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β-Branched acyclic nucleoside analogues as inhibitors of *Plasmodium falciparum* dUTPase

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ABSTRACT

We report a series of β-branched acyclic tritylated deoxyuridine analogues as inhibitors of *Plasmodium falciparum* deoxyuridine-5′-triphosphate nucleotidohydrolase (*Pf*dUTPase), an enzyme involved in nucleotide metabolism that acts as first line of defence against uracil incorporation into DNA. Compounds were assayed against both *Pf*dUTPase and intact parasites showing a correlation between enzyme inhibition and cellular assays. β-Branched acyclic uridine analogues described here showed equal or slightly better potency and selectivity compared with previously reported analogues. The best inhibitor gave a K_i of 0.5 μM against *Pf*dUTPase with selectivity greater than 200-fold compared to the corresponding human enzyme and sub-micromolar growth inhibition of *P. falciparum* (EC_{50} 0.6 μM). A crystal structure of the complex of *Pf*dUTPase with one of the inhibitors shows that this acyclic derivative binds to the active site in a similar manner to that previously reported for a tritylated cyclic deoxyuridine derivative.
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1. Introduction

Malaria is a widespread infectious disease endemic in 106 countries, and there are an estimated 236 million cases every year, causing nearly a million deaths. The disease burden is heaviest in African children under five years of age and pregnant women.[1] Malaria is caused by *Plasmodium* parasites that infect and destroy red blood cells, leading to fever, severe anaemia, cerebral malaria and, if untreated, death.[2] *Plasmodium falciparum* is responsible for most of the severe clinical malaria cases and it has developed resistance to commonly employed anti-malarial chemotherapeutics.[3] There is a need to develop new drugs against new molecular targets with minimal cross-resistance to existing therapeutic agents.[4]

The ubiquitous enzyme deoxyuridine 5′-triphosphate nucleotidohydrolase (dUTPase), involved in nucleotide metabolism, catalyses the hydrolysis of dUTP to dUMP in the presence of magnesium ions. The enzyme has two major roles in maintaining DNA integrity. Firstly, it provides dUMP, the substrate for thymidylate synthase, involved in the only biosynthetic route for dTTP in the malarial parasite. Secondly, it maintains a low dUTP/dTTP ratio reducing erroneous uracil incorporation into DNA by DNA polymerases, since these enzymes do not discriminate between dUTP and dTTP.[5] dUTPase has been proved to be essential for cell viability in several organisms[6,7] and recent evidence showed that inhibition of dUTPase is a viable strategy in cancer chemotherapy, particularly in combination with TS inhibitors like 5-fluorouracil (5-FU).[8]

By taking advantage of the structural differences between the human and *Plasmodium* enzymes, we have previously reported the selective inhibition of *P. falciparum* dUTPase (*Pf*dUTPase) in the low micromolar range by 5′-tritylated deoxyuridine analogues,[9,10] which also showed selective antiplasmodial activity. More recently we demonstrated that tritylated acyclic uridine analogues can have equal or better antiplasmodial activities compared to cyclic analogues (Fig. 1).[11,12] *Pf*dUTPase is a promising target for the development of anti-malarial drugs, owing to its biological role, the structural differences with its human orthologue and the discovery of selective inhibitors of *Pf*dUTPase with antiplasmodial activity.

The program GRID is a computational procedure for determining energetically favourable binding sites for a probe on a protein of known structure.[13] In this project, GRID was used to probe the active site of *Pf*dUTPase for energetically favoured interactions.

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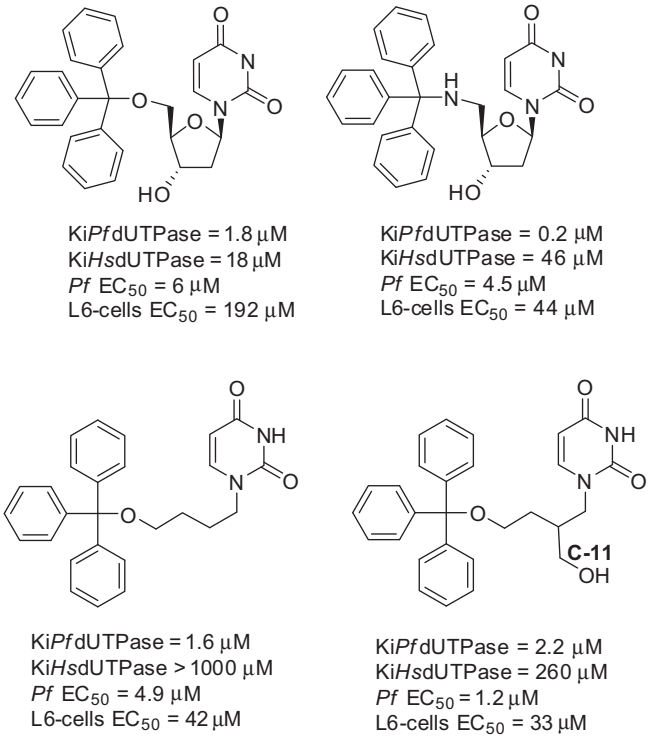


Figure 1.

Docking of a previously synthesised β-branched acyclic inhibitor (Fig. 1) and subsequent superimposition of this molecule into the GRID calculated structure, suggested that the presence of a carbonyl oxygen at the branch point (C-11) could lead to an extra hydrogen bond within the active site providing the conformation of the ligand did not change significantly with this modification (Fig. 2).

In this paper we extend our SAR analysis of β-branched acyclic nucleoside analogues with the introduction of an amide group at the branch point (Fig. 3), and we determined the crystal structure of a complex with one of these ligands in the *Pf*dUTPase active site. These compounds are designed to probe the active site of *Pf*dUTPase for energetically favourable interactions such as additional H-bonds. Furthermore, the presence of an amide bond would make the conformationally flexible acyclic inhibitor more rigid.

2. Chemistry

One of the key SAR features established so far for both cyclic and acyclic inhibitors of *Pf*dUTPase is the requirement for a group bearing two or three phenyl rings, with trityl, triphenylsilyl and *tert*-butyldiphenylsilyl being the groups of choice.[9,11] In this paper we report our results on three series of compounds to study the effect of O/NH and C/Si switch within the trityl group itself as well as the variation of substituents on the amide on the branch chain.

Michael addition of uracil to commercially available α-methylene γ-butyrolactone followed by aminolysis of the lactone **1** with the corresponding amine and subsequent tritylation of the resulting alcohol led to the desired trityloxy β-branched derivatives **3** and **4** (Scheme 1). For an initial screen, no attempts were made at producing enantiomerically pure samples, and the compounds were tested as racemic mixtures.

