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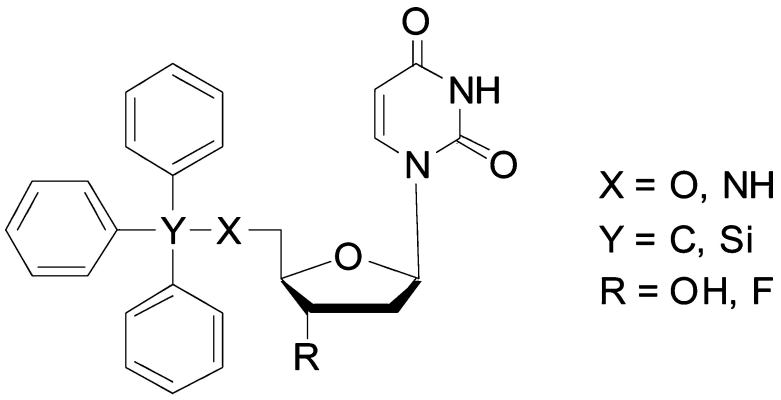

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Deoxyuridine Triphosphate Nucleotidohydrolase as a Potential Antiparasitic Drug Target

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This paper describes a structure−activity study to identify novel, small-molecule inhibitors of the enzyme deoxyuridine 5′-triphosphate nucleotidohydrolase (dUTPase) from parasitic protozoa. The successful synthesis of a variety of analogues of dUMP is described in which the substituents are introduced at the 3′- and 5′-positions, together with variation in the heteroatom at the 5′-position. The compounds were assayed against recombinant *Plasmodium falciparum* and *Leishmania major* enzymes and the human enzyme to give a measure of selectivity. The compounds were also tested in vitro against the intact parasites *P. falciparum* and *L. donovani*. A number of potent and selective inhibitors of the *P. falciparum* dUTPase that show drug-like properties and represent good leads for future development were identified. The best inhibitors included the compounds 5′-tritylamino-2′,5′-dideoxyuridine (**2j**) (K_i = 0.2 μM) and 5′-*O*-triphenylsilyl-2′,3′-didehydro-2′,3′-dideoxyuridine (**5h**) (K_i = 1.3 μM), with selectivity greater than 200-fold compared to the human enzyme. Structural features important for antiplasmodial activity were determined. The correlation observed between the inhibition of the enzyme and the inhibition of the parasite growth in vitro demonstrates that the *P. falciparum* dUTPase constitutes a valid and attractive novel target for the development of much-needed new antimalarial drugs.

Introduction

The enzyme deoxyuridine 5′-triphosphate nucleotidohydrolase (dUTPase, E.C. 3.6.1.23) is involved in nucleotide metabolism; it catalyses the hydrolysis of deoxyuridine triphosphate (dUTP) to deoxyuridine monophosphate (dUMP) and inorganic pyrophosphate in the presence of magnesium ions (Scheme 1). dUTPase is widespread in nature and has been found in a variety of prokaryotic and eukaryotic organisms as well as in many viruses.[1,2] It was shown to be essential for viability in *Escherichia coli*,[3] *Saccharomyces cerevisiae,*[4] and *Leishmania major*[5] and is probably essential for all cellular systems. The enzyme is thought to be crucial to DNA integrity in two ways. It prevents the build up of dUTP and ensures the provision of dUMP, the substrate for thymidylate synthase in the biosynthesis of deoxythymidine triphosphate (dTTP). As a result, a low dUTP/dTTP ratio is maintained, which greatly reduces uracil incorporation into DNA because DNA polymerases do not discriminate between dUTP and dTTP. Under normal circumstances, following dUTP misincorporation into DNA, uracil is excised and replaced by thymine through a repair process catalyzed by uracil−DNA glycosylase. However, when dUTP levels are abnormally high, repetitive cycles of introduction and excision of uracil take place, giving rise to DNA fragmentation and ultimately cell death.[2]

Structurally, dUTPases can be classified into three families according to their subunit composition as either monomers, dimers, or trimers. Trimeric dUTPases form the largest and most varied group. Currently, X-ray structures have been published for two bacterial,[6,7] one mammalian,[8] one protozoal,[9] and two retroviral[10,11] dUTPases. They reveal three identical active sites formed at the subunit interfaces, with each active site made up of residues contributed by all subunits and containing five highly conserved motifs.[12] Two subunits are involved in base and sugar recognition, and the third subunit has a flexible glycine-rich motif positioned in the C-terminal region, which is thought to close up on the active site and substrate during catalysis. From the kinetic point of view, trimeric dUTPases appear extremely exclusive toward dUTP; they can discriminate between the four nucleobases and are also selective toward the sugar moiety. Thus, other naturally occurring nucleotides such as dTTP, deoxycytosine triphosphate (dCTP), and uridine triphosphate (UTP) are very poor substrates.[13,14]

Monomeric dUTPases are encoded by mammalian herpesviruses and are thought to be evolutionarily related to the trimeric family with which they share catalytic properties and the same conserved motifs, although the motifs are ordered in a slightly different manner.[12]

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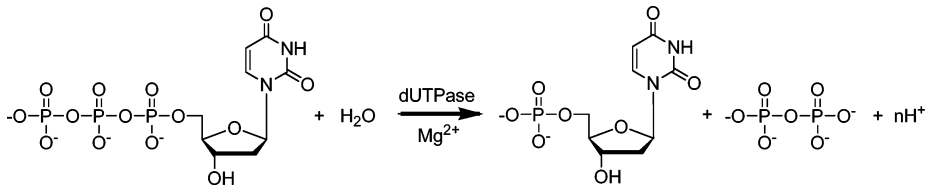
Scheme 1



Dimeric dUTPases were identified in the trypanosomatids *Trypanosoma cruzi*[15] and *Leishmania major*,[16] as well as in the bacterium *Campylobacter jejuni.*[17] They form a very distinct group: structurally they consist of two oligomers that lack the five conserved motifs found in the other classes of dUTPases; catalytically they show a broader specificity and are capable of hydrolyzing not only dUTP but also deoxyuridine diphosphate (dUDP).[5] To date, only the crystal structures of *T. cruzi* and *C. jejuni* have been elucidated. The *T. cruzi* protein reveals a rigid core that remains static, whereas upon nucleotide binding, mobile domains undergo extensive structural rearrangements.[18] The structure of the *C. jejuni* dUTPase has only been determined in a closed, substrate-bound conformation, which was found to resemble the *T. cruzi* structure in many ways.[19]

The dissimilarity in structure between the dimeric dUTPases (e.g., *L. major* and *T. cruzi*) and the human dUTPase and the essential nature of the enzyme suggest that dUTPase may be a drug target against parasitic protozoa. Furthermore, recent work shows that, despite a common overall topology, there are also structural differences between the *Plasmodium* and human dUTPases, even though the *Plasmodium* enzyme is trimeric.[9] This may be connected to an insertion of ~23 amino acids unique to the *Plasmodium* dUTPase.

Kinetic studies have demonstrated that dUTPases have high specificity for 2′-deoxyuridine compared to other nucleosides including deoxythymidine, deoxycytosine, and uridine.[20] This suggests that uracil is crucial for activity and that there should be no hydroxyl at the 2′-position. This is also supported by crystallographic data that consistently show a tight fitting of the uracil base within the active site, regardless of the type of dUTPase.[12,21] Therefore, we set out to initially focus on positions 3′ and 5′ of 2′-deoxyuridine. In particular, we decided to probe these positions with a range of lipophilic and hydrophilic substituents of varying steric bulk to build up a series of structure−activity relationships (SARs). Interestingly, dUMP has been shown to be an

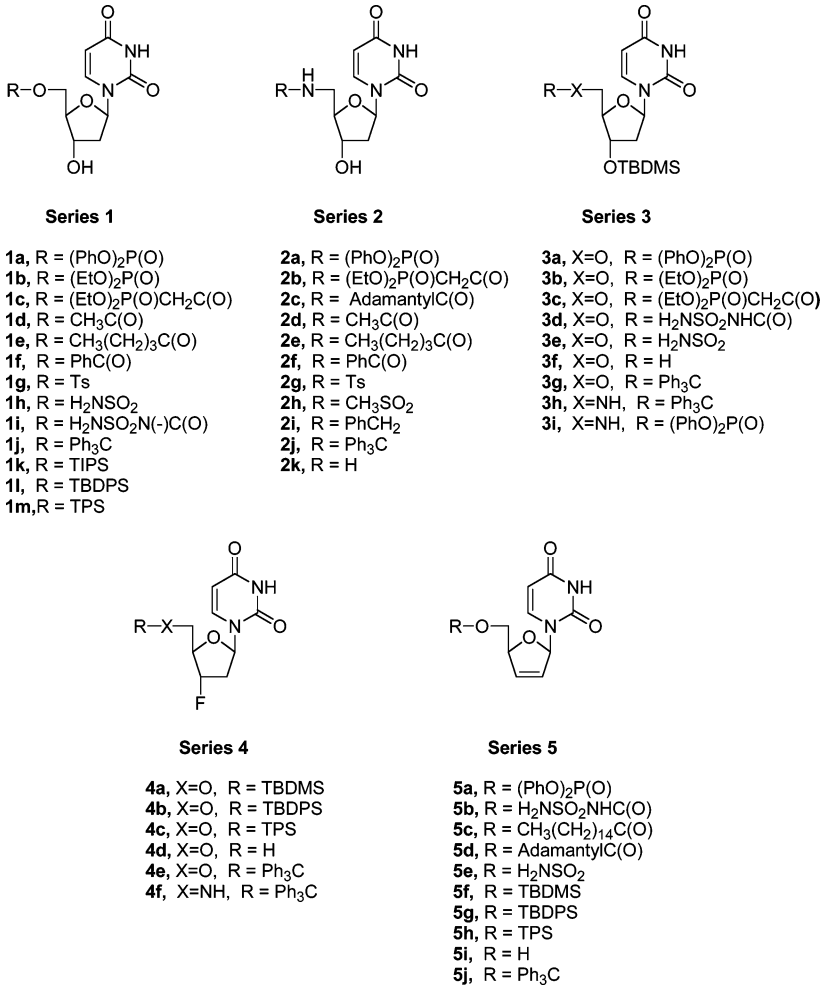
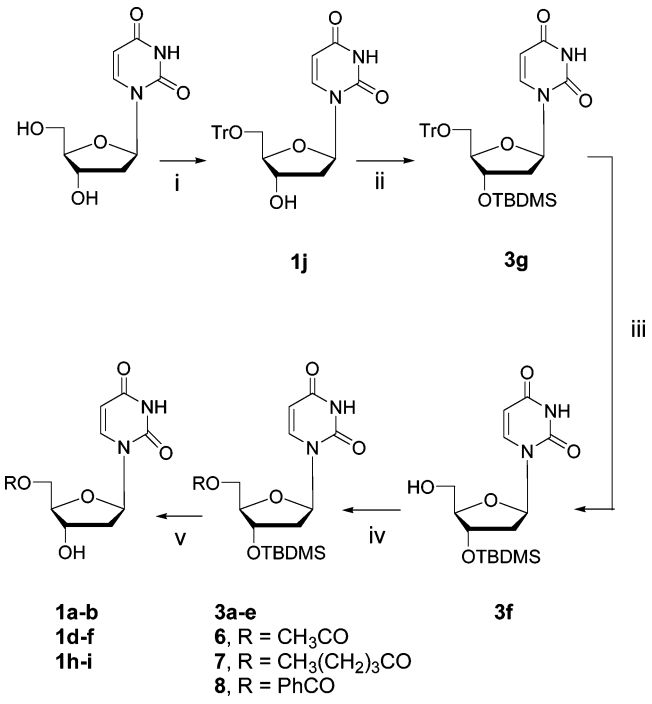


Figure 1. Compounds prepared and assayed. (TBDMS = *tert*-butyldimethylsilyl; TBDPS = *tert*-butyldiphenylsilyl; TIPS = triisopropylsilyl; TPS = triphenylsilyl; Ts = *p*-toluenesulfonyl).

