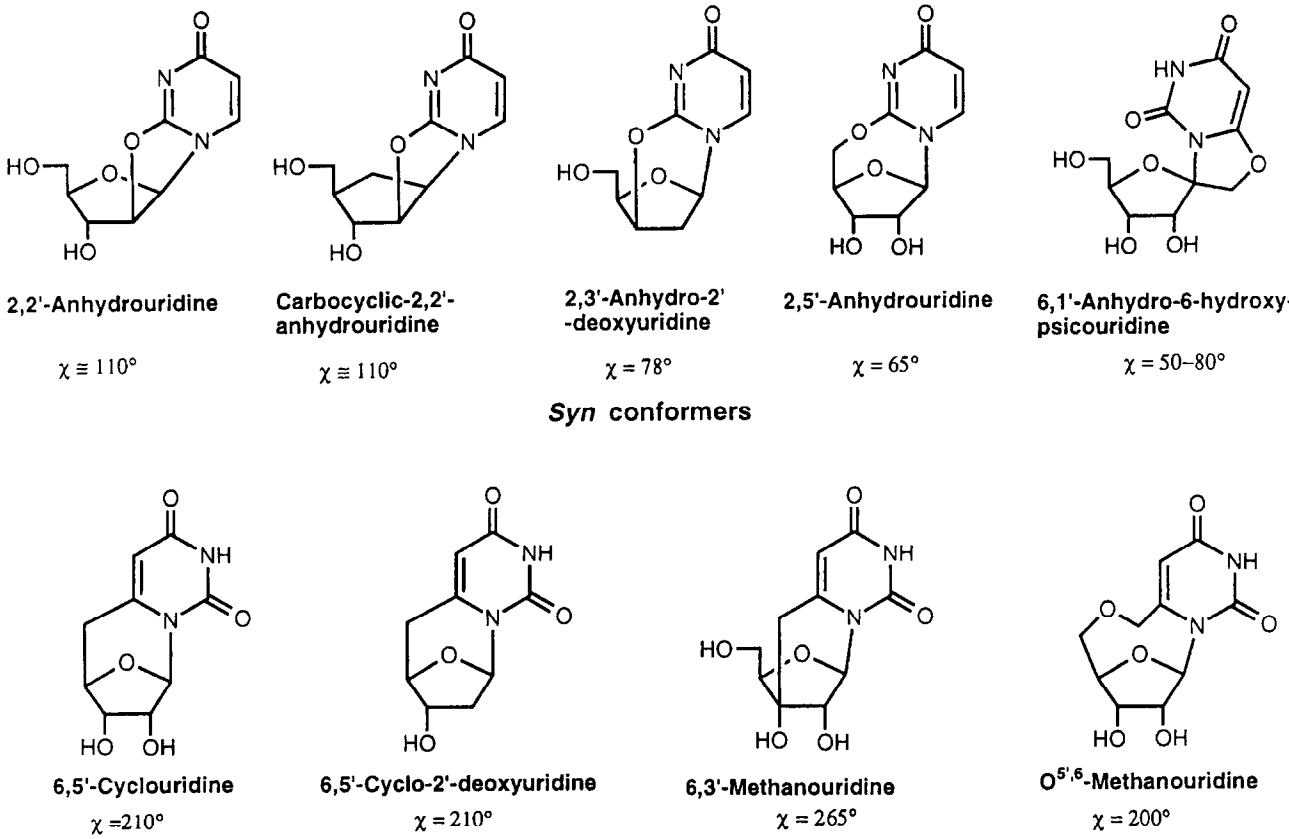


FIG. 4. Chemical structures of uridine analogues that are constrained in the *syn* or *anti* conformation around the *N*-glycosyl bond. The torsion angle (χ) around the *N*-glycosyl bond (*O4'-C1'-N1-C2*) of each compound is shown below its structure. The values for the torsion angle (χ) were obtained from X-ray of solid structures except for 6,1'-anhydro-6-hydroxypsicouridine and $O^{5',6}$-methanouridine where the angles were obtained from molecular modeling as previously described [50].

hydrophobic pocket in the binding site of UrdPase adjacent to C5 of the pyrimidine aglycon (see below). Furthermore, the double bond in the acyclo tail may prevent the hydrogen bonding at the 3'-position, which appears to enhance binding of ligands to UrdPase (see below).