The Michael addition of uracil to α-methylene butyrolactone was performed using an excess of uracil in the presence of DBU and dropwise addition of the lactone in order to minimise uracil dialkylation. The dialkylated derivative was readily separated by column chromatography and a mixture of monoalkylated uracil derivatives was obtained in 45% yield with a ratio 78/22 in favour of the desired N^1 alkylated uracil. The two isomers proved difficult to separate and the mixture was used in the next step without further purification. It is interesting to note the importance of the choice of base in the selectivity of this reaction. When the reaction was carried out under the same conditions but in the presence of

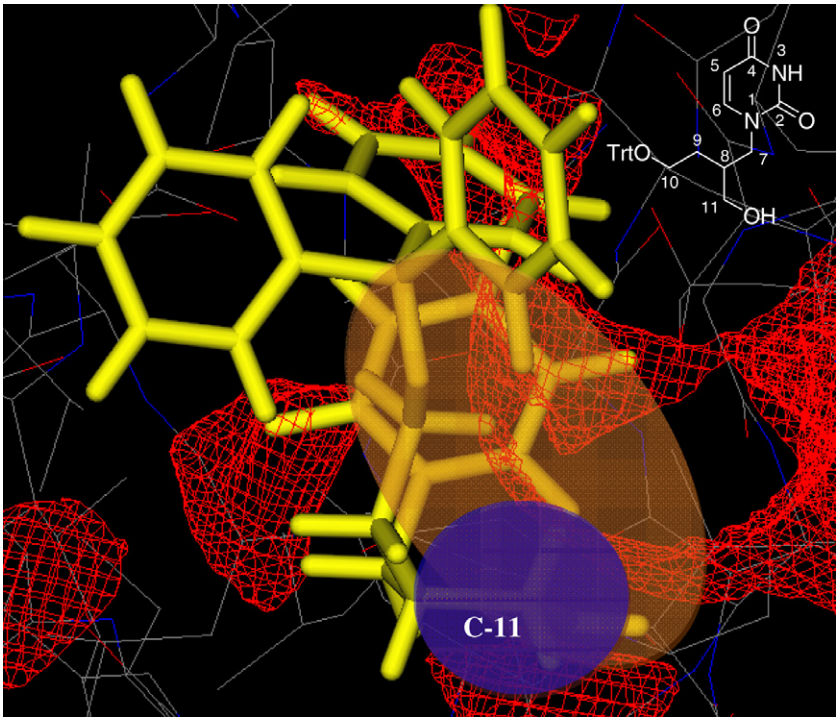


Figure 2. GRID calculations for O probe at an energy of −4.01 kcal. (Side chain highlighted in blue bubble and interaction area highlighted in orange bubble).

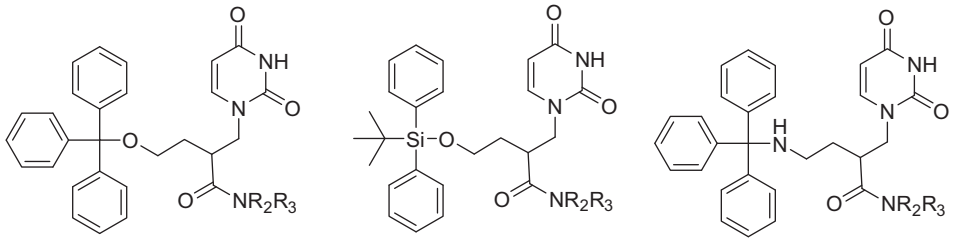
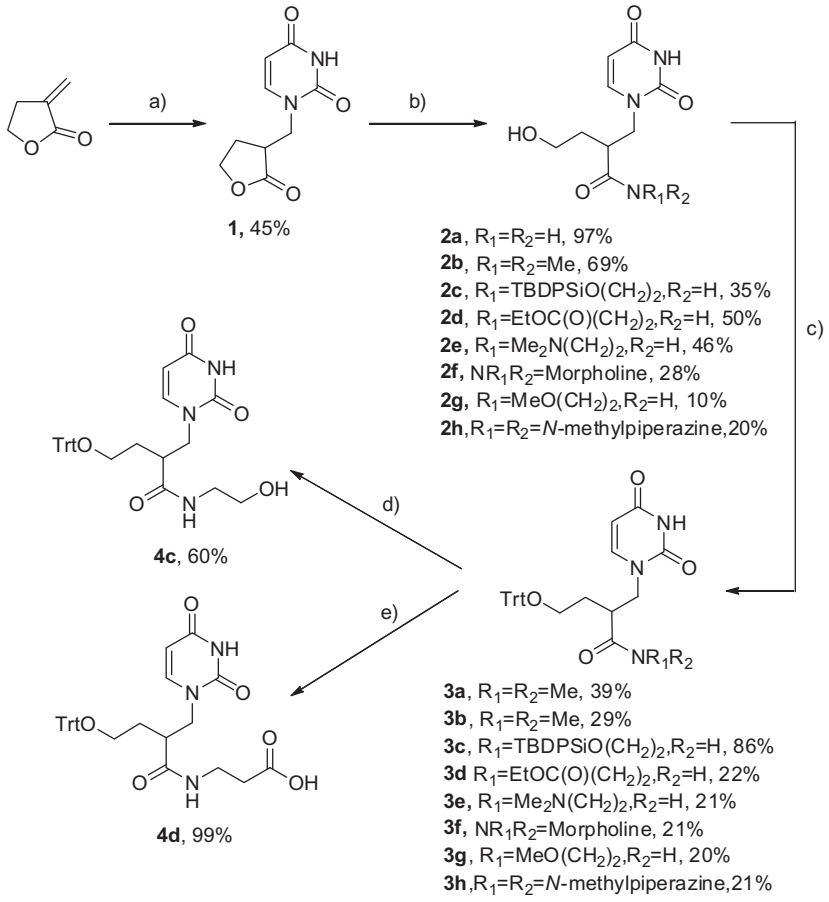


Figure 3.



Scheme 1. Reagents and conditions: (a) uracil, DBU, acetonitrile, reflux; (b) (i) amine (10 equiv), EtOH, rt (**2a**–**b**) or 100 °C (**2c**–**h**); (ii) PL-MIA, acetonitrile, rt or microwave irradiation; (c) TrtCl, DMAP cat, py, 100 °C, microwave irradiation; (d) TBAF, THF, rt; (e) LiOH (10 equiv), MeOH/water 4:1, rt.

DIPEA instead of DBU the reaction did not reach completion but it was remarkably selective. The only reaction product was N^1 alkylated uracil that was isolated in 15% yield.

The aminolysis step required an excess of amine[14] and, with the exception of **2a**–**b**, the reaction was heated at 100 °C for several days. A polymer supported scavenger, PL-MIA, was used to remove the excess of amine from the reaction crude prior to purification by column chromatography. The structure of **2a**–**h** was verified by two-dimensional NOESY NMR showing the coupling through space between 1′-H on the side chain and 6-H of the uracil. Compounds **2a**–**h** were tritylated in pyridine in the presence of catalytic DMAP at 100 °C under microwave irradiation.[11] Finally, **4c** was obtained by deprotection with TBAF in 60% yield and **4d** by ester hydrolysis with LiOH in methanol/water in quantitative yield.

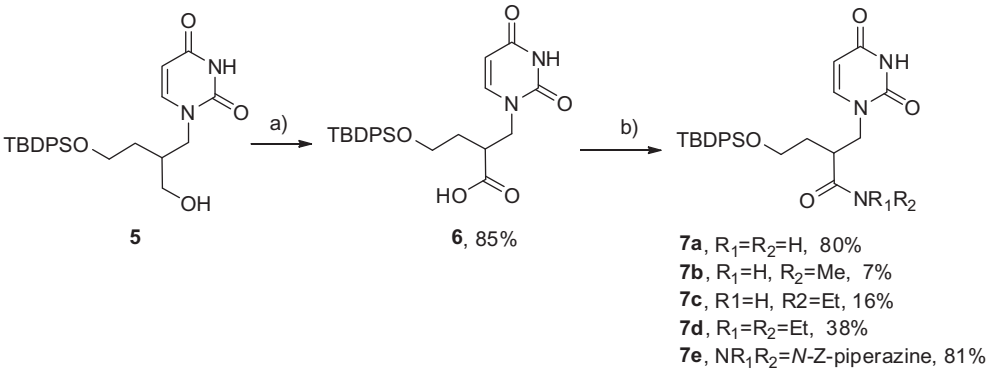
The silyloxy branched acyclic analogues were synthesised in two steps from the branched precursor **5**. The first step was the oxidation of the alcohol to acid using an excess of [bis(acetoxy)-iodo]benzene (BAIB) and catalytic TEMPO in a mixture 1:1 of acetonitrile and water.[15] Subsequent amide formation gave rise to compounds **7a**–**e** (Scheme 2).[16]

Finally, triphenylmethylamino derivatives were synthesised from intermediate **8**. The Staudinger reaction[17] was used to reduce the azide to the amine **9** that was then tritylated in the presence of DMAP in pyridine at 160 °C in a microwave reactor.[11] Cleavage of the benzyl carbonate was carried out by hydrogenolysis using 5% Pd/C at rt. Oxidation to acid **12** using BAIB and TEMPO as described above followed by amide coupling afforded compound **13** (Scheme 3).