Table 1. Derivatization of the 3′-Protected Deoxyuridine **3f** and Subsequent Removal of the 3′-TBDMS Group (Scheme 2)

	derivatization of compound **3f**			removal of 3′-TBDMS group		
R	compd	yield (%)	conditions	compd	yield (%)	conditions
$(PhO)_2PO$	**3a**	94	$(PhO)_2POCl$, pyridine, rt	**1a**	87	Dowex, MeOH, rt
$(EtO)_2PO$	**3b**	69	$(EtO)_2POCl$, pyridine, rt	**1b**	90	Dowex, MeOH, rt
$(EtO)_2POCH_2CO$	**3c**	77	$(EtO)_2POCH_2CO_2H$, EDCI, DMF, rt			
HN_2SO_2NHCO	**3d**	74	(a) $ClSO_2NCO$, CH_3CN, −20 °C (b) NH_3, CH_3CN, −20 °C	**1i**	66	TBAF, THF, rt
HN_2SO_2	**3e**	73	$ClSO_2NH_2$, DMA, 0 °C	**1h**	90	$(CH_3CN)_2PdCl_2$, acetone, rt
CH_3CO	**6**	74	CH_3COCl, pyridine/DCM, rt	**1d**	72	TBAF, THF, rt
$CH_3(CH_2)_3CO$	**7**	96	$CH_3(CH_2)_4COCl$, pyridine/DCM, rt	**1e**	68	TBAF, THF, rt
PhCO	**8**	99	PhCOCl, pyridine/DCM, rt	**1f**	80	Dowex, MeOH, rt

Scheme 2[a]



[a] (i) TrCl, pyridine, 50 °C, 97%; (ii) TBDMSCl, imidazole, DMF, 78%; (iii) Et_2AlCl, $CHCl_3$, 73%; for (iv) and (v), see Table 1.

inhibitor of the trypanosomid enzymes, suggesting that it may be possible to develop analogues of dUMP as potential inhibitors.

Chemistry

As a starting point, five series of compounds were made to investigate SARs (Figure 1). In Series 1, a variety of substituents were placed on the 5′-position. Compounds **1a** and **1b** represent phosphate esters of dUMP with bulky or small substituents. Compounds **1d** and **1f** are simple esters of varying lipophilicity. Compounds **1g** and **1h** are sulfonates, which should be isosteres for the phosphate group. **1j** is a simple ether, whereas **1k**−**m** bear a silyl ether moiety. Finally, **1c** and **1i** are isosteres of the diphosphate dUDP. A similar series of compounds was prepared in which the 5′-oxygen is replaced with an amine (Series 2). In the case of the acyl derivatives, the amide analogues are likely to be biologically more stable than are their ester counterparts, which may be subject to hydrolysis and enzymatic cleavage by cellular esterases. The conversion from ester (O) to amide (NH) also changes a hydrogen-bond acceptor to a hydrogen-bond donor and may be useful to provide more information on the interactions required at the enzyme active site. Series 3, 4, and 5 were designed to probe the 3′-position and comprise a selection of compounds in which the 5′-position is varied while the 3′-hydroxyl is either blocked as a *tert*-butyldimethylsilyl ether (Series 3), replaced by a fluorine isostere (Series 4), or removed altogether (Series 5). These modifications aimed to investigate the effects of increasing lipophilicity and steric hindrance, and of altering possibilities of hydrogen bonding at the 3′-position. In addition, in the case of the didehydrodideoxy analogues (Series 5), the presence of the double bond at the 2′−3′ position provides rigidity for the system.

Series 1 and 3. The preparation of the compounds from Series 1 and 3 (except **1g**, **1k**−**m**, and **3h**−**i**) is described in Scheme 2 and Table 1. In both of these series, the 5′-position is varied.

Initial studies revealed that the attempted phosphorylation of deoxyuridine following the methodology of Hes et al.[22] led to mixtures with substitution on the 3′- and 5′-positions despite lowering the temperature and increasing dilution. Therefore the 3′-position was protected in a three-step procedure: (1) tritylation of deoxyuridine at the 5′-position; (2) silylation with *tert*-butyldimethylsilyl chloride (TBDMSCl);[23] and (3) removal of the trityl protection by treatment with diethylaluminum chloride.[24]

The subsequent derivatization of the 5′-position with various RCl reagents in the presence of pyridine proceeded well to give phosphate and carboxylic esters (Table 1). Reacting the 3′-protected alcohol **3f** with diethylphosphonoacetic acid in the presence of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDCI) afforded the phosphonoacetate analogue **3c**. Reaction with chlorosulfonylisocyanate at −20 °C followed by treatment with ammonia produced the sulfamoylcarbamoyl analogue **3d**.[25] Conversion of **3f** to its sulfamoyl derivative **3e** was effected with sulfamoyl chloride in dimethylacetamide (DMA).[26]

For the final removal of the 3′-*tert*-butyldimethylsilyl (TBDMS) group, three different methods were employed, depending on the nature of the 5′-substituent (Table 1). It was found that when a bulky substituent is present at the 5′-position, desilylation was best carried out by treatment with Dowex sulfonic acid resin; **1a**, **1b**, and **1f** were obtained in this manner. Compounds containing an acid-sensitive ester functionality were treated with tetrabutylammonium fluoride (TBAF) instead, thus yielding **1d**, **1e**, and **1i**. However, for **1i**, difficulties were encountered in purifying the tetrabutylammonium salt obtained. To circumvent this problem, a third method of silyl deprotection was investi-

Table 2. Derivatization of the 5′-Amino Compounds **2k** and **13** (Scheme 3)

compd	R	conditions	yield(%)
2a	$(PhO)_2PO$	$(PhO)_2POCl$, pyridine/DMF, rt	19
2b	$(EtO)_2POCH_2CO$	$EtO)_2POCH_2CO_2H$, EDCI, DMF, rt	56
2c	adamantoyl	adamantoyl chloride, pyridine, rt	53
2d	CH_3CO	CH_3COCl, pyridine/DMF, rt	52
2e	$CH_3(CH_2)_3CO$	$CH_3(CH_2)_3COCl$, pyridine/DMF, rt	49
2f	PhCO	PhCOCl, pyridine/DMF, rt	34
2g	Ts	TsCl, pyridine/DMF, 0−4 °C	37
2h	CH_3SO_2	CH_3SO_2Cl, pyridine/DMF, 0 °C	51
2j	Ph_3C	Ph_3CCl, pyridine, 50 °C	49
4f	Ph_3C	Ph_3CCl, pyridine, 50 °C	32

gated, which employed palladium chloride in acetone;[27] the addition of hydrochloric acid was required to avoid the poisoning of the catalyst. This proved to be very effective for **1h** (90% yield). In the case of the phosphonoacetate analogue **1c**, the direct derivatization of deoxyuridine with diethylphosphonoacetic acid in the presence of EDCI offered an even better alternative. Similarly, the 5′-moieties for compounds **1k**−**m** were bulky enough to allow for a clean monosilylation of deoxyuridine under standard conditions[23] (74−85%). Compound **1g** could be obtained by direct tosylation[28] (Scheme 3).

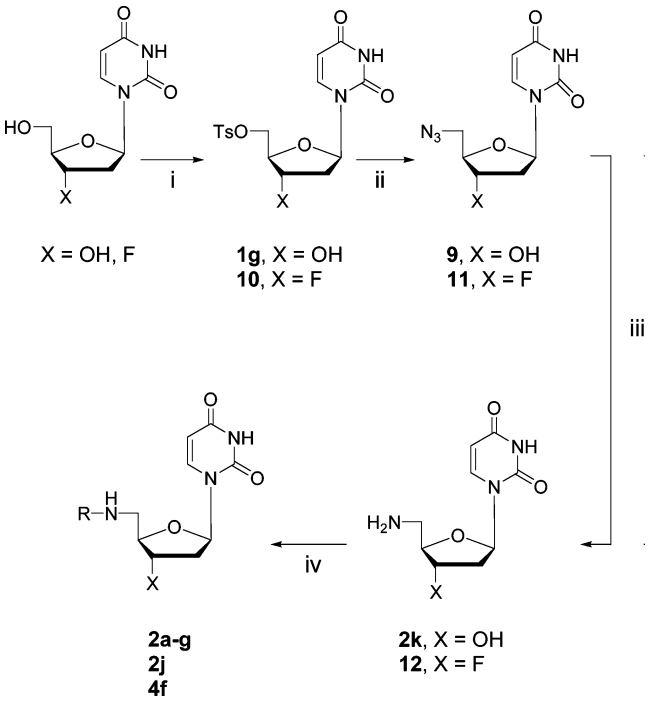
Compounds **3h** and **3i** were obtained in good yields by silylation of the 5′-protected amino analogues **2j** and **2a**, using TBDMSCl and *tert*-butyldimethylsilyl trifluoromethanesulfonate (TBDMSOTf), respectively.

Series 2. The preparation of the compounds from Series 2 (except **2i**, which is discussed later) is shown in Scheme 3 (see also Table 2). In this series, the 5′-oxygen is replaced by NH. Preparation of 5′-aminodeoxyuridine **2k** was achieved by selective tosylation of the 5′-position of deoxyuridine, displacement with azide, and hydrogenation, following a procedure adapted from Lin et al.[28] The amine **2k** was derivatized (Table 2) with a range of substituents similar to those for Series 1 to allow comparisons to be made. Because of the difference in reactivity between amine and alcohol functions, the selective transformation of the 5′-amino moiety should have proceeded smoothly without prior protection of the 3′-hydroxyl. However, this methodology failed to produce the 5′-benzylamino derivative **2i**, which was finally obtained in 45% yield from **1g** by the displacement of the tosylate with a large excess of benzylamine.

Series 4. The 3′-fluoro analogues (Series 4) were prepared from 2′,3′-dideoxy-3′-fluorouridine. Direct silylation or tritylation produced compounds **4a**−**c** and **4e** in 60−92% yield. The 5′-tritylamino derivative **4f** was prepared following a reaction sequence similar to that used for the amino compounds from Series 2 (Scheme 3), starting from 2′,3′-dideoxy-3′-fluorouridine instead of 2′-deoxyuridine.

Series 5. Considerable interest has been devoted to didehydrodideoxy nucleosides and their chemistry following their effective use as potential chemotherapeutic agents for the treatment of HIV-AIDS.[29] The synthetic route we chose for our 2′,3′-didehydro-2′,3′-dideoxyuridine (d4U) series (Series 5) is depicted in Scheme 4 and Table 3, using the methodology of Dudycz.[30] The desilylation of **5f** was best effected using TBAF.[23] Further derivatization included phosphorylation with chlorodiphenyl phosphate (**5a**), formation of the sulfamoylcarbamoyl and sulfamoyl analogues (**5b** and **5e**), esterification (**5c** and **d**), tritylation (**5j**), and introduction of bulky silyl moieties (**5g** and **h**) to probe further the importance of bulky substituents. The reaction conditions, given in Table 3, were similar to those employed for Series 1.