Binding of the α- and β-Anomers

The present results clearly demonstrate that the α-anomers of uridine (**2**), and its 2'-deoxyriboside (**32, 38**) and xyloside (**70, 73**) analogues do not bind to *T. gondii* UrdPase, when compared to their respective β-anomers (**1, 31, 37, 69, 72**), unless there is a large hydrophobic substitution at C5 of the pyrimidine moiety (e.g. α-5-benzyl-2'-deoxyuridine, **38**). Even then, the α-anomer bound to a lesser degree than its β-anomer (**37**). Therefore, it appears that the β-D-configuration of ribosides, deoxyribosides, and xylosides is the preferred configuration for ligands to bind to *T. gondii* UrdPase. On the other hand, α-lyxosides (**75, 77**) and α-[4-(hydroxy)tetrahydrofuran-2-*yl*]thymine (**149**) were better ligands than their β-anomers (**74, 76, 148**). Preliminary molecular modeling studies [49] indicate that the α- (**149**) rather than the β-anomer (**148**) of [4-(hydroxy)tetrahydrofuran-2-*yl*]thymine emulates the β-anomer of thymidine (**31**) or 2'-deoxyuridine (**23**). Similarly, the α-lyxosides (**75, 77**) resemble the β-anomers of uridine (**1**) and thymidine (**31**) due to the *trans* relationship between the hydroxyl groups on the pentose moiety with the pyrimidine ring. The binding of these α-anomers to UrdPase from *T. gondii* contrasts with the situation with mammalian UrdPase, where α-lyxosides do not bind to the enzyme [38]. This indicates that *T. gondii* UrdPase is more tolerant to modifications in the pentose moiety than the host enzyme.

Binding of D- and L-Enantiomers

β-L-Enantiomers of uridine (**3**), 2'-deoxyuridine (**24**), thymidine (**33**), arabinosyluracil (**62**), arabinosylthymine (**65**), and xylouracil (**71**) bound less or did not bind to UrdPase when compared to their respective D-enantiomers (**1, 23, 31, 61, 64, 69**). This indicates that the D-enantiomer is the preferred configuration of these nucleosides for binding to *T. gondii* UrdPase. On the other hand, β-L-2',3'-dideoxyuridine (**46**) bound better or as well as β-D-2',3'-dideoxyuridine (**45**) to *T. gondii* UrdPase. Assuming that the pyrimidine moieties of the β-L-(**46**) and β-D-(**45**) enantiomers of 2',3'-dideoxyuridine bind to the

TABLE 1. Inhibitory potencies of pyrimidine nucleosides and analogues for uridine phosphorylase from *Toxoplasma gondii*