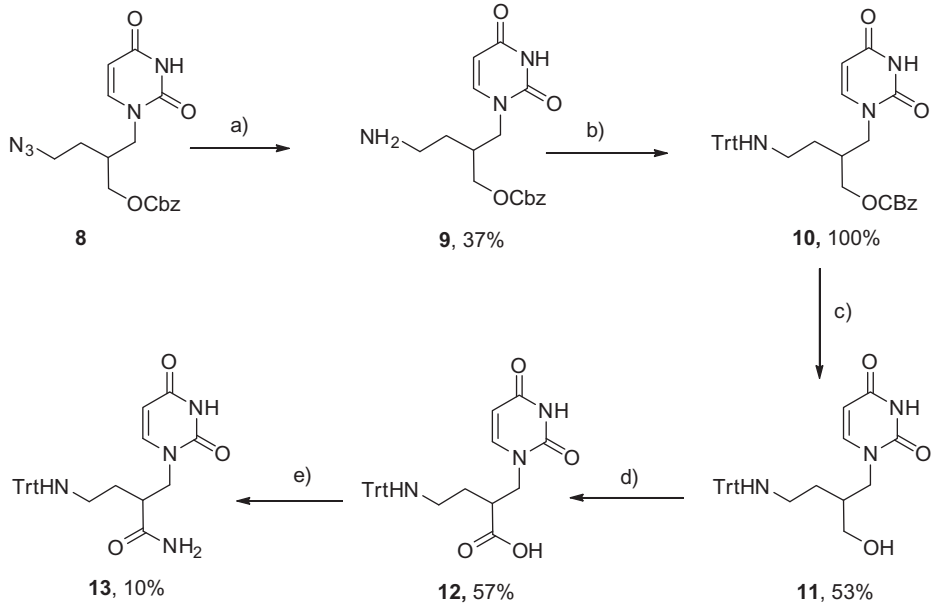
3. Results and discussion

3.1. Enzyme assays

Compounds were tested against the recombinant *Pf*dUTPase and recombinant human dUTPase to determine inhibition and selectivity (Table 1).



Scheme 2. Reagents and conditions: (a) BAIB, TEMPOcat, acetonitrile/water 1:1; (b) NHR_1R_2, DCC, DhbtOH, Et_3N, DMF, 0 °C to rt.



Scheme 3. Reagents and conditions: (a) (i) PhP_3, py, rt; (ii) NH_4OH, 50 °C; (b) Ph_3CCl, pyridine, DMAP, microwave, 160 °C; (c) H_2, 5% Pd/C, rt, MeOH; (d) BAIB, TEMPO cat, acetonitrile/water 1:1; (e) NH_4Cl, DCC, DhbtOH, Et_3N, DMF, 0 °C to rt.

The replacement of the β-hydroxymethyl branch on our lead compound (Fig. 1) for a β-amide branch led to compounds with similar or greater enzyme inhibition and selectivity. In particular, introduction of polar substituents on the amide presented the best K_i in the sub-micromolar range. The nature of the polar group in that position is important and includes acid **4d** (K_i = 0.2 μM), ester **3d** (K_i = 0.5 μM) and alcohol **4c** (K_i = 0.4 μM). However, basic amines **3e** (K_i = 7.8 μM) and **3h** (K_i = 5.6 μM) led to a drop in activity. Moreover, bulkier and more lipophilic groups also led to lower activities compared with smaller polar groups. For example, the removal of the TBDPS group from **3c** to give **4c** led to a more than 20-fold increase in the enzyme inhibition and improved selectivity.

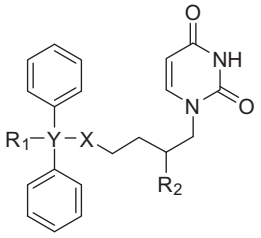
A second series of compounds explored the effect of the C/Si switch on the C-4 chain. In general, compounds with a TBDPS group inhibit selectively *Pf*dUTPase. Their activity decreases as lipophilicity and MW increases with primary and secondary amides **7a–c** more active than tertiary amides **7d–e**. Overall, in comparison to the trityloxy derivatives, the C/Si switch does not appear to improve *Pf*dUTPase inhibition. Finally, three compounds with a tritylamino group at the C-4 chain were tested for enzyme inhibition, but the O/NH switch within the trityl group does not increase inhibition of *Pf*dUTPase and has a detrimental effect on selectivity on this series of β-branched acyclic nucleoside analogues.

3.2. Cellular assays

Compounds were also assayed in vitro against the erythrocytic stages of the chloroquine resistant K1 strain of *P. falciparum* to evaluate antiplasmodial activity and against rat skeletal myoblasts (L6 cells) as a test for cytotoxicity (Table 1). All of the compounds, except for compound **6**, showed activities against the parasite in a relatively small range, with EC_{50} values varying from 0.3 to 8.8 μM. The reason for the lack of activity of this acid could be poor permeability caused by its negative charge. Additionally, there are three compounds **3c**, **7d** and **7e** that were not active against *Pf*dUTPase but showed in vitro activity against the parasite suggesting that they have a different mode of action.

For some compounds differences were observed between the extent of inhibition in the enzyme and cellular assays, which could be due to differences in cellular permeability. For example, acid **4d** showed similar levels of enzyme inhibition as the corresponding ethyl ester **3d**; however it is 6 fold less active against the parasite. Additionally, some compounds may be metabolised; for example a

Table 1
Inhibition of *P. falciparum* and human dUTPases and growth inhibition of *P. falciparum* and a mammalian cell line