Scheme 3[a]



[a] (i) Tosyl chloride, pyridine, 0−4 °C; (ii) NaN_3, DMF, 90 °C; (iii) H_2, 5% Pd/C, $EtOH/H_2O$; for (iv), see Table 2.

Biology

Enzyme Assays. Compounds were assayed against the recombinant *L. major*, *P. falciparum,* and human dUTPases (Table 4). There is a high degree of similarity/identity between the *L. major*, *T. cruzi*, and *T. brucei* enzymes, so the *L. major* enzyme should serve as a good model for the other trypanosomatid enzymes. Inhibition of the human enzyme was also studied and provided a measure of the selectivity of the compounds through the calculation of their selectivity index [SI = (K_i human)/(K_i parasite)].

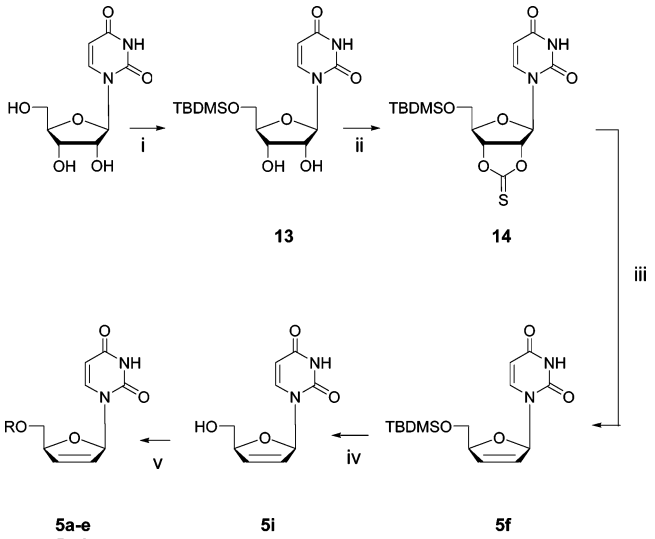
The enzyme assays were carried out using a continuous spectrophotometric method based on the fact that the hydrolysis of dUTP to dUMP is coupled to a pH and Mg^{2+}-dependent release of protons (Scheme 1). The reaction can therefore be followed by monitoring the pH change using a stopped-flow apparatus.[20]

Generally, better results were observed against the *Plasmodium* enzyme than against the *L. major* enzyme.

Table 3. Derivatization of the Didehydrodideoxyuridine **5i** (Scheme 4)

compd	R	conditions	yield (%)
5a	$(PhO)_2PO$	$(PhO)_2POCl$, pyridine, rt	12
5b	H_2NSO_2NHCO	(a) chlorosulfonylisocyanate, CH_3CN, −20 °C (b) NH_3 CH_3CN, −20 °C, rt	73
5c	$CH_3(CH_2)_{14}CO$	$CH_3(CH_2)_{14}COCl$, pyridine, rt	92
5d	adamantoyl	adamantoyl chloride, pyridine, rt	74
5e	H_2NSO_2	H_2NSO_2Cl, DMA, 0 °C	76
5j	Ph_3C	Ph_3CCl, pyridine, 50 °C	58
5g	TBDPS	TBDPSCl, imidazole, DMF, 0 °C	73
5h	TPS	TPSCl, pyridine, 0 °C	68

Scheme 4[a]



[a] (i) TBDMSCl, imidazole, DMF, 0 °C, 75%; (ii) 1,1′-thiocarbonylimidazole, $CHCl_3$, rt, 60% (iii) $(MeO)_3P$, 118 °C, 86%, (iv) TBAF, THF, rt, 78%; for (vii), see Table 3.

Indeed, inhibitors with K_i values in the 1−5 μM range (compounds **1j**, **1l**, **4e**, **5g**, **5h**, and **5j**) and even in the submicromolar range (compound **2j**, $K_i = 0.21$ μM) were obtained against *P. falciparum* dUTPase. Interestingly, we originally discovered the activity of compound **2j** during a directed screen of nucleoside analogues.[31] However, essentially no activity or poor activity was observed against the *L. major* dUTPase with the best K_i values in the 100 μM range (compounds **3a** and **3f**). In the case of the *L. major* enzyme, although the structural variations made affected the activity, the effects were variable, and no clear SARs could be found. On the other hand, interesting trends have emerged from the assays against the *P. falciparum* enzyme.

***P. falciparum* dUTPase.** Generally, enzyme inhibition ($K_i < 10$ μM) appears to be related to the presence, at the 5′-position, of a bulky lipophilic substituent containing two or three phenyl groups as well as an amine or ether linkage. Thus, preferred moieties were found to be either a trityl amine or ether or a triphenylsilyl (TPS) or *tert*-butyldiphenylsilyl (TBDPS) ether.

The following SARs can be seen:

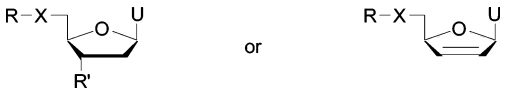
1. The presence of at least two aromatic ring substituents at the 5′-position is required for enzyme inhibition. Changing the tritylamino moiety **2j** to a benzylamino group **2i** results in a drastic loss of activity (more than 500-fold). The 5′-adamantylamido derivative **2c** is inactive against *P. falciparum* dUTPase ($K_i = 426$ μM), indicating that it is unlikely that steric bulk alone is sufficient for activity. However, compound **2c** bears an amide rather than an amino moiety at the 5′-position, which could also be significant. The data from the silyl series (compounds **1k**−**m**, **4a**−**c**, and **5f**−**h**) support the hypothesis that a bulky lipophilic group bearing an aromatic moiety is required at the 5′-position for the inhibition of *P. falciparum* dUTPase. Similar activity is observed for the 5′-triphenylsilyloxy (TPSO) and 5′-*tert*-butyldiphenylsilyloxy (TBDPSO) derivatives (**1m** and **1l**, $K_i = 2.8$ and 4.2 μM, respectively; **5h** and **5g**, $K_i = 1.3$ and 1.2 μM, respectively), whereas replacing more than one phenyl by alkyl groups (even bulky isopropyls) is detrimental. This is evidenced by the poor activity of the TBDMS derivative **5f** ($K_i = 10$ μM) and the triisopropylsilyl (TIPS) derivative **1k** ($K_i = 227$ μM) compared to that of their TPS and TBDPS analogues (**5h** and **5g**, and **1m** and **1l**, respectively).

2. The replacement of the carbon with a silicon in the trityl group increases inhibition of the enzyme. The introduction of a silicon atom (Ph_3C/Ph_3Si replacement) appears to produce as much as a 32-fold increase in enzyme selectivity while the same level of activity is retained (compare **1j** and **1m**; **5j** and **5h**). An exception to this is the 3′-fluoro analogues (**4e** and **4c**) in which the introduction of the TPS moiety results in a total loss of activity ($K_i = 5$ and 975 μM, respectively). However, in this case, it is suspected that premature degradation of the molecule might explain the loss of inhibitory effect.

3. The replacement of the 5′-oxygen with a 5′-nitrogen generally leads to an increase in enzyme inhibition. In the 3′-OH and 3′-OTBDMS deoxyuridine series (compare Series 1 and 3 with Series 2), this generally results in as much as a 10-fold increase in inhibitory activity, as evidenced by compounds **1j** (1.8 μM) and **2j** (0.2 μM); **1a** (238 μM) and **2a** (26 μM); and **3a** (597 μM) and **3i** (67 μM). Other cases are less significant, with the 5′-acetyl and the 5′-tosylate causing slight decreases in activity (0.4- and 0.9-fold, respectively) upon replacing O with NH, although neither compound is very active ($K_i > 140$ μM). Again, in the case of the 3′-fluoro compounds, **4e** has a K_i value of 5 μM, whereas its amino analogue **4f** shows a K_i value of 12 μM. As far as selectivity is concerned, data are limited, but in the case of compounds **1j** and **2j**, the O/NH replacement produces a striking 25-fold increase.

4. The requirements of the substituents at the 3′-position are less clear. The comparison of the 3′-OH and d4U series, in which there is no 3′-substituent (Series 1 and 5), shows essentially no significant effect on activity, although the K_i values obtained for the d4U analogues might tend to be slightly lower.

Table 4. Inhibition Constants K_i (μM) for Compounds against *P. falciparum, L. major*, and Human dUTPases and Selectivity Indexes for *P. falciparum*



compd	R	X	R′	*P. f.*[a]	*L. m.*[b]	human	SI *P. f.*[c]
1a	$(PhO)_2P(O)$	O	OH	238	>1 mM	135	0.6
1b	$(EtO)_2P(O)$	O	OH	316	>1 mM	>1 mM	>3
1c	$(EtO)_2P(O)CH_2P(O)$	O	OH	123	>1 mM	>1 mM	>8
1d	$CH_3C(O)$	O	OH	294	>1 mM	>1 mM	>3
1e	$CH_3(CH_2)_3C(O)$	O	OH	228	>1 mM	>1 mM	>4
1f	PhC(O)	O	OH	268	>1 mM	>1 mM	>3
1g	Ts	O	OH	142	>1 mM	>1 mM	>7
1h	H_2NSO_2	O	OH	>1 mM	707	>1 mM	na
1i	$H_2NSO_2N(-)C(O)$	O	OH	468	314	>1 mM	>2
1j	Ph_3C	O	OH	1.8	>1 mM	18	10
1k	TIPS	O	OH	227	>1 mM	>1 mM	>4
1l	TBDPS	O	OH	4.2	>1 mM	806	192
1m	TPS	O	OH	2.8	>1 mM	909	325
2a	$(PhO)_2P(O)$	NH	OH	26	>1 mM	>1 mM	>38
2b	$(EtO)_2P(O)CH_2P(O)$	NH	OH	324	>1 mM	>1 mM	>3
2c	adamantylC(O)	NH	OH	426	>1 mM	>1 mM	>2
2d	$CH_3C(O)$	NH	OH	>1 mM	>1 mM	>1 mM	na
2e	$CH_3(CH_2)_3C(O)$	NH	OH	189	>1 mM	>1 mM	>5
2f	PhC(O)	NH	OH	178	>1 mM	469	2.6
2g	Ts	NH	OH	>1 mM	>1 mM	>1 mM	1
2h	CH_3SO_2	NH	OH	178	>1 mM	>1 mM	>5
2i	$PhCH_2$	NH	OH	111	>1 mM	>1 mM	>9
2j	Ph_3C	NH	OH	0.2	304	46	230
2k	H	NH	OH	>1 mM	>1 mM	>1 mM	na
3a	$(PhO)_2P(O)$	O	OTBDMS	597	116	>1 mM	>1.7
3b	$(EtO)_2P(O)$	O	OTBDMS	340	>1 mM	>1 mM	>3
3c	$(EtO)_2P(O)CH_2P(O)$	O	OTBDMS	217	>1 mM	nd	na
3d	$H_2NSO_2NHC(O)$	O	OTBDMS	244	>1 mM	>1 mM	>4
3e	H_2NSO_2	O	OTBDMS	88	>1 mM	324	3.7
3f	H	O	OTBDMS	648	114	119	0.2
3g	Ph_3C	O	OTBDMS	515	454	>1 mM	>1.9
3h	Ph_3C	NH	OTBDMS	313	>1 mM	>1 mM	>3
3i	$(PhO)_2P(O)$	NH	OTBDMS	67	>1 mM	232	3.5
4a	TBDMS	O	F	628	>1 mM	>1 mM	>1.6
4b	TBDPS	O	F	89	>1 mM	808	9.1
4c	TPS	O	F	975	>1 mM	>1 mM	na
4d	H	O	F	nd	nd	nd	
4e	Ph_3C	O	F	5.0	177	457	91
4f	Ph_3C	NH	F	12	>1 mM	>1 mM	>83
5a	$(PhO)_2P(O)$	O	—[d]	99	>1 mM	>1 mM	>10
5b	$H_2NSO_2NHC(O)$	O	—[d]	82	>1 mM	799	9.7
5c	$CH_3(CH_2)_{14}C(O)$	O	—[d]	256	>1 mM	350	1.4
5d	adamantylC(O)	O	—[d]	58	433	298	0.5
5e	H_2NSO_2	O	—[d]	254	>1 mM	>1 mM	>4
5f	TBDMS	O	—[d]	10	>1 mM	56.4	5.6
5g	TBDPS	O	—[d]	1.2	178	>1 mM	>833
5h	TPS	O	—[d]	1.3	350	>1 mM	>769
5i	H	O	—[d]	298	>1 mM	>1 mM	>3
5j	Ph_3C	O	—[d]	1.9	798	157	83