	Compound	Source	Apparent K_i (μM)	Ratio†
	Ribosides			
1.	Uridine (β-D-uridine)	SIG	279.0 ± 0.8	1.00
2.	α-D-Uridine	SYN	‡	
3.	β-L-Uridine	SYN	6304.7 ± 450.9	0.04
4.	3-Deazauridine	SIG	‡	
5.	4-Aminouridine (cytidine)	SIG	‡	
6.	4-Thiouridine	SIG	96.5 ± 2.91	2.89
7.	5-Bromouridine	ALD	264.7 ± 34.8	1.05
8.	5-Fluorouridine	SIG	274.8 ± 43.2	1.02
9.	5-Iodouridine	SIG	344.6 ± 51.3	0.81
10.	5-Methyluridine	SIG	317.3 ± 16.7	0.88
11.	5-Methyl-2-thiouridine	SIG	939.4 ± 67.9	0.30
12.	5-(*E*)-5-(2-Bromovinyl)uridine	SIG	186.8 ± 12.2	1.49
13.	5-Benzyluridine	SYN	16.1 ± 1.24	17.3
14.	5-(Phenylselenenyl)uridine	SYN	8.85 ± 0.32	31.5
15.	5-(Phenylthiomethyl)uridine	SYN	56.6 ± 2.88	4.93
16.	5-(Benzyloxybenzyl)uridine	SYN	4.18 ± 0.30	66.8
17.	6-Azauridine	SIG	‡	
18.	6-Carboxyuridine (orotidine)	SIG	6057.0 ± 839	0.05
19.	6-Oxyuridine (barbituric acid riboside)	SIG	120.4 ± 6.25	2.32
20.	Pseudouridine	SIG	‡	
21.	2′-O-Methyluridine	SIG	‡	
22.	2′,3′-Dibenzoyluridine	SIG	‡	
	2′-,3′- or 5′-Deoxyribosides			
23.	2′-Deoxyuridine (β-D-2′-deoxyuridine)	SIG	1414.5 ± 207.5	0.20
24.	β-L-2′-Deoxyuridine	SYN	3327.7 ± 166.5	0.08
25.	2′-Azido-2′-deoxyuridine	SIG	82.1 ± 28.5	3.41
26.	2′-Azido-2′-deoxycytidine	SIG	‡	
27.	2′-Chloro-2′-deoxy-4-thiouridine	SIG	2295.9 ± 249.8	0.12
28.	5-Bromo-2′-deoxyuridine	SIG	1281.7 ± 66.8	0.22
29.	5-Fluoro-2′-deoxyuridine	SIG	1180.0 ± 259.6	0.24
30.	5-Iodo-2′-deoxyuridine	SIG	1156.8 ± 363.7	0.24
31.	5-Methyl-2′-deoxyuridine (β-D-thymidine)	SIG	613.0 ± 132.7	0.46
32.	α-D-Thymidine	SYN	‡	
33.	β-L-Thymidine	SYN	‡	
34.	Trifluorothymidine	SIG	2572.0 ± 124.7	0.11
35.	5-Ethyl-2′-deoxyuridine	SIG	396.9 ± 43.0	0.70
36.	5-(*E*)-5-(2-Bromovinyl)-2′-deoxyuridine	SIG	348.4 ± 31.0	0.80
37.	5-Benzyl-2′-deoxyuridine	SYN	38.4 ± 3.6	7.27
38.	α-D-5-(Benzyl)-2′-deoxyuridine	SYN	1430.0 ± 185.3	0.20
39.	5′-Azido-5-benzyl-2′-deoxyuridine	SYN	423.1 ± 20.2	0.66
40.	5-(Phenylselenenyl)-2′-deoxyuridine	SYN	26.4 ± 2.36	10.6
41.	5-(Benzyloxybenzyl)-2′-deoxyuridine	SYN	14.0 ± 1.5	19.9
42.	3′-Deoxyuridine (β-D-3′-deoxyuridine)	SIG	2459.7 ± 579.7	0.11
43.	5′-Deoxyuridine (β-D-5′-deoxyuridine)	SIG	2576.4 ± 262.3	0.11
44.	5′-Deoxy-5-fluorouridine	HL	1854.0 ± 229.1	0.15
	2′,3′-Dideoxyribosides			
45.	2′,3′-Dideoxyuridine (β-D-2′,3′-dideoxyuridine)	SIG	688.4 ± 80.7	0.41
46.	β-L-2′,3′-Dideoxyuridine	SYN	584.3 ± 53.8	0.48
47.	5-Bromo-2′,3′-dideoxyuridine	ALD	2789.1 ± 201.8	0.10
48.	5-Methyl-2′,3′-dideoxyuridine (3′-deoxythymidine)	SIG	6422.3 ± 811.0	0.04
49.	3′-Amino-2′,3′-dideoxyuridine (AmddU)	JPS	763.8 ± 61.0	0.37
50.	3′-Amino-3′-deoxythymidine (AMT)	JPS	527.1 ± 73.6	0.53
51.	3′-Azido-2′,3′-dideoxyuridine (AzddU)	SIG	1620.4 ± 254.4	0.17
52.	3′-Azido-3′-deoxythymidine (AZT)	SIG	9390.0 ± 2139.0	0.03
53.	3′-Fluoro-3′-deoxythymidine (FLT)	ALD	‡	
54.	2′,3′-Didehydro-2′,3′-dideoxyuridine (D4U)	SYN	1562.9 ± 135.0	0.18
55.	2′,3′-Didehydro-3′-deoxythymidine (D4T)	JPS	2840.0 ± 24.4	0.10

(continued)

TABLE 1. Inhibitory potencies of pyrimidine nucleosides and analogues for uridine phosphorylase from *Toxoplasma gondii*