Compd	R_1	Y	X	R_2	dUTPase		In vitro		
					P. falciparum K_i (μM)	*Human* K_i (μM)	*P. falciparum*[a] EC_{50} (μM)	L6-cells[b] EC_{50} (μM)	SI_{cell}
3a	Ph	C	O	$CONH_2$	1.3	>100	2.1		
3b	Ph	C	O	$CONMe_2$	2.7	>100	1.2	47	39
3c	Ph	C	O	$CONH(CH_2)_2OSiPh_2{}^tBu$	>10	>100	0.3	>120	400
3d	Ph	C	O	$CONH(CH_2)_2CO_2Et$	0.5	>100	0.6	158	263
3e	Ph	C	O	$CONH(CH_2)_2NMe_2$	7.8	>100	0.8	20	25
3f	Ph	C	O	CO-morpholine	2.1	>100	1.4	67	48
3g	Ph	C	O	$CONH(CH_2)_2OMe$	1.4	>100	1.0	47	47
3h	Ph	C	O	CO-*N*-methylpiperazine	5.6	>100	1.3	43	33
4c	Ph	C	O	$CONH(CH_2)_2OH$	0.4	>100	1.3	>175	135
4d	Ph	C	O	$CONH(CH_2)_2CO_2H$	0.2	>100	3.4	166	49
6	*t*-Bu	Si	O	CO_2H	1.6	>100	>10	188	19
7a	*t*-Bu	Si	O	$CONH_2$	5.0	>100	5.0	63	13
7b	*t*-Bu	Si	O	CONHMe	3.6	>100	8.8	165	19
7c	*t*-Bu	Si	O	CONHEt	5.7	>100	5.0	51	10
7d	*t*-Bu	Si	O	$CONEt_2$	20	>100	2.7	48	18
7e	*t*-Bu	Si	O	CO-*N*-Cbz-piperazine	98	>100	0.7	19	27
10	Ph	C	N	CH_2OCbz	6.5	>100	1.9	22	12
12	Ph	C	N	CO_2H	7.2	90	1.3	51	39
13	Ph	C	N	$CONH_2$	5.5	98	1.1	177	161

[a] *Plasmodium falciparum* K1 chloroquine resistant strain.
[b] Cytotoxicity on rat L6 myoblasts. Controls: for *P. falciparum*, chloroquine, EC_{50} = 0.1 μM; for cytotoxicity, podophyllotoxin, EC_{50} = 0.012 μM. The EC_{50} values are the means of two independent assays.

silanol group generated by hydrolysis could be the actual parasite growth inhibitor. Thus, whereas compound **3c** containing a silyl protecting group, was four times more active against *P. falciparum* than deprotected alcohol **4c**, it showed less activity in the enzyme assay. In this case the silyl group may have a dual role, enhancing cell permeation and upon hydrolysis, inside the cell, generating two active metabolites, **4c** and silanol.

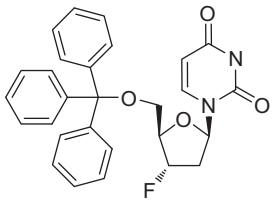
In terms of selectivity it is interesting to note the difference between enzyme selectivity and cell selectivity for compounds **6**, **7a**–**d** and **10**. Although they showed very high levels of selectivity towards *Pf*dUTPase with no inhibition of the human enzyme, their selectivity at the cellular level (SI ranges from 10 to 27) is low, suggesting some general toxic effect.

Finally, trityloxy derivatives show the best parasite growth inhibition and cell selectivity indexes. In particular, **3c** and **3d** are the compounds with the best in vitro activities (0.3 and 0.6 μM, respectively) and good selectivity to parasitic over mammalian cells (SI_{cell} 400 and 263, respectively).

3.3. Crystal structure of the 4c complex

*Pf*dUTPase was co-crystallised with the **4c** ligand, and the structure refined to a resolution of 2.1 Å. The asymmetric unit contains a trimer, and the overall fold of the enzyme is the same as that reported earlier[10] for the complex with the trityl ligand, compound **14** (Fig. 4), and will not be discussed further. The N-terminus is well ordered, from residue Met1. There is a disordered loop with no visible density from residue 58 to 81 (the latter number varies slightly over the three protomers), as seen in the earlier complex and corresponding to the low complexity region in the sequence. This loop does not affect the structure around the active site. As seen in many structures of dUTPases, the C-terminus of each protomer is disordered with no visible density: in chains A and C there is density up to residue 165, while chain B is only ordered up to residue 155. The ordering of residues 156–165 in Chains A and C appears to be an artefact of the crystal packing, with these residues in both protomers interacting with a symmetry related trimer in a similar manner. Residues 155–165 can be seen pointing away from the body of the trimer—the neighbouring trimers which stabilise these conformations are not shown for simplicity, Figure 5(a). In contrast this region in protomer B has no symmetry contacts and is more representative of what is likely to be the conformation in solution.

The ligand is bound to the active site in all three subunits, in a closely similar manner. Here we focus on the binding to subunit B, Figure 5(b). There is excellent electron density for the uridine and trityl groups. The acyclic linker between them also has good density and its position is clear. The uridine points down into a largely hydrophobic pocket, with its O4 atom forming an H-bond to the enzyme (not shown) and to a buried water molecule (W) at the base of the pocket. The trityl groups stack against a hydrophobic



14

Figure 4. The structure of ligand previously co-crystallised with *Pf*dUTPase (1VYQ).[10]

surface on the left of the figure. The extended arm on the acyclic linker in **4c** is less well ordered with lower electron density and substantially higher temperature factors. The arm points away from the linker towards the solvent region, packing against the hydrophobic surface shown on the bottom right. While there are no H-bonds between the arm and the protein, which probably explains its relative mobility, the packing against the surface is presumed to contribute to the ligand binding energy. The uridine and trityl groups occupy essentially identical positions to those seen in the structure of the complex with compound **14**[10] (Fig. 6).

4. Conclusions

We have described the synthesis of a new series of β-branched acyclic analogues of deoxyuridine with the introduction of an amide bond at the branch point. These compounds were designed to target *Pf*dUTPase focusing on the effects in activity of O/NH and C/Si switch within the trityl group itself as well as the variation of substituents on the amide on branch chain. In general the compounds reported here show relatively similar activity against *Pf*dUTPase, varying from K_i 0.2 to 8 μM, with just three exceptions. This is probably due to the main interactions of the ligands with the enzyme being through the uracil ring and trityl group. The group attached to the amide points away from the protein towards solvent and does not appear to have significant interactions with the protein, accounting for the relatively small variation in activity across the series.

Our SAR study has led to the identification of the trityloxy derivatives as the most promising series both in terms of synthetic accessibility and biological activity and selectivity. The compounds in this series showed equal or even slightly better antiplasmodial properties compared to the cyclic and acyclic analogues previously reported. Moreover, crystallographic data for compound **4c** represents the first crystal structure of an acyclic analogue of deoxyuridine bound to *Pf*dUTPase and shows this derivative forms the same interactions in the active site as the cyclic analogues.

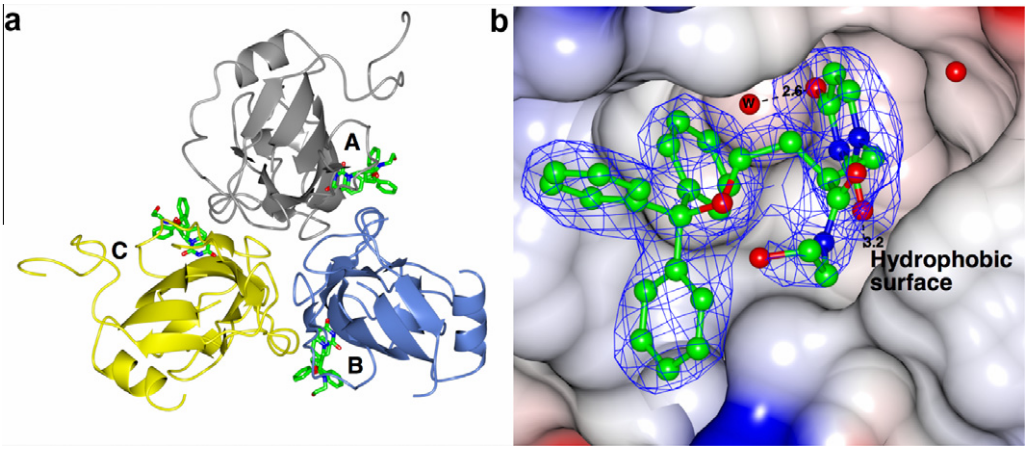


Figure 5. The structure of the **4c** complex. (a) The trimer is shown as ribbons coloured by chain. The ligand, shown as cylinders, is bound to all three active sites. The C-terminus points away from the main body of the trimer and is ordered by packing against neighbouring trimers (not shown). (b) Close up of the surface around **4c** (shown as cylinders) bound to the active site of chain B. The figure was made using CCP4mg.[18]