[a] *Plasmodium falciparum*. [b] *Leishmania major*. [c] SI *P. f.* = selectivity index for *P. falciparum* defined as (K_i Human)/(K_i *P. falciparum*); nd = not determined; na = not relevant. Some of the data for compounds **1j**, **1k**, **1m**, **2j**, **4e**, and **4f** have been reported previously.[9] [d] d4U series.

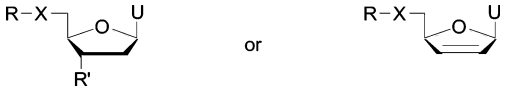
The comparison of the 3′-OH and 3′-F series (Series 1, 2, and 4) does not provide any obvious trend. Although the presence of a fluorine instead of the hydroxyl at the 3′-position seems to lower the activity, the extent of this effect varies considerably with the nature of the group at the 5′-position: from an insignificant decrease (5′-Ph_3CO derivatives **1j** and **4e**), to a 20-fold decrease (5′-TBDPSO derivatives **1l** and **4b**) or 60-fold decrease (5′-Ph_3CNH derivatives **2j** and **4f**), to possibly even more, as in the case of the 5′-TPSO derivatives **1m** and **4c** (K_i = 2.8 and 975 μM, respectively). However, in this latter case a more complex scenario involving the possible hydrolysis of the TPS moiety may have to be considered.

The silylation of the 3′-OH produces a total loss of activity against *P. falciparum* dUTPase (compare Series 1 and 2 to Series 3), particularly for the 5′-Ph_3CNH (**2j** and **3h**) and 5′-Ph_3CO (**1j** and **3g**) derivatives for which the K_i values increase from 0.2 to 313 and 1.8 to 515 μM, respectively. In instances in which compounds are not active anyway, the effect varies and is far less marked (2- to 4-fold variations).

Overall, the above results suggest that a bulky lipophilic group (such as TBDMS) is not tolerated at the 3′-position and that hydrogen bonding involving the 3′-OH may be important for activity.

In Vitro Assays. The assays were conducted against the clinically relevant forms of the parasites: the

Table 5. Growth Inhibition [IC_{50} (μM) Values] of Compounds against *L. donovani*, *P. falciparum*, *T. b. rhodesiense*, *T. cruzi,* and a Mammalian Cell Line



compd	R	X	R′	*P. f.*[a]	*L. d.*[b]	*T. b.*[c]	*T. c.*[d]	cytotoxicity[e]
1a	$(PhO)_2P(O)$	O	H	>11	>65	52	>196	>196
1b	$(EtO)_2P(O)$	O	H	>14	>82	37	>247	198
1c	$(EtO)_2P(O)CH_2P(O)$	O	H	98	>74	123	>222	168
1d	$CH_3C(O)$	O	H	>19	>111	13	273	64
1e	$CH_3(CH_2)_3C(O)$	O	H	>16	96	147	>284	>289
1f	PhC(O)	O	H	>15	>90	117	>271	>271
1g	Ts	O	H	>13	>79	44	>236	>236
1h	H_2NSO_2	O	H	>163	>98	145	>293	nd
1i	$H_2NSO_2N(-)C(O)$	O	H	20	>65	90	>194	nd
1j	Ph_3C	O	H	6	>64	24	306	192
1k	TIPS	O	H	>13	nd	8.0	58	25
1l	TBDPS	O	H	6.6	nd	3.0	45	23
1m	TPS	O	H	1.1	nd	15	71	nd
2a	$(PhO)_2P(O)$	NH	H	>11	>65	>196	>65	>196
2b	$(EtO)_2P(O)CH_2P(O)$	NH	H	78	29	41	>222	nd
2c	adamantylC(O)	NH	H	>13	nd	>231	>231	nd
2d	$CH_3C(O)$	NH	H	>19	>112	194	>335	>335
2e	$CH_3(CH_2)_3C(O)$	NH	H	>16	>97	149	>289	>289
2f	PhC(O)	NH	H	>15	>91	120	>272	>272
2g	Ts	NH	H	>13	>79	102	>236	>236
2h	CH_3SO_2	NH	H	>16	>98	147	>295	>295
2i	$PhCH_2$	NH	H	>16	nd	17	>83	nd
2j	Ph_3C	NH	H	4.5	nd	48	44	44
2k	H	NH	H	nd	nd	nd	nd	nd
3a	$(PhO)_2P(O)$	O	OTBDMS	6	>17	12	10	>157
3b	$(EtO)_2P(O)$	O	OTBDMS	>10	63	35	41	108
3c	$(EtO)_2P(O)CH_2P(O)$	O	OTBDMS	18	>58	40	75	nd
3d	$H_2NSO_2NHC(O)$	O	OTBDMS	37	>51	12	>152	nd
3e	H_2NSO_2	O	OTBDMS	40	>7	104	160	nd
3f	H	O	OTBDMS	>15	>88	83	>263	>263
3g	Ph_3C	O	OTBDMS	1	17	25	247	>154
3h	Ph_3C	NH	OTBDMS	1.8	nd	2.7	97	7.8
3i	$(PhO)_2P(O)$	NH	OTBDMS	4	<6	28	6	18
4a	TBDMS	O	F	>14	>87	59	37	nd
4b	TBDPS	O	F	8.8	nd	29	32	16
4c	TPS	O	F	1.0	nd	4.5	84	86
4d	H	O	F	>21	>391	100	>130	>391
4e	Ph_3C	O	F	2.0	nd	47	29	35
4f	Ph_3C	NH	F	5.3	>63	3.6	47	30
5a	$(PhO)_2P(O)$	O	–[f]	54	nd	68	>204	nd
5b	$H_2NSO_2NHC(O)$	O	–[f]	>150	>90	40	>270	262
5c	$CH_3(CH_2)_{14}C(O)$	O	–[f]	>11	>67	113	>201	nd
5d	adamantylC(O)	O	–[f]	>13	>81	177	>81	nd
5e	H_2NSO_2	O	–[f]	163	nd	>311	>311	nd
5f	TBDMS	O	–[f]	51	nd	73	110	nd
5g	TBDPS	O	–[f]	3.0	nd	55	33	67
5h	TPS	O	–[f]	1.0	13	1.6	42	85
5i	H	O	–[f]	214	nd	72	170	nd
5j	Ph_3C	O	–[f]	>11	>66	55	44	nd

[a] *Plasmodium falciparum*. [b] *Leishmania donovani*. [c] *Trypanosoma cruzi*. [d] *Trypanosoma brucei rhodesiense*. [e] Rat L-6 cells; nd = not determined. Controls: for *P. f.*, chloroquine, IC_{50} = 0.1 μM; for *L. d.*, miltefosine, IC_{50} = 1.2 μM; for *T. c*., benznidazole, IC_{50} = 1.44 μM; for *T. b. rhod.*, melarsoprol, IC_{50} = 0.007 μM; for cytotoxicity, podophyllotoxin, IC_{50} = 0.012 μM. Some of the data for compounds **1j**, **1k**, **1m**, **2j**, **4e**, and **4f** have been reported previously.[9] [f] d4U series.

erythrocytic stages of the chloroquine-resistant K1 strain of *P. falciparum* (the parasite responsible for the most acute form of malaria), the amastigote form of *Leishmania donovani* (the organism causing visceral leishmaniasis), the trypomastigote form of *Trypanosoma brucei rhodesiense* (the causative agent of African trypanosomiasis), and the amastigote form of *Trypanosoma cruzi* (the causative agent of Chagas' disease). In vitro screening procedures are detailed in the Experimental Section. The data for these assays are shown in Table 5.

Plasmodium falciparum. The most active compounds against the *Plasmodium* parasite were as follows: **1m**, **3g**, **4c**, and **5h** with IC_{50} = 1 μM and then **1j**, **1l**, **2j**, **3a**, **3h**, **3i**, **4b**, **4e**, **4f**, and **5g** with IC_{50} values between 1 and 10 μM. Interestingly, all these compounds possess a trityl, TPS, or TBDPS group at the 5′-position, except analogues **3a** and **3i**, which bear a 5′-$(PhO)_2P(O)$ group. Therefore, our study shows that in vitro activity is clearly related to the presence of a phenyl-containing bulky lipophilic group at the 5′-position. On the other hand, structural requirements regarding the 3′-position remain unclear as active compounds are found across all five series studied. Only in the case of derivatives **3a** and **3i** does the nature of the 3′-moiety (TBDMSO) appear to be essential for

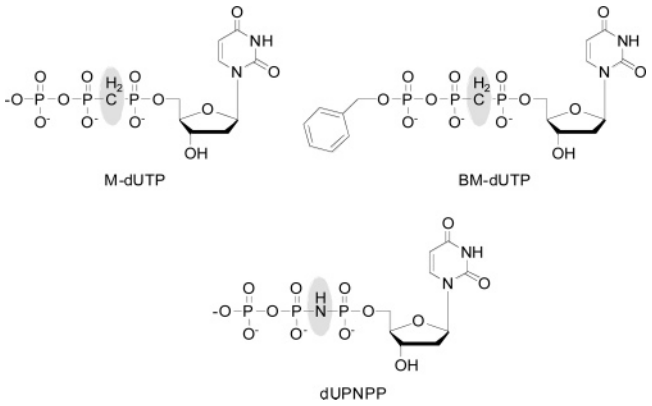


Figure 2. Examples of inhibitors of dUTPase.

parasite growth inhibition. Indeed, although both compounds possess a $(PhO)_2P(O)$ group at the 5′-position, so do the inactive analogues **1a**, **2a**, and **5a**. The only feature specific to **3a** and **3i** is that they both belong to the 3′-OTBDMS series.

Leishmania donovani. Essentially, none of the compounds were notably active against the *L. donovani* parasite, with the best IC_{50} values obtained for **5h** (13 μM) and **3g** (17 μM). Although these two compounds possess very similar 5′-moieties (Ph_3SiO and Ph_3CO), this feature is also shared by analogues that were found to be inactive (e.g., **1j** and **5j**).