Compound		Source	Apparent K_i (μM)	Ratio†
56.	3′-Thia-2′,3′-dideoxyuridine	SYN	‡	
57.	5-Fluoro-3′-thia-2′,3′-dideoxyuridine (FTU)	SYN	6751.4 ± 1301.3	0.04
	2′,5′-Dideoxyribosides			
58.	2′,5′-Dideoxyuridine (β-D-2′,5′-dideoxyuridine)	ALD	1000.5 ± 103.5	0.28
59.	5-Methyl-2′,5′-dideoxyuridine (5′-deoxythymidine)	ALD	‡	
60.	4′-Amino-5′-deoxythymidine	CAL	‡	
	Arabinosides			
61.	Arabinosyluracil (β-D-arabinosyluracil)	SIG	2503.5 ± 566.2	0.11
62.	β-L-Arabinosyluracil	SYN	3726.7 ± 457.5	0.07
63.	4-Aminoarabinosyluracil (arabinosylcytosine)	SIG	4385.3 ± 1758.9	0.06
64.	5-Methylarabinosyluracil (β-D-arabinosylthymine)	SIG	3706.7 ± 524.3	0.08
65.	β-L-Arabinosylthymine	SYN	4589.3 ± 408.2	0.06
66.	5-Benzylarabinosyluracil	SYN	217.7 ± 12.6	1.28
67.	5-(Benzyloxybenzyl)arabinosyluracil	SYN	27.0 ± 2.5	10.3
68.	β-D-3′-Deoxyarabinosyluracil	SYN	4847.2 ± 388.2	0.06
	Xylosides			
69.	β-D-Xylouracil	SYN	637.9 ± 54.7	0.44
70.	α-D-Xylouracil	SYN	‡	
71.	β-L-Xylouracil	SYN	2334.3 ± 236.8	0.12
72.	β-D-Xylothymine	SYN	742.9 ± 53.8	0.38
73.	α-D-Xylothymine	SYN	‡	
	Lyxosides			
74.	β-D-Lyxouracil	SYN	‡	
75.	α-D-Lyxouracil	SYN	7011.6 ± 2060.4	0.04
76.	β-D-Lyxothymine	SYN	‡	
77.	α-D-Lyxothymine	SYN	1952.7 ± 187.5	0.14
	Acyclonucleosides			
78.	Acyclouridine	SYN	140.4 ± 26.0	1.99
79.	5-Fluoroacyclouridine	SYN	253.0 ± 32.4	1.10
80.	5-Methylacyclouridine (acyclothymidine)	SYN	83.1 ± 18.6	3.36
81.	5-Ethylacyclouridine	SYN	65.2 ± 9.4	4.28
82.	5-(Phenylselenenyl)acyclouridine (PSAU)	SYN	9.4 ± 0.7	29.7
83.	5-(Phenylthio)acyclouridine (PTAU)	SYN	9.2 ± 0.4	30.5
84.	5-Benzylacyclouridine (BAU)	SYN	11.9 ± 1.1	23.5
85.	*m*-Hydroxy BAU	SYN	23.2 ± 3.6	12.0
86.	*m*-Methoxy-BAU	SYN	10.7 ± 0.6	26.1
87.	2-Thio-BAU	SYN	890.9 ± 192.1	0.31
88.	Aminomethyl-BAU (AM-BAU)	SYN	16.6 ± 1.6	16.8
89.	Deoxyamino-BAU (DA-BAU)	SYN	18.1 ± 1.3	15.4
90.	5-(Benzyloxybenzyl)acyclouridine (BBAU)	SYN	4.1 ± 1.1	68.1
91.	Aminomethyl-BBAU (AM-BBAU)	SYN	4.3 ± 0.3	64.9
92.	Hydroxymethyl-BBAU (HM-BBAU)	SYN	2.2 ± 0.4	126.8
93.	5-(Benzyloxybenzyloxybenzyl)acyclouridine (BBBAU)	SYN	4.7 ± 0.5	59.4
94.	5-(Benzyloxybenzyl)barbituric acid acyclonucleoside (BBBA)	SYN	3.5 ± 0.6	79.7
95.	6-(Phenylselenenyl)acyclouridine	SYN	2201.4 ± 153.6	0.13
96.	2-Thio-6-(phenylselenenyl)acyclouridine)	SYN	1662.2 ± 201.2	0.17
97.	5-Bromo-6-(phenylselenenyl)acyclouridine	SYN	140.8 ± 22.5	1.98
98.	5-Chloro-6-(phenylselenenyl)acyclouridine	SYN	240.3 ± 24.5	1.16
99.	5-Fluoro-6-(phenylselenenyl)acyclouridine	SYN	764.3 ± 39.9	0.37
100.	5-Methyl-6-(phenylselenenyl)acyclouridine	SYN	760.2 ± 57.9	0.37
101.	Acyclopseudouridine	SYN	‡	
102.	Thioacylcopseudouridine	SYN	810.1 ± 81.7	0.34
103.	1-Methylacyclopseudouridine	SYN	2215.6 ± 191.8	0.13
104.	5-Methylacyclopseudouridine	SYN	2215.6 ± 191.8	0.13
105.	4-Aminoacyclopseudouridine (acyclopseudocytidine)	SYN	3227.1 ± 440.5	0.09
106.	1-Methylacyclopseudocytidine	SYN	5311.8 ± 784.2	0.05
107.	1-Allyloxymethyluracil	SYN	332.7 ± 25.6	0.84