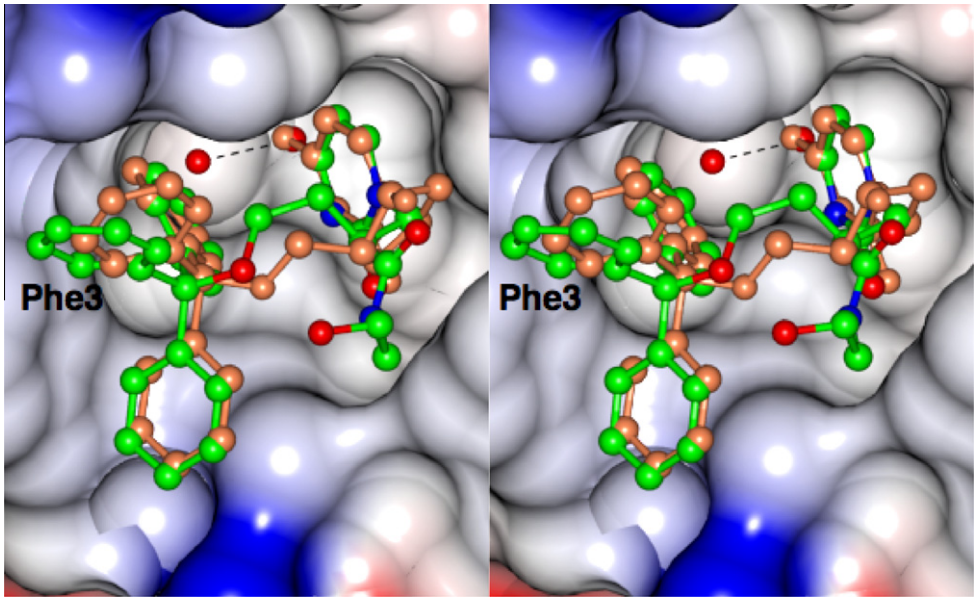


Figure 6. Stereoview of the superposition of the structure of **4c** (green) with that of the original ligand **14** (orange).

5. Experimental section

5.1. Chemistry

Solvents and reagents were purchased from commercial suppliers and used without further purification. Dry solvents were purchased in sure sealed bottles stored over molecular sieves. Intermediates **5** and **8** were kindly provided by Medivir AB, Sweden. Reactions were performed in predried apparatus under an atmosphere of argon unless otherwise stated. Reactions using microwave irradiation were carried out in a Biotage Initiator microwave. Normal phase TLC was carried out on pre-coated silica plates (Kieselgel 60 F_{254}, BDH) with visualisation via UV light and/or ninhydrin solution. Flash chromatography was performed using Combiflash Companion and prepacked column (silica gel and C18 reverse phase) purchased from Redisep (Presearch) or Sylicycle (Anachem) or a Flasmaster II using ISOLUTE SI columns purchased from Argonaut. Preparative HPLC was performed using a Gilson (321-Pump, 153-UV–vis Detector) equipped with a Gilson liquid handler for injection and fraction collection and XBridge Prep C18, 5 μm, ODB, 19x100 mm column (Waters) with 0.1% ammonia in water (solvent A) and acetonitrile (solvent B) as mobile phase. Melting points (mp) were measured on a Gallenkamp melting point apparatus and were uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance DPX500 spectrometer or on a Bruker Avance DPX300 using the applied solvent simultaneously as internal standard. Deuterated solvents were purchased from Goss. Chemical shifts (δ) are given in ppm together with the multiplicity, relative frequency, coupling constants (J, Hz) and assignment. Low resolution electrospray (ES) mass spectra were recorded on a Bruker MicroTof mass spectrometer, run in positive or negative ion mode. High resolution mass spectra were performed on a Bruker MicroTof mass spectrometer at University of Dundee or, alternatively, on a Waters ZQ4000 and a Finningan MAT 95XP at EPSRC National Mass Spectrometry Service centre in the Chemistry department, University of Wales Swansea, Swansea, Wales, UK. LC–MS analysis and chromatographic separation were conducted with a Brucker MicroTof mass spectrometer using an Agilent HPLC 1100 with a diode array detector in series. The column used was a Waters XBridge column (5 × 50 mm) and the compounds were eluted with a gradient of 5–95% acetonitrile/water + 0.1% ammonia.

5.1.1. 1-[(2-Oxotetrahydrofuran-3-yl)methyl]uracil (1)

Uracil (1 g, 8.92 mmol) was suspended in acetonitrile (100 ml) and DBU (0.88 ml, 5.90 mmol) was added. The resulting white suspension was refluxed and α-methylene-γ-butyrolactone (0.52 ml, 5.90 mmol) were added dropwise over 10 min. The suspension was refluxed for 4 h and quenched with acetic acid (10 drops). Solvents were removed under vacuum to obtain a white solid. The reaction crude was purified by column chromatography on a Redisep 40 g disposable flash column using CH_2Cl_2/MeOH 40:1 and then CH_2Cl_2/MeOH 20:1 as eluent. After removing solvents a white solid was obtained (0.560 g, 45% yield) containing a mixture of N-1 and N-3 alkylated uracil derivatives on a ratio of 78:22. This mixture was used for the next step without further purification.

Pure **1** can be obtained by the alternative protocol below:

Uracil (1 g, 8.92 mmol) was suspended in acetonitrile (100 ml) and diisopropylethylamine (1.5 ml, 8.92 mmol) was added. The resulting white suspension was refluxed and α-methylene-γ-butyrolactone (0.52 ml, 5.90 mmol) were added dropwise over 10 min. The suspension was refluxed for overnight and quenched with acetic acid (10 drops). Solvents were removed under vacuum to obtain a white solid. The reaction crude was purified by column chromatography on a Combiflash Companion using a Silicyle 40 g disposable flash column and the following gradient: 3 min hold CH_2Cl_2, 18 min ramp to 10% MeOH in CH_2Cl_2, 3 min hold 10% MeOH in CH_2Cl_2. After removing solvents the title compound was obtained as a white solid (189 mg, 15% yield). Purity by LC–MS (UV chromatogram, 190–450 nm): 99%. R_f = 0.5 (10% MeOH in CH_2Cl_2); ^{1}H NMR (500 MHz; DMSO-d_6): δ 11.33 (br s, 1H, N*H*), 7.63 (d, 1H, J = 7.9 Hz, C*H*N), 5.57 (d, 1H, J = 7.9 Hz, C*H*CO), 4.33 (dt, 1H, J = 8.9 Hz, J = 2.6 Hz, C*H*HN), 4.17 (dt, 1H, J = 8.9 Hz, J = 6.6 Hz, C*H*HN), 4.04 (dd, 1H, J = 13.9 Hz, J = 6.4 Hz, C*H*HO), 3.79 (dd, 1H, J = 13.9 Hz, J = 8.1 Hz, C*H*HO), 3.17–3.08 (m, 1H, CH_2CHCH_2), 2.31–2.25 (m, 1H, $CHCHHCH_2$), 2.06–1.98 (m, 1H, $CHCHHCH_2$); ^{13}C NMR (125 MHz; DMSO-d_6): δ 176.9 (C), 163.6 (C), 151.0 (C), 145.9 (CH), 100.8 (CH), 66.5 (CH_2), 47.4 (CH_2), 38.1 (CH), 26.3 (CH_2); LRMS (ES+) m/z 211.08 [$(M+H)^+$, 100%].