Trypanosoma brucei rhodesiense. The most active compounds (**1l**, **3h**, **4c**, **4f**, and **5h**) showed IC_{50} values in the 1−5 μM range; they have in common a phenyl-containing, bulky lipophilic substituent at the 5′-position (trityl, TPS, or TBDPS). The next best inhibitors, with IC_{50} values in the 5−20 μM range (**1d**, **1k**, **1m**, **2i**, **3a**, and **3d**), do not appear to share any obvious structural features.

Trypanosoma cruzi. The best results against the *T. cruzi* parasite were obtained for **3i** (6 μM) and **3a** (10 μM), with all of the other compounds assayed showing IC_{50} values above 30 μM. These two analogues are strikingly similar, both bearing a $(PhO)_2PO$ unit at the 5′-position as well as a TBDMS ether at the 3′-position.

Discussion

As mentioned earlier, prior to our SAR study, very few inhibitors of dUTPase had been reported and were not suitable drug candidates. Examples are shown in Figure 2. These compounds are nonhydrolysable analogues of dUTP, resulting from the replacement of the α−β bridging oxygen atom by a methylene or an amino group, and were developed essentially for crystallographic purposes and the analysis of enzyme kinetics.[32,33]

In our initial study, we have considered a broad range of compounds with the intention of identifying suitable leads for the development of antiparasitic drugs. Starting from isosteres of dUMP (including sulfonate, sulfamoyl, and phosphate analogues) and analogues of dUDP (sulfamoylcarbamoyl and phosphonoacetate derivatives) to simpler carboxylic esters, amides, ethers, and amines, we also selected substituents of varying bulk and lipophilicity. Interestingly, among this broad selection of compounds, the most promising results were obtained essentially for simple lipophilic ethers and amines. This is despite the fact that dUTPase binds polar and hydrophilic molecules.

Furthermore, very different results were obtained from the screening of our compounds against *P. falciparum* on one hand and the related organisms *Leishmania* and *Trypanosoma* on the other hand. This is not so surprising based on the structural and catalytic differences between dimeric and trimeric dUTPases.

Leishmania* and *Trypanosoma. Because none of the inhibitors prepared were found to effectively target *L. major* dUTPase (which also serves as a model for the other trypanosome dUTPases), it was surprising to find even a few compounds that showed some activity against the *L. donovani* and related organisms *T. cruzi* or *T. b. rhodesiense*. In addition, the IC_{50} data obtained for mammalian cell lines do not suggest general cytotoxicity. Therefore, it seems very likely that the in vitro activity observed results from the inhibition of a molecular target other than the intended dUTPase enzyme.

Plasmodium falciparum. By contrast, a good correlation between inhibition of *P. falciparum* dUTPase and inhibition of parasite growth is found for compounds **1j**, **1l**, **1m**, **2j**, **4e**, **4f**, **5g**, and **5h**. In addition, these compounds showed good selectivity for the *Plasmodium* dUTPase over the human enzyme (with selectivity indexes around 100 or above, except for **1j**, for which SI = 10) as well as no major in vitro toxic effect on mammalian cell lines (IC_{50} values from 20 to 200 μM corresponding to cellular toxicity indexes from 4 to 85). These encouraging initial results demonstrate that dUTPase is a valid target for the development of novel selective antimalarial drugs and provide valuable information regarding SAR, particularly regarding the 5′-position. Thus, activity is closely related to the presence, at the 5′-position of deoxyuridine, of a bulky aromatic group, such as a trityl, TPS, or TBDPS. Although replacing the 5′-oxygen with NH appears to be very beneficial at the enzyme level (both for activity and selectivity), in vitro the effect is less noticeable. The introduction of a silicon atom (Ph_3C/Ph_3Si replacement) seems to produce an increase in enzyme selectivity (compare **1j** and **1m**, **5j** and **5h**).

Two compounds (**5f** and **5j**, which are interestingly both d4U derivatives) failed to inhibit the *Plasmodium* parasite, although they showed selective activity against the *Plasmodium* dUTPase enzyme. Possible explanations could be either poor cellular penetration or a lack of stability in the in vitro assay medium.

Conversely, compounds **3a**, **3g**, **3h**, **3i**, **4b**, and **4c** were all found to inhibit parasite growth while showing neither cellular toxicity on mammalian cell lines nor any activity against the *Plasmodium* dUTPase. Interestingly, all these compounds contained a silyl group at either the 5′- or the 3′-position, which suggests that a silyl moiety contributes to in vitro activity. Because the actual molecular target for these compounds is unknown, it is difficult to speculate about their mode of action. One could even envision that the compounds do not act as inhibitors themselves, but undergo hydrolysis, thereby generating a silanol moiety that could be the actual inhibitor. Preliminary experiments support this idea; for example, triphenylsilanol was found to inhibit the growth of *P. falciparum* in vitro (IC_{50} = 0.3 μM)

while not inhibiting the *Plasmodium* dUTPase (K_i > 1 mM). Furthermore, it has been reported in the literature that in many cases silicon isosteres showed better physiological properties (e.g., cell penetration, tissue penetration, bioavailability) than did their parent drugs.[34,35]

Conclusion

We have reported the synthesis of analogues of dUMP in which a variety of substituents were introduced at the 5′- and 3′-positions, together with variation in the heteroatom at the 5′-position. The compounds were designed with the intention of targeting dUTPase, an enzyme involved in nucleotide metabolism, which has been shown to be essential for DNA replication and cell viability. Our SAR study has led to the identification of novel, selective, small-molecule inhibitors of *P. falciparum*, a causative organism of malaria, while little success has been encountered so far with respect to *Leishmania* and *Trypanosoma* parasites, which cause other devastating tropical diseases.

We have discovered new inhibitors of *P. falciparum* dUTPase that are selective, nontoxic, more drug-like than all the previously reported inhibitors, and easy and cheap to prepare. Furthermore, a good correlation between inhibition of the enzyme and antiparasitic activity is generally observed. The best results were obtained for analogues **2j** (enzyme inhibition: $K_i = 0.2$ μM with SI = 232; antiparasitic activity: $IC_{50} = 4.5$ μM with SI = 10) and **5h** (enzyme inhibition: $K_i = 1.3$ μM with SI > 769; antiparasitic activity: $IC_{50} = 1$ μM with SI = 85), which represent good drug leads.

The broad SAR study we have developed has also highlighted key features that are important for activity and selectivity. Thus, the requirement for a trityl-type moiety at the 5′-position was clearly identified. Crystallographic studies have shown that selective binding to *P. falciparum* dUTPase is achieved through favorable interactions between the trityl group and the side-chains of Phe and Ile residues that are unique to *Plasmodium*.[9] In addition, selectivity at the enzyme level is enhanced either by the O/NH switch at the 5′-position or by the introduction of a silyl moiety at the 5′-position. Silyl derivatives were also shown to produce the best antiparasitic activity.

The SARs with respect to the 5′-position are now better understood, but the requirements at the 3′-position still remain unclear.

Globally, we have discovered selective inhibitors of *P. falciparum* and have chemically validated the *Plasmodium* dUTPase as a target for the development of new antimalarial drugs. As a novel target, dUTPase is all the more attractive in light of the alarming spread of resistance to currently used drugs, particularly chloroquine. Furthermore, the pathway in which dUTPase acts is the same as that of well-established targets such as thymidylate synthase and dihydrofolate reductase, which may offer opportunities for drug combination therapy.

Experimental Section

Chemistry. Chemicals were purchased from Aldrich, Sigma, Lancaster, Acros, or Avocado and were used without further purification. 2′,3′-Dideoxy-3′-fluorouridine was provided by Medivir AB. Dry solvents were generally purchased from Fluka in sure-seal bottles and stored over molecular sieves. Reactions were performed in a predried apparatus under an atmosphere of nitrogen unless otherwise stated. Thin-layer chromatography (TLC) was performed on Merck silica gel $60F_{254}$ plates. Column chromatography was carried out with the use of either Fisons matrix silica 60 (35−70 micron) or prepacked ISOLUTE SI columns purchased from Argonaut. ^1H, ^{13}C, and ^{19}F NMR were recorded on a Bruker Avance DPX 300 spectrometer, operating at 300, 75, and 282 MHz, respectively, with tetramethylsilane as the internal standard and deuterated solvents purchased from Goss unless stated otherwise. Infrared (IR) spectra were recorded on a Perkin-Elmer 1600 FT-IR spectrometer. Melting points (mp) were measured on a Gallenkamp melting point apparatus and were not corrected. Low-resolution mass spectra were recorded on a Fisons VG Platform II spectrometer. High-resolution mass spectra were determined by the EPSRC Mass Spectrometry Centre, Swansea, UK. Microanalyses were carried out by MEDAC Ltd.

5′-*O*-Triphenylsilyl-2′-deoxyuridine (1m). A solution of triphenylsilyl chloride (TPSCl) (0.437 g, 1.48 mmol) in dry pyridine (4 mL) was added dropwise to a solution of 2′-deoxyuridine (0.278 g, 1.22 mmol) in dry pyridine (4 mL) cooled in an ice−salt bath. The reaction mixture was kept at 0 °C for 1 h. The reaction was quenched with CH_3OH (50 μL). The solvent was removed in vacuo, and the crude product was purified by flash column chromatography (ISOLUTE SI column) eluting with 0 → 10% CH_3OH in $CHCl_3$. Compound **1m** was obtained as a white crystalline solid (0.506 g, 85%) from the fractions, with $R_f = 0.30$ (10% $CH_3OH/CHCl_3$). mp 83−85 °C; ^1H NMR ($CDCl_3$) δ 2.25 (1H, m, 2′-C*H*H), 2.44 (1H, m, 2′-CH*H*), 2.95 (1H, bs, 3′-OH), 3.93−4.27 (3H, m, 5′-H and 4′-H), 4.60 (1H, m, 3′-H), 5.19 (1H, d, J = 8.2 Hz, 5-H), 6.41 (1H, t, J = 6.4 Hz, 1′-H), 7.35−7.73 (15H, m, Ph-H), 7.80 (1H, d, J = 8.1 Hz, 6-H), 9.46 (1H, bs, 3-NH); ^{13}C NMR ($CDCl_3$) δ 41.6 (2′-CH_2), 63.8 (5′-CH_2), 71.7 (3′-CH), 85.3 (1′-CH), 87.3 (4′-CH), 102.7 (5-CH), 128.6 (Ph-CH), 131.1 (Ph-CH), 133.3 (Ph-C), 135.8 (Ph-CH), 140.5 (6-CH), 150.9 (2-C), 163.9 (4-C); MS (ES^+) *m/z* 509 ($[M + Na]^+$, 100%); HRMS (ES^+) calcd for $C_{27}H_{26}N_2O_5SiNa^+$ $(M + Na)^+$ 509.1503, found 509.1504; Anal. ($C_{27}H_{26}N_2O_5Si$, 0.32 HCl) C, H, N.