(continued)

TABLE 1. (*continued*) Inhibitory potencies of pyrimidine nucleosides and analogues for uridine phosphorylase from *Toxoplasma gondii*

Compound		Source	Apparent K_i (μM)	Ratio†
108.	5-Methyl-1-allyloxymethyluracil	SYN	5665.2 ± 573.2	0.05
	Carbocyclic nucleosides			
109.	Carbocyclic uridine	SYN	‡	
110.	5-Methyl-carbocyclic uridine	SYN	‡	
111.	5-Ethynyl-carbocyclic uridine	SYN	‡	
112.	5-Methyl-2′-deoxy-carbocyclic uridine (carbocyclic thymidine)	SYN	‡	
113.	5-Ethyl-2′-deoxy-carbocyclic uridine	SYN	‡	
114.	5-Ethynyl-2′-deoxy-carbocyclic uridine	SYN	‡	
115.	2,2′-Anhydro-carbocyclic uridine	SYN	3363.8 ± 695.4	0.08
116.	2,2′-Anhydro-5-methyl-carbocyclic uridine	SYN	2103.6 ± 290.5	0.13
117.	2,5′-Anhydro-carbocyclic thymidine	SYN	‡	
118.	3′-Amino-3′-deoxy-carbocyclic uridine	SYN	‡	
119.	3′,4′-Didehydro-carbocyclic thymidine	SYN	‡	
	Anhydronucleosides			
120.	2,2′-Anhydrouridine (β-D-2,2′-anhydrouridine)	SIG	1.58 ± 0.13	176.6
121.	β-L-2,2′-Anhydrouridine	SYN	‡	
122.	5-Ethyl-2,2′-anhydrouridine	SYN	10.8 ± 0.94	25.8
123.	5-Benzyl-2,2′-anhydrouridine	SYN	0.22 ± 0.02	1,268
124.	5-(Benzyloxybenzyl)-2,2′-anhydrouridine	SYN	0.06 ± 0.003	4,650
125.	5-Aza-6-thio-2,2′-anhydrouridine	SYN	3210.1 ± 742.2	0.09
126.	5-Aza-5-methyl-6-thio-2,2′-anhydrouridine	SYN	‡	
127.	5-Aza-6-allylthio-2,2′-anhydrouridine	SYN	‡	
128.	5-Aza-6-proparagylthio-2,2′-anhydrouridine	SYN	3557.8 ± 542.6	0.08
129.	α-D-2,2′-Anhydroxylouracil	SYN	‡	
130.	β-D-3′-Deoxy-2,2′-anhydrouridine	SYN	1631.1 ± 158.4	0.17
131.	β-L-3′-Deoxy-2,2′-anhydrouridine	SYN	‡	
132.	5-Methyl-2′-deoxy-2,3′-anhydrouridine (2,3′-anhydrothymidine)	SIG	‡	
133.	5-Benzyl-2′-deoxy-2,3′-anhydrouridine	SYN	‡	
134.	5-(Benzyloxybenzyl)-2′-deoxy-2,3′-anhydrouridine	SYN	‡	
135.	2,5′-Anhydrouridine	SYN	511.9 ± 57.7	0.55
136.	6,1′-Anhydro-6-hydroxypsicouridine	SYN	931.7 ± 61.9	0.30
	Cyclonucleosides			
137.	6,5′-Cyclouridine	SYN	‡	
138.	4-Thio-6,5′-cyclouridine	SYN	‡	
139.	5-Bromo-6,5′-cyclouridine	SYN	‡	
140.	5-Methyl-6,5′-cyclouridine	SYN	‡	
141.	5-Hydroxyethyl-6,5′-cyclouridine	SYN	‡	
142.	2′-Deoxy-6,5′-cyclouridine	SYN	‡	
143.	5-Bromo-2′-deoxy-6,5′-cyclouridine	SYN	‡	
144.	6,3′-Methanouridine	SYN	‡	
145.	4-Thio-6,3-methanouridine	SYN	‡	
146.	$O^{5'-6}$-Methanouridine	SYN	6639.7 ± 517.8	0.04
	Other nucleoside analogues			
147.	Dioxolanethymidine	SYN	‡	
148.	β-[4-(Hydroxy)tetrahydrofuran-2-*yl*]thymine	SYN	‡	
149.	α-[4-(Hydroxy)tetrahydrofuran-2-*yl*]thymine	SYN	946.5 ± 82.7	0.29
150.	5-Fluoro-1-(tetrahydro-2-furyl)uracil (Ftorafur)	SIG	450.6 ± 49.8	0.62
151.	Deoxyglucosylthymine	SIG	199.9 ± 21.2	1.40