5.1.2. 1-(2-Amido-4-hydroxybutyl)uracil (2a)

Lactone **1** (189 mg, 0.9 mmol) was suspended in 7 N NH_3 in methanol (4 ml) and the reaction was stirred at room temperature for 3 h. Further 7 N NH_3 in methanol (4 ml) was added and the reaction mixture was stirred at room temperature for further 2 h. The solvents were removed under vacuum. The product was purified by column chromatography on a Combiflash Companion using a Redisep 4 g disposable flash column and the following gradient: 3 min hold at 100% CH_2Cl_2, 15 min ramp to 20% MeOH in CH_2Cl_2, 3 min hold at 20% MeOH in CH_2Cl_2. After removing solvents the title compound was obtained as a white solid (198 mg, 97%). Purity by LC–MS (UV chromatogram, 190–450 nm): 99%; R_f = 0.3 (20% MeOH in CH_2Cl_2); ^{1}H NMR (500 MHz; DMSO-d_6): δ 11.22 (br s, 1H, N*H*), 7.43 (br s, 1H, N*H*), 7.41 (d, J = 7.9 Hz, 1H, C*H*N), 6.95 (br s, 1H, N*H*), 5.49 (d, 1H, J = 7.9 Hz, C*H*CO), 4.48 (t, 1H, J = 5.2 Hz, O*H*), 3.82 (dd, 1H, J = 13.4 Hz, J = 5.2 Hz, C*H*HN), 3.60 (dd, 1H, J = 13.4 Hz, J = 9.5 Hz, C*H*HN), 3.45–3.35 (m, 2H, CH_2OH), 2.76–2.71 (m, 1H, CH_2CHCH_2), 1.65–1.58 (m, 1H, CH_2CHHCH), 1.51–1.45 (m, 1H, CH_2CHHCH); ^{13}C NMR (125 MHz; DMSO-d_6): δ 174.3 (C), 163.7 (C), 150.8 (C), 146.2 (CH), 100.3 (CH), 58.5 (CH_2), 50.0 (CH_2), 41.6 (CH), 33.01 (CH_2); LRMS (ES+) m/z 211.0 [$(M-NH_2)^+$, 66%], 228.10 [$(M+H)^+$, 100%], 455.19 [$(2\,M+H)^+$, 8%].

5.1.3. 1-(2-Dimethylamido-4-hydroxybutyl)uracil (2b)

To lactone **1** (182 mg, 0.86 mmol) in anhydrous ethanol (2.5 ml), dimethylamine (5.6 M in ethanol, 1.5 ml, 8.6 mmol) was added and the reaction was stirred at room temperature overnight. Further dimethylamine (5.6 M in ethanol, 1.5 ml, 8.6 mmol) was added and the mixture was stirred at room temperature for further 4 h. Ethanol and the excess of amine were removed under vacuum. The product was purified by column chromatography on a Redisep 12 g disposable flash column using 10% MeOH in CH_2Cl_2 as eluent. After removing solvents the title compound was obtained as a white solid (152 mg, 45%). Purity by LC–MS (UV chromatogram, 190–450 nm) >99%; R_f = 0.3 (10% MeOH in CH_2Cl_2); ^{1}H NMR (500 MHz; DMSO-d_6): δ 11.30 (br s, 1H, NH), 7.45 (d, J = 7.9 Hz, 1H, C*H*N), 5.50 (d, 1H, J = 7.9 Hz, C*H*CO), 4.58 (t, 1H, J = 4.9 Hz, OH), 3.81 (dd, 1H, J = 13.4 Hz, J = 6.2 Hz, C*H*HN), 3.63 (dd, 1H, J = 13.4 Hz, J = 8.3 Hz, C*H*HN), 3.44–3.38 (m, 2H, CH_2CHCH_2 and C*H*HOH), 3.28–3.22 (m, 1H, C*H*HOH), 2.97 (s, 3H, CH_3), 2.80 (s, 3H, CH_3), 1.70–1.63 (m, 1H, CH_2CHHCH), 1.52–1.46 (m, 1H, CH_2CHHCH); ^{13}C NMR (125 MHz; DMSO-d_6): δ 172.2 (C), 163.7 (C), 150.9 (C), 146.3 (CH), 100.3 (CH), 58.1 (CH_2), 50.5 (CH_2), 36.7 (CH_3), 36.2 (CH_3), 35.1 (CH), 32.9 (CH_2); LRMS (ES+) m/z 256.13 [$(M+H)^+$, 100%].

5.1.4. 1-[2-(*tert*-Butyldiphenylsilyloxyethylamido)-4-hydroxybutyl]uracil (2c)

Lactone **1** (250 mg, 1.19 mmol) was dissolved in anhydrous ethanol (6.2 ml) and 2-(*tert*-butyldiphenylsilanyloxy)-ethylamine (3.558 g, 11.9 mmol) was added. The reaction was stirred for

4 days at room temperature and then heated at 100 °C for 30 min under microwave irradiation. Ethanol was removed under vacuum, the resulting residue was dissolved in acetonitrile (10 ml) and PL-MIA (5 g, 2.58 mmol/g, 12.9 mmol) was added. The mixture was gently stirred for 18 h and then filtered to remove the scavenger. The compound was further purified by column chromatography on a Redisep 12 g disposable flash column using CH_2Cl_2/MeOH 40:1 as eluent. After removing solvents the title compound was obtained as a white solid (210 mg, 35%). Purity by LC–MS (UV chromatogram, 190–450 nm): 96%; R_f = 0.4 (10% MeOH in CH_2Cl_2); ^{1}H NMR (500 MHz; $CDCl_3$): δ 7.65–7.62 (m, 4H, H-Ar), 7.54 (br s, 1H, NH), 7.41–7.34 (m, 6H, H-Ar), 7.19 (d, J = 7.7 Hz, 1H, C*H*N), 5.43–5.42 (m, 1H, C*H*CO), 3.97 (dd, 1H, J = 13.3 Hz, J = 3.7 Hz, CHC*H*HN), 3.78–3.55 (m, 6H, CHC*H*HN, SiO-CH_2–CH_2, CH_2CHCH_2, CH_2OH), 3.27–3.17 (m, 2H, CH_2CH_2N), 1.84–1.79 (m, 1H, CH_2CHHCH), 1.74–1.69 (m, 1H, CH_2CHHCH), 1.02 (s, 9H, CH_3); ^{13}C NMR (125 MHz; $CDCl_3$): δ 172.9 (C), 151.1 (C), 146.0 (CH), 135.6 (C), 133.3 (C), 129.8 (C), 127.7 (C), 101.5 (CH), 62.6 (CH_2), 59.6 (CH_2), 51.6 (CH_2), 41.9 (CH), 41.8 (CH_2), 32.8 (CH_2), 26.9 (CH_3), 19.2 (C); LRMS (ES+) m/z 510.25 [(M+H)$^+$, 100%].