2′,5′-Dideoxyuridine 5′-*N*-Diphenylphosphoramidate (2a). Chlorodiphenyl phosphate (0.18 mL, 0.88 mmol) was added dropwise to a solution of 5′-amino-2′,5′-dideoxyuridine (**2k**) (0.20 g, 0.88 mmol) in dry DMF/pyridine (4:1) (20 mL). After being stirred overnight at room temperature, the solvent was reduced in vacuo, and the crude product was purified by flash column chromatography eluting with 5 → 20% CH_3OH in $CHCl_3$. Compound **2a** was obtained as a white solid (75 mg, 19%) from the fractions, with $R_f = 0.58$ (20% $CH_3OH/CHCl_3$). mp 175−177 °C; ^1H NMR (300 MHz, CD_3OD) δ 2.06−2.27 (2H, m, 2′-H), 3.24−3.36 (2H, m, 5′-H), 3.84−3.89 (1H, m, 4′-H), 4.23−4.28 (1H, m, 3′-H), 5.61 (1H, d, J = 8.1 Hz, 5-H), 6.15 (1H, t, J = 6.8 Hz, 1′-H), 7.19−7.35 (6H, m, Ph-H), 7.37−7.42 (4H, m, Ph-H), 7.65 (1H, d, J = 8.1 Hz, 6-H); ^{13}C NMR (75 MHz, CD_3OD) δ 40.8 (2′-CH_2), 45.0 (5′-CH_2), 72.9 (3′-CH), 87.2 (1′-CH), 87.9 (4′-CH), 103.2 (5-CH), 122.0 (d, J = 4.7 Hz, Ph-CH), 126.8 (Ph-CH), 131.3 (Ph-CH), 143.0 (6-CH), 152.0 (2-C), 157.3 (Ph-C), 166.5 (4-C); ^{31}P NMR (121 MHz, CD_3OD) δ 2.5; IR (KBr) 3398, 1724, 1667, 1488, 1237, 1196 cm^{-1}; MS (CI) *m/z* 477 ($[M + NH_4]^+$, 5%), 460 ($[M + H]^+$, 100%); HRMS (ES^+) calcd for $C_{21}H_{23}N_3O_7P^+$ $(M + H)^+$ 460.1268, found 460.1265.

5′-Pentanoylamino-2′,5′-dideoxyuridine (2e). A procedure identical to the one used for **2a** was employed. Compound **2e** was obtained as a white solid (0.27 g, 49%) from the amine **2k** (0.40 g, 1.76 mmol) and valeryl chloride (0.21 mL, 1.76 mmol). Flash column chromatography was carried out using 3 → 8% CH_3OH in $CHCl_3$. R_f (10% $CH_3OH/CHCl_3$) 0.48; mp 168−171 °C; ^1H NMR (300 MHz, CD_3OD) δ 0.94 (3H, t, J = 7.3 Hz, $CH_2CH_2CH_2CH_3$), 1.30−1.42 (2H, m, $CH_2CH_2CH_2CH_3$), 1.61 (2H, m, $CH_2CH_2CH_2CH_3$), 2.21−2.30 (4H, m, 2′-H and $CH_2CH_2CH_2CH_3$), 3.46 (2H, m, 5′-H), 3.93 (1H, m, 3′-H), 4.26 (1H, m, 4′-H), 5.73 (1H, d, J = 8.1 Hz, 5-H), 6.19 (1H, m t, J

= 6.8 Hz, 1′-H), 8.10 (1H, d, J = 8.1 Hz, 6-H); ^{13}C NMR (75 MHz, CD_3OD) δ 14.5 ($CH_2CH_2CH_2\mathit{CH_3}$), 23.8 ($CH_2CH_2\mathit{CH_2}$-$CH_3$), 29.6 ($CH_2\mathit{CH_2}CH_2CH_3$), 37.2 ($\mathit{CH_2}CH_2CH_2CH_3$), 40.6 (2′-$CH_2$), 42.8 (5′-$CH_2$), 73.5 (3′-CH), 87.1 (1′-CH), 87.6 (4′-CH), 103.2 (5-CH), 143.0 (6-CH), 152.51 (2-C), 166.6 (4-C), 177.0 (*C*ONH); IR (KBr) 3332, 1727, 1667, 1639 cm^{-1}; MS (CI) *m/z* 312 ([M + H]$^+$, 7%), 329 ([M + NH_4]$^+$, 2%); HRMS (ES$^+$) calcd for $C_{14}H_{22}N_3O_5^+$ (M + H)$^+$ 312.1554, found 312.1554; Anal. ($C_{14}H_{21}O_5N_3$) C, H, N.

5′-Tritylamino-2′,5′-dideoxyuridine (2j). The amine **2k** (0.200 g, 0.88 mmol) was taken up in dry pyridine (5 mL), and the mixture was sonicated for a few minutes. Trityl chloride (0.278 g, 1.00 mmol) was added, and the reaction mixture was stirred at 50 °C overnight. The reaction was quenched with H_2O (20 mL), and the crude mixture was extracted with dichloromethane (DCM) (3 × 10 mL). The combined organic layers were washed with H_2O (10 mL), dried over $MgSO_4$, and concentrated in vacuo. The crude oil was purified by flash column chromatography (ISOLUTE SI column) eluting with 0 → 10% CH_3OH in $CHCl_3$. The fractions with R_f = 0.28 (10% $CH_3OH/CHCl_3$) yielded compound **2j** as a white solid (0.202 g, 49%). mp 132−134 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.07 (2H, m, 2′-H), 2.28−2.53 (2H, m, 5′-H), 2.73 (1H, dd, J = 3.5, 12.1 Hz, $CH_2N\mathit{H}$), 2.97 (1H, bs, 3′-OH), 4.19 (1H, m, 4′-H), 4.33 (1H, m, 3′-H), 5.72 (1H, d, J = 8.1 Hz, 5-H), 6.36 (1H, t, J = 6.4 Hz, 1′-H), 7.14 (1H, d, J = 8.1 Hz, 6-H), 7.23−7.43 (9H, m, Ph-H), 7.57 (6H, m, Ph-H), 9.47 (1H, bs, 3-NH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 40.8 (2′-CH_2), 46.6 (5′-CH_2), 71.1 ($Ph_3\mathit{C}$), 73.0 (3′-CH), 85.4 (1′-CH), 86.7 (4′-CH), 103.2 (5-CH), 127.0 (Ph-CH), 128.4 (Ph-CH), 129.0 (Ph-CH), 139.8 (6-CH), 145.8 (Ph-C), 150.7 (2-C), 163.7 (4-C); MS (ES$^+$) *m/z* 492 ([M + Na]$^+$, 23%), 243 (Ph_3C^+, 100%), 470 ([M + H]$^+$, 4%); HRMS (ES$^+$) calcd for $C_{28}H_{28}N_3O_4^+$ (M + H)$^+$ 470.2074, found 470.2076; Anal. ($C_{28}H_{27}N_3O_4$, 0.3 HCl, 0.1 H_2O) C, H, N, Cl.

3′-*O*-*tert*-Butyldimethylsilyl-5′-*O*-sulfamoyl-2′-deoxyuridine (3e). A solution of sulfamoyl chloride (1.55 g, 13.47 mmol) in dry DMA (10 mL) was added dropwise at 0 °C to a stirred solution of **3f** (2.30 g, 6.73 mmol) in dry DMA (10 mL). After being stirred for 3 h, the reaction mixture was poured into cold brine (40 mL) and extracted with $CHCl_3$ (3 × 100 mL). The combined organic layers were washed with H_2O (2 × 100 mL), dried over Na_2SO_4, and reduced in vacuo. The resulting crude product was purified by flash column chromatography eluting with 3 → 10% CH_3OH in $CHCl_3$. The fractions with R_f = 0.33 (10% $CH_3OH/CHCl_3$) yielded compound **3e** as a white solid (2.02 g, 71%). mp 163−165 °C; ^{1}H NMR (300 MHz, CD_3OD) δ 0.05 [6H, s, Si($C\mathit{H_3}$)$_2$], 0.86 [9H, s, C($C\mathit{H_3}$)$_3$], 2.05−2.13 (1H, m, 2′-C*H*H), 2.17−2.27 (1H, m, 2′-CH*H*), 3.95 (1H, m, 3′-H), 4.12 (2H, m, 5′-H), 4.36 (1H, m, 4′-H), 5.62 (1H, d, J = 8.1 Hz, 5-H), 6.13 (1H, t, J = 6.8 Hz, 1′-H), 7.56 (1H, d, J = 8.1 Hz, 6-H); ^{13}C NMR (75 MHz, CD_3OD) δ −4.6 (Si$\mathit{CH_3}$), −4.5 (Si$\mathit{CH_3}$), 18.0 [C(CH$_3$)$_3$], 26.0 [C($\mathit{CH_3}$)$_3$], 39.2 (2′-CH_2), 68.3 (5′-CH_2), 72.2 (3′-CH), 84.0 (1′-CH), 84.7 (4′-CH), 102.5 (5-CH), 140.9 (6-CH), 150.7 (2-C), 163.4 (4-C); IR (KBr) 1712, 1694, 1650, 1378, 1142 cm^{-1}; MS (CI) *m/z* 439 ([M + NH_4]$^+$, 100%), 422 ([M + H]$^+$, 80%); HRMS (ES$^+$) calcd for $C_{15}H_{28}N_3O_7SSi^+$ (M + H)$^+$ 422.1412, found 422.1421.

3′-*O*-*tert*-Butyldimethylsilyl-2′,5′-dideoxyuridine 5′-*N*-Diphenylphosphoramidate (3i). TBDMSOTf (0.11 mL, 0.48 mmol) was added dropwise at 0 °C to a solution of **2a** (0.20 g, 0.44 mmol) in dry DMF (10 mL) and dry pyridine (0.1 mL, 0.96 mmol). The reaction was stirred for 2.5 h, H_2O (5 mL) was added, and the mixture was extracted with CH_2Cl_2 (3 × 50 mL). The organic layers were dried over Na_2SO_4 and reduced in vacuo. Further purification by flash column chromatography, eluting with 5 → 15% CH_3OH in $CHCl_3$, yielded compound **3i** as a pale yellow viscous liquid (0.15 g, 60%). R_f (15% $CH_3OH/CHCl_3$) 0.61; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.00 [6H, s, Si($C\mathit{H_3}$)$_2$], 0.83 [9H, s, C($C\mathit{H_3}$)$_3$], 2.00−2.19 (2H, m, 2′-H), 3.14−3.39 (2H, m, 5′-H), 3.83 (1H, m, 4′-H), 4.26 (1H, m, 3′-H), 5.56 (1H, d, J = 8.1 Hz, 5-H), 6.03 (1H, t, J = 6.8 Hz, 1′-H), 7.09−7.21 (6H, m, Ph-H), 7.21−7.36 (4H, m, Ph-H), 7.55 (1H, d, J = 8.1 Hz, 6-H); ^{13}C NMR (75 MHz, $CDCl_3$) δ −4.5 (Si$\mathit{CH_3}$), −4.3 (Si$\mathit{CH_3}$), 18.3 [C(CH$_3$)$_3$], 26.1 [C($\mathit{CH_3}$)$_3$], 40.8 (2′-CH_2), 43.5 (5′-CH_2), 72.4 (3′-CH), 86.5 (1′-CH), 86.6 (4′-CH), 102.9 (5-CH), 120.7 (d, J = 9.8 Hz, Ph-CH), 125.5 (Ph-CH), 130.2 (d, J = 1.5 Hz, Ph-CH), 141.2 (6-CH), 150.6 (2-C), 151.3 (d, J = 22.5 Hz, Ph-C), 164.0 (4-C); ^{31}P NMR (121 MHz, $CDCl_3$) δ 2.4; IR (neat) 1697, 1681, 1257, 1192 cm^{-1}; MS (CI) *m/z* 575 ([M + H]$^+$, 5%), 347 ([M-$(PhO)_2PO$ + Na]$^+$, 30%); HRMS (ES$^+$) calcd for $C_{27}H_{37}O_7N_3SiP^+$ (M + H)$^+$ 574.2133, found 574.2128.