* Values are means ± SD from at least three determinations.
† Ratio: apparent K_i for uridine/apparent K_i for the compound.
‡ Less than 10% inhibition at an inhibitor concentration of 0.9 mM.

active site of the enzyme in the same fashion, then apart from the 5′-hydroxyl group, only the furanose ring oxygen is available for hydrogen bonding (see Figs. 1 and 2). Addition of hydroxyl groups at C2′ and C3′ of the inverted furanose ring (see Fig. 1) may cause steric congestion and, therefore, the markedly reduced binding of β-L-uridine **(3)**. Removing a hydroxyl group from C2′ or C3′ of the furanose ring of β-L-uridine **(3)**, as in β-L-deoxyuridine **(24)**, β-L-arabinosyluracil **(62)**, or β-L-xylouracil **(71)**, appears to relieve some of this steric congestion, hence, the improved binding of these β-L-enantiomers when compared with β-L-uridine **(3)**. The better or equal binding of the β-L- **(46)** and β-D- **(45)** enantiomers of 2′,3′-dideoxyuridine also suggests that the *trans* relationship between the 5′-hydroxyl

group of the pentose and the pyrimidine moiety could be the preferred configuration for the binding of 2',3'-dideoxynucleosides to *T. gondii* UrdPase.

Effect of the 2'-Hydroxyl Group

Table 1 shows that 2'-deoxyribosides **(23, 28–31, 36, 37, 40, 41)** bound weaker to UrdPase than their corresponding ribosides **(1, 7–10, 12–14, 16).** It also shows that replacement of the 2'-position hydroxyl group of uridine **(1)** with a methoxy (i.e. 2'-O-methyluridine, **21**) or a benzoyl (2',3'-dibenzoyluridine, **22**) group abolished binding. Replacement of the 2'-hydroxyl group with a chloro group (i.e. 2'-chloro-2'-deoxy-4-thiouridine, **27**) reduced the binding of 4-thiouridine **(6)** by 24-fold. These results can be explained presumably by involvement of the 2'-hydroxyl group in ribosides in hydrogen bonding with a histidine residue in UrdPase as suggested by Bose and Yamada [70]. However, this suggestion does not explain the very strong binding of 2'-azido-2'-deoxyuridine **(25)** or the 2,2'-anhydro- **(120, 122–124)** compounds which lack the 2'-hydroxyl group. On the other hand, arabinosyluracil **(61),** which has a 2'-hydroxyl group, but is *cis* to the pyrimidine ring, bound poorly to UrdPase. The strong binding of the 2,2'-anhydrouridines **(120, 122–124)** suggests that the *syn* orientation outweighs the contribution of hydrogen bonding by the 2'-hydroxyl group in the active site of the enzyme. As for the poor binding of the arabinosides **(61, 64, 66, 67),** it can be attributed to the significant changes in their conformation from that of their respective ribosides **(1, 10, 13, 16)** [40, 71, 72] or the intolerance of the enzyme for a 2'-hydroxyl group in the *ara* configuration which also favors the *anti* form of the arabinosides [40, 72].