5.1.5. 1-[2-(*N*,*N*-dimethylaminoethylamido)-4-hydroxybutyl)uracil (2e)

Lactone **1** (133 mg, 0.63 mmol) was dissolved in anhydrous ethanol (2 ml) and *N*,*N*-dimethylethylenediamine (689 μl, 6.33 mmol) was added. The reaction was heated at 100 °C overnight. Ethanol was removed under vacuum and the compound was purified by column chromatography on a Combiflash Companion using a Redisep 12 g disposable flash column and the following gradient: 1 min hold at 100% CH_2Cl_2, 15 min ramp to 20% MeOH–NH_3 in CH_2Cl_2, 5 min hold at 20% MeOH–NH_3 in CH_2Cl_2. After removing solvents the title compound was obtained as a white solid (86 mg, 46%). Purity by LC–MS (UV chromatogram, 190–450 nm): 99%; R_f = 0.4 (20% MeOH–NH_3 in CH_2Cl_2); ^{1}H NMR (500 MHz; $CDCl_3$): δ 8.31 (br s, 1H, NH), 7.25 (d, 1H, J = 7.9 Hz, C*H*N), 5.67 (d, 1H, J = 7.9 Hz, C*H*CO), 4.00 (dd, 1H, J = 13.4 Hz, J = 4.3 Hz, CHC*H*HN), 3.78–3.68 (m, 2H, CHC*H*HN and CH_2CHHN), 3.64–3.59 (m, 1H, CH_2CHHN), 3.49–3.46 (m, 1H, CH_2CHHO), 3.33–3.39 (m, 1H, CH_2CHHO), 3.13 (dt, 1H, J = 14.9 Hz, J = 5.0 Hz, CH_2CHCH_2), 2.64–2.60 (m, 1H, CH_2CHHNMe_2), 2.50–2.45 (m, 1H, CH_2CHHNMe_2), 2.30 (s, 6H, CH_3), 1.81–1.76 (m, 2H, CH_2CH_2CH); ^{13}C NMR (125 MHz; $CDCl_3$): δ 173.0 (C), 164.0 (C), 153.0 (C), 145.3 (CH), 102.8 (CH), 59.1 (CH_2), 58.7 (CH_2), 51.6 (CH_2), 44.3 (CH), 41.9 (CH_2), 36.5 (CH_3), 33.1 (CH_2); LRMS (ES+) m/z 299.17 [(M+H)$^+$, 100%].

5.1.6. 1-[2-Morpholinamido-4-hydroxybutyl)uracil (2f)

Lactone **1** (150 mg, 0.71 mmol) was dissolved in anhydrous ethanol (2.5 ml) and morpholine (622 μl, 7.14 mmol) was added. The reaction was heated at 90 °C for 48 h. Ethanol was removed under vacuum and the compound was purified by column chromatography on a Combiflash Companion using a Silicyle 12 g disposable flash column and the following gradient: 1 min hold CH_2Cl_2, 15 min ramp to 20% MeOH in CH_2Cl_2, 5 min hold 20% MeOH in CH_2Cl_2. After removing solvents the title compound was obtained as a white solid (59 mg, 28%). Purity by LC–MS (UV chromatogram, 190–450 nm): 95%; R_f = 0.2 (10% MeOH in CH_2Cl_2); ^{1}H NMR (500 MHz; CD_3OD-d_3): δ 7.47 (d, 1H, J = 7.8 Hz, C*H*N), 5.63 (d, 1H, J = 7.8 Hz, C*H*CO), 4.01 (dd, 1H, J = 13.4 Hz, J = 5.4 Hz, CH_2CHHN), 3.79 (dd, 1H, J = 13.4 Hz, J = 9.1 Hz, CH_2CHHN), 3.71–3.48 (m, 11H, CH_2CHCH_2, $CH_2CH_2OCPh_3$, CHCH_2N and CH_2CH_2O), 1.90–1.84 (m, 1H, CH_2CHHCH), 1.74–1.68 (m, 1H, CH_2CHHCH); ^{13}C NMR (75 MHz; CD_3OD-d_3): δ 173.8 (C), 166.7 (C), 152.8 (C), 148.2 (CH), 101.9 (CH), 68.0 (CH_2), 67.9 (CH_2), 60.1 (CH_2), 52.9 (CH_2), 47.7 (CH_2), 43.8 (CH_2), 37.7 (CH), 34.2 (CH_2); LRMS (ES+) m/z 298.14 [(M+H)$^+$, 100%].

5.1.7. 1-[2-(Methoxyethylamido)-4-hydroxybutyl]uracil (2g)

Lactone **1** (100 mg, 0.48 mmol) was dissolved in anhydrous ethanol (2.5 ml) and 2-methoxyethylamine (410 μl, 4.76 mmol) was added. The reaction was heated at 90 °C overnight. Ethanol was removed under vacuum, the resulting residue was dissolved in acetonitrile (15 ml) and PL-MIA (2.76 g, 2.58 mmol/g, 7.12 mmol) was added. The suspension was heated at 55 °C under microwave irradiation for 15 min and then filtered to remove the scavenger. Solvents were removed and the compound was further purified by column chromatography using a Silicyle 12 g disposable flash column and the following gradient: 1 min hold CH_2Cl_2, 15 min ramp to 20% MeOH in CH_2Cl_2, 5 min hold at 20% MeOH in CH_2Cl_2. After removing solvents the title compound was obtained as a white solid (13 mg, 10%). Purity by LC–MS (UV chromatogram, 190–450 nm): 99%; ^{1}H NMR (300 MHz; MeOD-d_3): δ 7.30 (d, 1H, J = 7.9 Hz, C*H*N), 5.49 (d, 1H, J = 7.8 Hz, C*H*CO), 3.90 (dd, 1H, J = 13.5 Hz, J = 4.8 Hz, CH_2CHHN), 3.61 (dd, 1H, J = 13.5 Hz, J = 10.3 Hz, CH_2CHHN), 3.52–3.38 (m, 2H, $CH_2CH_2OCH_3$), 3.30–3.13 (m, 7H, CH_2CH_2OH, CH_2CH_2N, CH_3), 2.82 (qd, 1H, J = 14.5 Hz, J = 4.8 Hz, CH_2CHCH_2), 1.77–1.51 (m, 2H, CH_2CH_2CH); ^{13}C NMR (75 MHz; MeOD-d_3): δ 173.8 (C), 166.8 (C), 152.7 (C), 147.8 (CH), 101.9 (CH), 71.9 (CH_2), 60.3 (CH_2), 58.9 (CH_3), 52.2 (CH_2), 43.8 (CH), 40.3 (CH_2), 34.0 (CH_2); LRMS (ES$^+$) m/z 286.14.14 [(M+H)$^+$, 100%].