5′-*tert*-Butyldiphenylsilyloxy-2′,3′-dideoxy-3′-fluorouridine (4b). A solution of *tert*-butyldiphenylsilyl chloride (TBDPSCl) (0.238 g, 0.87 mmol) and imidazole (0.116 g, 1.70 mmol) in dry DMF (2 mL) was added dropwise at 0 °C to a cooled solution of 2′,3′-dideoxy-3′-fluorouridine (0.176 g, 0.77 mmol) in dry DMF (2 mL). The reaction mixture was stirred at 0 °C for 2 h and then at room temperature for 40 h. The reaction was quenched by the addition of H_2O (5 mL). The crude mixture was extracted with $CHCl_3$ (10 mL, 5 mL). The combined organic layers were washed with saturated $NaHCO_3$, dried over $MgSO_4$, and concentrated in vacuo. The crude oil was chromatographed on a silica gel column (ISOLUTE SI) eluted with a gradient of 0 → 5% CH_3OH in $CHCl_3$. Compound **4b** was obtained as a white solid (0.331 g, 92%) from the fractions with R_f = 0.63 (10% $CH_3OH/CHCl_3$). mp 140−141 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.17 [9H, m, C($C\mathit{H_3}$)$_3$], 2.24 (1H, m, 2′-C*H*H), 2.78 (1H, m, 2′-CH*H*), 4.00 (2H, m, 5′-H), 4.38 (1H, d, J = 26.7 Hz, 4′-H), 5.34 (1H, dd, J = 4.9, 53.8 Hz, 3′-H), 5.56 (1H, d, J = 8.1 Hz, 5-H), 6.51 (1H, m, 1′-H), 7.43−7.60 (6H, m, Ph-H), 7.65−7.74 (4H, m, Ph-H), 7.27 (1H, d, J = 8.1 Hz, 6-H), 9.11 (1H, bs, 3-NH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 19.7 [C(CH$_3$)$_3$], 27.4 [C($\mathit{CH_3}$)$_3$], 39.7 (d, J = 21.3 Hz, 2′-CH_2), 64.1 (d, J = 10.9 Hz, 5′-CH_2), 85.4 (1′-CH), 85.6 (d, J = 24.7 Hz, 4′-CH), 94.7 (d, J = 178.7 Hz, 3′-CH), 103.2 (5-CH), 128.5 (Ph-CH), 128.6 (Ph-CH), 130.7 (Ph-CH), 132.2 (Ph-C), 132.8 (Ph-C), 135.7 (Ph-CH), 136.0 (Ph-CH), 140.0 (6-CH), 150.6 (2-C), 163.5 (4-C); ^{19}F NMR (282 MHz, $CDCl_3$) δ-175.5 (m, 3′-F); MS (ES$^-$) *m/z* 467 ([M − H]$^-$, 53%), 75 (100%); HRMS (ES$^+$) calcd for $C_{25}H_{29}N_2O_4FNaSi^+$ (M + Na)$^+$ 491.1773, found 491.1764; Anal. ($C_{25}H_{29}N_2O_4F$, 0.08 HCl) C, H, N, Cl.

5′-*O*-Triphenylsilyl-2′,3′-dideoxy-3′-fluorouridine (4c). A procedure identical to the one used for **1m** was employed. Compound **4c** was obtained as a white solid (0.274 g, 60%) from the reaction of 3′-fluoro-2′,3′-dideoxyuridine (0.214 g, 0.93 mmol) with TPSCl (0.332 g, 1.12 mmol) in dry pyridine (7 mL). Flash column chromatography (ISOLUTE SI column) was carried out using 0 → 10% CH_3OH in $CHCl_3$. R_f (10% $CH_3OH/CHCl_3$) 0.66; mp 171−172 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.19 (1H, m, 2′-C*H*H), 2.67 (1H, m, 2′-CH*H*), 4.11 (2H, m, 5′-H), 4.36 (1H, d, J = 27.1 Hz, 3′-H), 5.20−5.45 (2H, m, 4′-H and 5-H), 6.50 (1H, dd, J = 5.4, 9.1 Hz, 1′-H), 7.41−7.75 (16H, m, 6-H and Ph-H), 9.04 (1H, bs, 3-NH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 39.5 (d, J = 20.7 Hz, 2′-CH_2), 64.3 (d, J = 11.5 Hz, 5′-CH_2), 85.2 (1′-CH), 85.4 (d, J = 24.7 Hz, 4′-CH), 94.9 (d, J = 178.7 Hz, 3′-CH), 103.1 (5-CH), 128.8 (Ph-CH), 131.2 (Ph-CH), 133.0 (Ph-C), 135.7 (Ph-CH), 140.1 (6-CH), 150.7 (2-C), 163.5 (4-C); ^{19}F NMR (282 MHz, $CDCl_3$) δ −175.1 (m, 3′-F); MS (ES$^-$) *m/z* 487 ([M − H]$^-$, 31%), 75 (100%); HRMS (ES$^+$) calcd for $C_{27}H_{25}N_2O_4FNaSi^+$ (M + Na)$^+$ 511.1460, found 511.1448; Anal. ($C_{27}H_{25}N_2O_4FSi$, 0.15 HCl) C, H, N, Cl.

5′-Tritylamino-3′-fluoro-2′,3′,5′-trideoxyuridine (4f). A procedure identical to the one used for **2j** was employed. Compound **4f** was obtained as a pale yellow solid (91 mg, 32%) from the reaction of 5′-amino-3′-fluoro-2′,3′,5′-trideoxyuridine (**12**) (0.137 g, 0.59 mmol) and trityl chloride (0.199 g, 0.66 mmol) in dry pyridine (4 mL). Flash column chromatography (ISOLUTE SI column) was carried out using 0 → 5% CH_3OH in $CHCl_3$. R_f (10% $CH_3OH/CHCl_3$) 0.36; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.87−2.13 (2H, m, 2′-H), 2.28 (1H, dd, J = 8.1, 12.0 Hz, $CH_2N\mathit{H}$), 2.57−2.78 (2H, m, 5′-H), 4.48 (1H, dm, $J \approx$ 25 Hz, 4′-H), 5.11 (1H, dd, J = 5.3, 53.7 Hz, 3′-H), 5.71 (1H, d, J = 8.1 Hz, 5-H), 6.37 (1H, dd, J = 5.6, 8.7 Hz, 1′-H), 6.98 (1H, d, J = 8.1 Hz, 6-H), 7.23−7.43 (9H, m, Ph-H), 7.53 (6H, m, Ph-H), 9.39 (1H, s, 3-NH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 38.5 (d, J = 21.8 Hz, 2′-CH_2), 46.1 (d, J = 9.2 Hz, 5′-CH_2), 71.1 ($Ph_3\mathit{C}$), 85.2 (d, J = 25.3 Hz, 4′-CH), 85.5 (1′-CH), 94.4 (d, J = 179.9 Hz, 3′-CH), 103.6 (5-CH), 127.1 (Ph-CH), 128.5 (Ph-CH),

128.9 (Ph-CH), 139.3 (6-CH), 145.7 (Ph-C), 150.5 (2-C), 163.4 (4-C); ^{19}F NMR (282 MHz, $CDCl_3$) δ −175.7 (m, 3′-F); MS (ES^+) *m/z* 494 ($[M + Na]^+$, 92%), 243 (Ph_3C^+, 93%); HRMS (ES^+) calcd for $C_{28}H_{26}N_3O_3FNa^+$ $(M + Na)^+$ 494.1850, found 494.1866; Anal. ($C_{28}H_{26}N_3O_3F$, 1.5 HCl) C, H, N, Cl.

2′,3′-Didehydro-2′,3′-dideoxyuridine 5′-Diphenyl Phosphate (5a). Chlorodiphenyl phosphate (0.30 mL, 1.43 mmol) was added dropwise to a solution of compound **5i** (0.25 g, 1.19 mmol) in dry pyridine (10 mL), and the reaction was stirred overnight at room temperature. H_2O (3 mL) was added, and the reaction mixture was extracted with $CHCl_3$ (2 × 100 mL). The organic extracts were washed with H_2O (100 mL), dried over Na_2SO_4, and reduced in vacuo. Further purification was carried out by flash column chromatography eluting with 0 → 5% CH_3OH in $CHCl_3$. Compound **5a** was obtained as a colorless oil (37 mg, 7%) from the fractions, with R_f = 0.40 (15% $CH_3OH/CHCl_3$). ^{1}H NMR (300 MHz, CD_3OD) δ 4.40−4.53 (2H, m, 5′-H), 5.03 (1H, m, 4′-H), 5.31 (1H, d, J = 8.1 Hz, 5-H), 5.91 (1H, m, 1′-H), 6.36 (1H, m, 2′-H), 6.87 (1H, m, 3′-CH), 7.12−7.22 (6H, m, Ph-H), 7.30−7.50 (5H, m, 6-H and Ph-H); ^{13}C NMR (75 MHz, CD_3OD) δ 70.9 (d, J = 6.8 Hz, 5′-CH_2), 86.5 (d, J = 6.0 Hz, 4′-CH), 91.8 (1′-CH), 103.3 (5-CH), 121.46, 121.52, 121.56, and 121.63 (Ph-CH), 127.48 and 127.50 (Ph-CH), 128.5 (2′-CH), 131.47 and 131.55 (Ph-CH), 135.0 (3′-CH), 142.6 (6-CH), 152.1 (Ph-C), 153.0 (2-C), 166.1 (4-C); ^{31}P NMR (121 MHz, CD_3OD) δ −11.3; IR (film) 1697, 1684, 1264, 1194 cm^{-1}; MS (CI) *m/z* 460 ($[M + NH_4]^+$, 100%), 443 ($[M + H]^+$, 70%); HRMS (ES^+) calcd for $C_{21}H_{20}N_2O_7P$ $(M + H)^+$ 443.1008, found 443.1001.

5′-*O*-Sulfamoylcarbamoyl-2′,3′-didehydro-2′,3′-dideoxyuridine (5b). A solution of **5i** (0.36 g, 1.72 mmol) in CH_3CN (15 mL) was reacted with chlorosulfonylisocyanate (0.16 mL, 1.89 mmol) at −20 °C for 2 h. The mixture was then treated at −20 °C with a saturated solution of NH_3 in dry CH_3CN added dropwise until the solution had reached a pH of 11. The reaction mixture was allowed to warm and was stirred at room temperature for 2 h. Compound **5b** precipitated out and, after filtration and drying, was obtained as a white solid (0.47 g, 74%). R_f (20% $CH_3OH/CHCl_3$) 0.25; mp 145−146 °C (dec); ^{1}H NMR (300 MHz, DMSO-d_6) δ 4.02 (2H, m, 5′-H), 4.96 (1H, m, 4′-H), 5.56 (1H, d, J = 8.1 Hz, 5-H), 6.12 (1H, m, 1′-H), 6.42 (1H, m, 2′-H), 6.83 (1H, m, 3′-H), 7.57 (2H, bs, N*H*$_2$), 7.76 (1H, d, J = 8.1 Hz, 6-H); ^{13}C NMR (75 MHz DMSO-d_6) δ 64.8 (5′-CH_2), 85.2 (4′-CH), 89.3 (1′-CH), 102.4 (5-CH), 126.3 (2′-CH), 134.8 (3′-CH), 142.0 (6-CH), 151.2 (2-C), 158.8 (COO), 163.6 (4-C); IR (film) 1732, 1723, 1694, 1672, 1620, 1377, 1162 cm^{-1}; MS (ES^+) *m/z* 333 ($[M + H]^+$, 80%); HRMS (ES^+) calcd for $C_{10}H_{16}N_5O_7S^+$ $(M + NH_4)^+$ 350.0765, found 350.0765.