Effect of the 3'-Hydroxyl Group

It was shown previously that the presence of a hydroxyl group in the proper *ribo* configuration at the 3'-position is essential for the binding of β-nucleosides to UrdPase from mammalian sources [40, 55, 71, 73]. In sharp contrast to the situation with the mammalian UrdPase [40, 71], the absence of the 3'-hydroxyl group (e.g. 3'-deoxyuridine, **42**), or change in its *ribo* configuration (e.g. xylouracil, **69**), reduced but did not prevent binding to *T. gondii* UrdPase. Nevertheless, the increase in binding of HM-BBAU **(92)** over the parent compound, BBAU **(90),** could be attributed to the availability of a second terminal hydroxyl group capable of hydrogen bonding on the acyclo tail of HM-BBAU. It has been suggested that the terminal hydroxyl on the acyclo tail of acyclopyrimidines is located at the same site as the 3'-hydroxyl group of uridine [55, 57, 71]. Therefore, the presence of the 3'-hydroxyl group seems to enhance, but is not essential for a ligand to bind to *T. gondii* UrdPase. However, this finding does not explain the lack of binding of the 2,3'-anhydro-2'-deoxyuridines **(132–134),** which are fixed in the *syn* range required for binding, but which lack the 3'-hydroxyl group.

Effect of the 5'-Hydroxyl Group

The present results suggest that the 5'-hydroxyl group enhances, but is not essential for binding of nucleoside ligands to *T. gondii* UrdPase. Table 1 shows that 5'-deoxyuridine **(43),** 5'-deoxy-5-fluorouridine **(44)** and 2,5'-anhydrouridine **(135),** which lack the 5'-hydroxyl group, bound to the enzyme. Nevertheless, the absence of the 5'-hydroxyl group in 5'-deoxyuridine **(43)** and 5'-deoxy-5-fluorouridine **(44)** significantly reduced their binding when compared with their riboside counterparts, uridine **(1)** and 5-fluorouridine **(8),** respectively. The absence of the 5'-hydroxyl group can also explain, at least in part, the lack of strong binding expected from 2,5'-anhydrouridine **(135),** an analogue held in the favored *syn* conformation. However, it is not known why further modifications to C5' in the pentose (i.e. 5'-amino-2',5'-dideoxyuridine, **60**) or C5 in the aglycon (i.e. 5'-deoxythymidine, **59**) abolished binding. In addition to the absence of the 5'-hydroxyl group, another factor(s) may be responsible for the lack of strong binding of 2,5'-anhydrouridine **(135),** 5'-amino-2',5'-dideoxyuridine **(60)** or 5'-deoxythymidine **(59).** Among these, as discussed above, are changes in conformation of the pentose moiety and/or the required relative orientation of the aglycon to the pentose moiety.

Effect of the Combined Absence of 2'-, 3'-, and 5'-Hydroxyl Groups

It is interesting to note that the reduced binding of 2'-deoxyuridine **(23)** resulting from the absence of a hydroxyl group at C2' is somewhat diminished by removal of the hydroxyl group at C3' (e.g. 2',3'-dideoxyuridine, **45**). The poor binding of 3'-deoxyuridine **(42)** is also improved by removal of the hydroxyl group at C2' (e.g. 2',3'-dideoxyuridine, **45**). Similarly, the binding of 5'-deoxyuridine **(43)** is improved by removal of the hydroxyl group at C2' (i.e. 2',5'-dideoxyuridine, **58**). However, the better binding of 2',3'-dideoxyuridine **(45)** or 2',5'-dideoxyuridine **(58)** over the single substituted deoxyuridines (i.e. 2'-deoxyuridine, **23;** 3'-deoxyuridine, **42;** 5'-deoxyuridine, **43**), was reduced sharply or abolished when the compounds were substituted at C5 with a bromo (e.g. 5-bromo-2',3'-dideoxyuridine, **47**) or a methyl (e.g. 5-methyl-2',3'-dideoxyuridine, **48;** 5-methyl-2',5'-dideoxyuridine, **59**) group, and/or at C3' with an azido (e.g. 3'-azido-2',3'-dideoxyuridine, **51;** 3'-azido-3'-deoxythymidine, **52**), fluoro (e.g. 3'-fluoro-3'-deoxythymidine, **53**), thia (e.g. 3'-thia-2',3'-dideoxyuridine, **56**), or oxo (e.g. dioxolanethymidine, **147**) group, or by eliminating the hydrogens at the 2'- and 3'-positions (e.g. 2',3'-didehydro-2',3'-dideoxyuridine, **54;** 2',3'-didehydro-3'-deoxythymidine, **55**). Only an amino group, a group that can be involved in hydrogen bonding, at C3' (e.g. 3'-amino-2',3'-dideoxyuridine, **49;** 3'-amino-3'deoxythymidine, **50**) allowed these analogues to bind as well as the unsubstituted parent compound, 2',3'-dideoxyuridine **(45)** regardless of

the 5-position substitution. The reason for this interaction between substituents at C3′ of the pentose moiety and C5 on the pyrimidine ring is unclear. The poorer binding of 2′,3′-didehydro-2′,3′-dideoxyuridine **(54)** when compared with its saturated counterpart, 2′,3′-dideoxyuridine **(45)**, could be attributed to reduced furanose ring puckering [74].

Effect of 5-Position Substitution

The enhancement of binding of uridine **(1)**, 2′-deoxyuridine **(23)**, arabinosyluracil **(61)**, acyclouridine **(78)**, and 2,2′-anhydrouridine **(120)** by increasing the hydrophobicity at the 5-position of the aglycon [ethyl **(35, 81)** < bromovinyl **(12, 36)** < benzyl **(13, 37, 66, 84, 123)** < *m*-methoxybenzyl **(86)** < phenylselenenyl **(14, 40, 82)** < *m*-benzyloxybenzyl **(16, 41, 67, 90, 124)**] is in agreement with the suggestion that a hydrophobic pocket exists on *T. gondii* UrdPase, adjacent to the binding site of the 5-position of the pyrimidine ring [7] similar to that proposed for UrdPase from other sources, [40, 55–58, 71, 75]. However, the binding of 5-(benzyloxybenzyloxybenzyl)acyclouridine **(93)** to *T. gondii* UrdPase (Table 1), but not the mammalian enzyme [57], suggests that the hydrophobic pocket on *T. gondii* UrdPase is larger than that on the mammalian enzyme. Similar results were reported for UrdPase from the parasitic helminth *Schistosoma mansoni* [58].

The contribution of the 5-position to the binding of ligands to UrdPase seems to be of far greater importance than the role of the 2′-hydroxyl group in the *ribo* configuration. This is inferred from the finding that the lack of binding of arabinosyluracil **(61)** is overcome by adding a hydrophobic group at the 5-position [i.e. 5-benzylarabinosyluracil, **66**; 5-(benzyloxybenzyl)arabinosyluracil, **67**]. It is also evident from the fact that while the unsubstituted 2′-deoxyriboside (2′-deoxyuridine, **23**) binds approximately 5-fold less than the riboside (uridine, **1**), 5-benzyl-2′-deoxyuridine **(37)** binds approximately 2-fold less than 5-benzyluridine **(13)**.

In conclusion, we have identified 5-(benzyloxybenzyl)-2,2′-anhydrouridine **(124)** as a powerful inhibitor of *T. gondii* UrdPase with an apparent K_i value of 60 ± 3 nM. Additionally, the present results indicate that the presence of a true *N*-glycosyl bond and the capacity to adopt the *high syn* or *syn* conformation are among the critical structural and conformational requirements for a nucleoside ligand to bind to *T. gondii* UrdPase. The presence and/or proper orientation of a 2′-, 3′-, and/or 5′-hydroxyl group on the pentose, and an *N*-glycosyl bond in the β-configuration enhances but is not essential for binding. The present results also indicate that *T. gondii* UrdPase differs from the host enzyme in at least three aspects. First, the parasite, but not the host UrdPase, can participate in hydrogen bonding with *N*3 of the pyrimidine ring of nucleoside ligands. Second, the parasite enzyme has a larger hydrophobic pocket adjacent to C5 of the pyrimidine moiety than in the host enzyme [57, 58]. Third, *T. gondii* UrdPase can accommodate modifications in the pentose moiety which cannot be tolerated by the host enzyme. Most prominent among these modifications is the absence and/or lack of *ribo* orientation of the 3′-hydroxyl group which is a requirement for a ligand to bind to mammalian UrdPase [40]. These differences between the parasite and host enzymes can be useful in designing specific inhibitors or "subversive" substrates for *T. gondii* UrdPase.

We thank Brian Kopicki for excellent technical assistance. This investigation was supported by Grants AI-29849 and AI-31702 awarded by the NIAID, DHHS.

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