5′-*O*-(1-Adamantoyl)-2′,3′-didehydro-2′,3′-dideoxyuridine (5d). A procedure similar to the one used for **5a** was employed. Compound **5d** was obtained as a white solid (0.48 g, 74%) from the reaction of **5i** (0.37 g, 1.74 mmol) with 1-adamantanecarbonyl chloride (0.38 g, 1.92 mmol). Flash column chromatography was carried out using 0 → 3% CH_3OH in $CHCl_3$. R_f (10% $CH_3OH/CHCl_3$) 0.41; mp 175−178 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.76 (4H, m, adamantyl-CH_2), 1.93 (6H, m, adamantyl-CH_2), 2.07 (5H, m, adamantyl-CH), 4.21 (1H, dd, J = 4.1, 12.5 Hz, 5′-C*H*H), 4.53 (1H, dd, J = 4.2, 12.5 Hz, 5′-CH*H*), 5.12 (1H, m, 4′-H), 5.80 (1H, d, J = 8.1 Hz, 5-H), 5.98 (1H, m, 1′-H), 6.31 (1H, m, 2′-H), 7.02 (1H, m, 3′-H), 7.56 (1H, d, J = 8.1 Hz, 6-H); ^{13}C NMR (300 MHz, $CDCl_3$) δ 28.2 (adamantyl-CH), 28.3 (adamantyl-CH), 36.6 (adamantyl-CH_2), 36.8 (adamantyl-CH_2), 36.9 (adamantyl-CH_2), 38.9 (adamantyl-CH_2), 39.1 (adamantyl-CH_2), 39.3 (adamantyl-CH_2), 41.3 (adamantyl-C), 64.9 (5′-CH_2), 85.2 (1′-CH), 90.4 (4′-CH), 103.2 (5-CH), 127.7 (2′-CH), 133.6 (3′-CH), 140.5 (6-CH), 151.0 (2-C), 163.9 (4-C), 177.7 (*C*OO); IR (KBr) 1732, 1713, 1697, 1620 cm^{-1}; MS (ES^+) *m/z* 395 ($[M + Na]^+$, 100%); HRMS (ES^+) calcd for $C_{20}H_{24}N_2O_5Na^+$ $(M + Na)^+$ 395.1577, found 395.1580.

5′-*O*-Triphenylsilyl-2′,3′-didehydro-2′,3′-dideoxyuridine (5h). A procedure similar to the one used for **1m** was employed. Compound **5h** was obtained as a white solid (0.476 g, 68%) from the reaction of **5i** (0.316 g, 1.50 mmol) with TPSCl (0.891 g, 3.02 mmol) added in two portions. Flash column chromatography (ISOLUTE SI column) was carried out using 0 → 5% CH_3OH in $CHCl_3$. R_f (10% $CH_3OH/CHCl_3$) 0.57; mp 73−74 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.04 (1H, dd, J = 2.2, 11.7 Hz, 5′- C*H*H), 4.19 (1H, dd, J = 2.5, 11.7 Hz, 5′- CH*H*), 4.78 (1H, dd, J = 1.9, 8.1 Hz, 5-H), 4.98 (1H, m, 4′-H), 5.90 (1H, d, J = 5.7 Hz, 1′-H), 6.33 (1H, dd, J = 1.4, 4.5 Hz, 2′-H), 7.12 (1H, m, 3′-H), 7.40−7.65 (15H, m, Ph-H), 7.80 (1H, d, J = 8.1 Hz, 6-H), 8.99 (1H, bs, 3-NH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 64.7 (5′-CH_2), 87.4 (1′-CH), 90.0 (4′-CH), 102.6 (5-CH), 127.1 (2′-CH), 128.6 (Ph-CH), 131.0 (Ph-CH), 133.3 (Ph-C), 134.9 (3′-CH), 135.8 (Ph-CH), 141.5 (6-CH), 151.2 (2-C), 163.6 (4-C); MS (ES^+) *m/z* 491 ($[M + Na]^+$, 36%), 119 (100%); HRMS (ES^+) calcd for $C_{25}H_{28}N_3OSi^+$ $(M + NH_4)^+$ 446.1894, found 446.1887; Anal. ($C_{27}H_{27}N_2O_4Si$, 0.25 HCl) C, H, N, Cl.

Biological Assays

Enzyme Purification and Inhibition Assays. Both recombinant *P. falciparum* and human dUTPases were expressed in *E. coli* BL21 (DE3) cells, which had been transformed with the pET11*Pfdut*[9] and pET3*Hudut* (kindly provided by P. O. Nyman, Lund University, Sweden) expression vectors, respectively. For the dUTPase purification, the same procedure was used for both the human and the *Plasmodium* enzymes. Cell pellets from a 2-L IPTG-induced culture were resuspended in 40 mL of buffer A [20 mM sodium acetate (pH 5.5), 50 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT] and 20 μM PMSF. The cells were lysed by sonication, and the cell extract was cleared by centrifugation at 15 000 rpm for 45 min. The supernatant was loaded onto a 50-mL phosphocellulose (Whatman P-11) column at 4 °C and was eluted with a 50 mM−2 M NaCl gradient in buffer A. The enzyme was then dialyzed against buffer A prior to gel filtration chromatography on a Superdex 200 HA 10/30 column at 4 °C. Purified fractions contained dUTPase of ∼96% purity.

Nucleotide hydrolysis was monitored by mixing the enzyme and substrate with a rapid kinetic accessory (Hi-Tech Scientific) attached to a spectrophotometer (Cary 50) and connected to a computer for data acquisition and storage as described in ref 5. Protons, released through the hydrolysis of nucleotides, were neutralized by a pH indicator in a weak buffered medium with similar pK_a and were monitored spectrophotometrically at the absorbance peak of the basic form of the indicator. The ratio between the indicator and the buffer concentration was 50:2000 (μM), and the absorbance changes were kept within 0.1 units. The indicator/buffer pair used was red cresol/bicine (pH 7.5−8.5, 573 nm). Assay mixes contained 30 nM of *Pf*dUTPase, 50 μM dUTP, 5mM $MgCl_2$, and 2.5 mM DTT, 1.25 mg/mL BSA, and 100 mM KCl. V_{max} and K_{Mapp} were calculated by fitting the resulting data to the integrated Michaelis−Menten equation. The apparent K_M values were plotted against inhibitor concentration, and K_i values (Table 4) were obtained according to eq I.

$$K_{Mapp} = \frac{K_M}{K_i}[I] + K_M \qquad \text{(I)}$$

In Vitro Assays. All in vitro assays were carried out twice independently and in duplicate.

***Plasmodium falciparum*.** In vitro activity against the erythrocytic stages of *P. falciparum* was determined by using a ^{3}H-hypoxanthine incorporation assay[36,37]

using the chloroquine and pyrimethamine resistant K1 strain and the standard drugs chloroquine (Sigma C6628) and artemisinin (Arteannuin, Qinghaosu; Sigma 36,159−3). Compounds were dissolved in DMSO at 10 mg/mL and added to parasite cultures incubated in RPMI 1640 medium without hypoxanthine, supplemented with HEPES (5.94 g/L), $NaHCO_3$ (2.1 g/L), neomycin (100 U/mL), Albumax^R (5 g/L), and washed human red cells A^+ at 2.5% haematocrit (0.3% parasitaemia). Serial doubling dilutions of each drug were prepared in 96-well microtiter plates and incubated in a humidified atmosphere at 37 °C; 4% CO_2, 3% O_2, 93% N_2.

After 48 h, 50 μL of ^{3}H-hypoxanthine (= 0.5 μCi) was added to each well of the plates. The plates were incubated for an additional 24 h under the same conditions. The plates were then harvested with a Betaplate cell harvester (Wallac, Zurich, Switzerland), and the red blood cells were transferred onto a glass fiber filter and then washed with distilled water. The dried filters were inserted into a plastic foil with 10 mL of scintillation fluid and were counted in a Betaplate liquid scintillation counter (Wallac, Zurich, Switzerland). IC_{50} values were calculated from sigmoidal inhibition curves using Microsoft Excel.

Leishmania donovani. Mouse peritoneal macrophages were seeded in RPMI 1640 medium with 10% heat-inactivated fetal bovine serum (FBS) into Lab-tek 16-chamber slides. After 24 h, *L. donovani* amastigotes were added at a ratio of 3:1 (amastigotes to macrophages). The medium containing free amastigotes was replaced by fresh medium 4 h later. The next day, the medium was replaced by fresh medium containing different drug concentrations. The slides were incubated at 37 °C under a 5% CO_2 atmosphere for 96 h. Then the medium was removed, and the slides were fixed with methanol and stained with Giemsa. The ratio of infected to noninfected macrophages was determined microscopically and expressed as a percentage of the control, and the IC_{50} value was calculated by linear regression.

Trypanosoma cruzi. Rat skeletal myoblasts (L-6 cells) were seeded in 96-well microtiter plates at 2000 cells/well/100 μL in RPMI 1640 medium with 10% FBS and 2 mM of L-glutamine. After 24 h, 5000 trypomastigotes of *T. cruzi* [Tulahuen strain C2C4 containing the β-galactosidase (Lac Z) gene] were added in aliquots of 100 μL per well with 2× of a serial drug dilution. The plates were incubated at 37 °C in 5% CO_2 for 4 days. Then the substrate CPRG/Nonidet was added to the wells. The color reaction, which developed during the following 2−4 h, was read photometrically at 540 nm. IC_{50} values were calculated from the sigmoidal inhibition curve.

Trypanosoma brucei rhodesiense. Minimum essential medium (50 μL), supplemented according to Baltz et al.[38] with 2-mercaptoethanol and 15% heat-inactivated horse serum, was added to each well of a 96-well microtiter plate. Serial drug dilutions were added to the wells. Then 50 μL of trypanosome suspension (*T. b. rhodesiense* STIB 900) was added to each well, and the plate was incubated at 37 °C under a 5% CO_2 atmosphere for 72 h. Ten microliters of Alamar Blue (Trinova, Giessen, Germany) was then added to each well, and incubation was continued for an additional 2−4 h.[39] The plates were read in a microplate fluorescence scanner (Spectramax Gemini XS by Molecular Devices) using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. IC_{50} values were calculated from the sigmoidal inhibition curve.

Cytotoxicity. Rat skeletal myoblasts (L-6 cells) were seeded in 96-well microtiter plates in RPMI 1640 medium with 10% FBS and 2 mM L-glutamine at a density of 4×10^4 cells/mL. After 24 h, the medium was removed and replaced by fresh medium containing a serial drug dilution, and the plate was incubated at 37 °C under a 5% CO_2 atmosphere for 72 h. Ten microliters of Alamar Blue (Trinova, Giessen, Germany) was then added to each well, and incubation was continued for an additional 2−4 h. The plates were read in a microplate fluorescence scanner (Spectramax Gemini XS by Molecular Devices) using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. IC_{50} values were calculated from the sigmoidal inhibition curve.

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Supporting Information Available: Experimental procedures and analytical data for compounds not included in the Experimental Section and a table of elemental analysis data obtained for selected compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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