5.1.8. 1-(2-Amido-4-trityloxybutyl)uracil (3a)

The alcohol **2a** (186 mg, 0.82 mmol) was dissolved in pyridine (3 ml) and tritylchloride (343 mg, 1.23 mmol) and DMAP (0.3% w/w) were added. The reaction was irradiated in the microwave for 30 min at 100 °C. The solvents were removed under vacuum. The product was purified by column chromatography on a Combiflash Companion using a Redisep 12 g disposable flash column and the following gradient: 3 min hold at 100% CH_2Cl_2, 15 min ramp to 10% MeOH in CH_2Cl_2, 3 min hold at 10% MeOH in CH_2Cl_2. After removing solvents the title compound was obtained as white solid (112 mg, 29%). Purity by LC–MS (UV chromatogram, 190–450 nm) >99%; R_f = 0.9 (20% MeOH in CH_2Cl_2); ^{1}H NMR (500 MHz; $CDCl_3$): δ 10.57 (br s, 1H, N*H*), 7.46–7.21 (m, 15H, *H*-Ar and C*H*N), 6.30 (br s, 1H, N*H*), 6.08 (br s, 1H, N*H*), 5.53 (d, 1H, J = 7.9 Hz, C*H*CO), 3.99 (dd, 1H, J = 13.4 Hz, J = 4.3 Hz, C*H*HN), 3.59 (dd, 1H, J = 13.4 Hz, J = 10.7 Hz, C*H*HN), 3.29–3.25 (m, 1H, C*H*HO), 3.15–3.05 (m, 2H, C*H*HO and CH_2CHCH_2), 1.91–1.84 (m, 1H, CH_2CHHCH), 1.78–1.71 (m, 1H, CH_2CHHCH); ^{13}C NMR (125 MHz; $CDCl_3$): δ 175.6 (C), 164.9 (C), 151.2 (C), 146.4 (CH), 143.9 (C), 128.6 (C), 127.9 (C), 127.1 (C), 101.4 (CH), 87.0 (C), 60.9 (CH_2), 51.4 (CH_2), 42.3 (CH), 30.7 (CH_2); LRMS (ES$^-$) m/z 468.2 [(M−H)$^-$, 100%]; HRMS (ES$^-$) found 468.1948 [M−H]$^-$, $C_{28}H_{26}N_3O_4^+$ requires 468.1929.

5.1.9. 1-(2-Dimethylamido-4-trityloxybutyl)uracil (3b)

Alcohol **2b** (140 mg, 0.55 mmol) was dissolved in pyridine (1.5 ml) and tritylchloride (229 mg, 0.82 mmol) and DMAP (0.3% w/w) were added. The reaction was irradiated in the microwave for 3 × 15 min at 100 °C. The solvents were removed under vacuum. The white solid reaction crude was purified by column chromatography on a Redisep 12 g disposable flash column using CH_2Cl_2/MeOH 40:1 and then CH_2Cl_2/MeOH 20:1 as eluent. After removing solvents the title compound was obtained as a white solid (107 mg, 39%). Purity by LC–MS (UV chromatogram, 190–450 nm) >99%; R_f = 0.5 (10% MeOH in CH_2Cl_2); ^{1}H NMR (500 MHz; DMSO-d_6): δ 11.34 (br s, 1H, N*H*), 7.43 (d, J = 7.9 Hz, 1H, C*H*N), 7.40–7.24 (m, 15H, H-Ar), 5.50 (d, J = 7.9 Hz, 1H, C*H*CO), 3.81 (dd, J = 13.3 Hz, J = 6.4 Hz, 1H, C*H*HN), 3.60 (dd, J = 13.3 Hz, J = 8.1 Hz, 1H, C*H*HN), 3.52–3.50 (m, 1H, CH_2CHCH_2), 3.03–2.99 (m, 1H, C*H*HO), 2.85 (s, 3H, CH_3), 2.82–2.77 (m, 1H, C*H*HO), 2.69 (s, 3H, CH_3), 1.83–1.79 (m, 1H, CH_2CHHCH), 1.67–1.63 (m, 1H, CH_2CHHCH); ^{13}C NMR (125 MHz; $CDCl_3$): δ 172.8 (C), 163.5 (C),

150.6 (C), 146.2 (CH), 143.9 (C), 128.6–127.9 (C), 127.1 (C), 101.2 (CH), 87.0 (C), 61.0 (CH_2), 52.2 (CH_2), 37.51–37.47 (CH_3), 35.8 (CH), 31.1 (CH_2); LRMS (ES+) *m*/*z* 243.0 [$(Ph_3C)^+$, 100%]; 512.2 [$(M+NH_4)^+$, 5%]; HRMS (ES^+) found 515.2652 [$M+NH_4]^+$, $C_{30}H_{35}N_4O_4^+$ requires 515.2653.

5.1.10. 1-[2-(*tert*-Butyldiphenylsilyloxyethylamido)-4-trityloxybutyl]uracil (3c)

Alcohol **2c** (200 mg, 0.39 mmol) was dissolved in pyridine (1.5 ml) and tritylchloride (164 mg, 0.59 mmol) and DMAP (0.3% w/w) were added. The reaction was irradiated in the microwave for 4 × 15 min at 100 °C. The solvents were removed under vacuum. The white solid reaction crude was purified by column chromatography on a Redisep 12 g disposable flash column using CH_2Cl_2/MeOH 40:1 and then CH_2Cl_2/MeOH 20:1 as eluent. After removing solvents the title compound was obtained as a white solid (255 mg, 86%). Purity by LC–MS (UV chromatogram, 190–450 nm): 93%; R_f = 0.5 (10% MeOH in CH_2Cl_2); ^{1}H NMR (500 MHz; $CDCl_3$): δ 9.08 (br s, 1H, NH), 7.50 (d, *J* = 7.4 Hz, 1H, C*H*N), 7.33–7.09 (m, 25H, H-Ar), 5.82 (br s, 1H, NH), 5.38 (d, 1H, *J* = 7.4 Hz, C*H*CO), 3.91 (dd, *J* = 13.9 Hz, *J* = 3.9 Hz, 1H, CHC*H*HN), 3.55–3.50 (m, 1H, CHC*H*HN), 3.43–3.40 (m, 2H, SiO–CH_2–CH_2), 3.23–3.17 (m, 1H, CH_2CHCH_2), 3.16–3.12 (m, 1H, $CH_2CH_2OCPh_3$), 2.98–2.91 (m, 2H, CH_2CH_2N), 2.85–2.79 (m, 1H, $CH_2CH_2OCPh_3$), 1.77–1.67 (m, 2H, CH_2CH_2CH), 0.92 (s, 9H, CH_3); ^{13}C NMR (125 MHz; $CDCl_3$): δ 172.4 (C), 163.7 (C), 150.8 (C), 145.9 (CH), 143.9 (C), 135.5 (C), 133.2 (C), 129.9 (C), 128.5 (C), 127.92 (C), 127.86 (C), 127.1 (C), 101.3 (CH), 86.9 (C), 62.4 (CH_2), 60.8 (CH_2), 51.3 (CH_2), 43.3 (CH), 41.4 (CH_2), 30.9 (CH_2), 26.8 (CH_3), 19.1 (C). LRMS (ES^+) *m*/*z* 243.0 [$(Ph_3C)^+$, 100%].

5.1.11. 1-[2-(Carboethoxyethylamido)-4-trityloxybutyl]uracil (3d)

To β-Alanine ethyl ester hydrochloride (1.18 g, 7.70 mmol) in anhydrous ethanol (20 ml), PL-DIPAM MP-resin (4.4 g, 7.70 mmol) was added and the suspension was gently stirred at room temperature overnight. The basic resin was filtered off and washed with ethanol. The filtrate was concentrated under vacuum to approximately 5 ml and lactone **1** (162 mg, 0.77 mmol) was added. The reaction was heated at 100 °C for 3 days. Ethanol was removed under vacuum, the resulting residue was dissolved in acetonitrile (10 ml) and PL-MIA (3.6 g, 2.58 mmol/g, 9.23 mmol) was added. The mixture was gently stirred for 48 h and then filtered to remove the scavenger. The compound was further purified by column chromatography on the Combiflash Companion using a Redisep 40 g disposable flash column and the following gradient: 5 min CH_2Cl_2, 20 min to 20% MeOH in CH_2Cl_2, 5 min 20% MeOH in CH_2Cl_2. After removing solvents a mixture of alcohol **2d** and β-alanine ethyl ester was obtained (129 mg) that was used without further purification for the next step.

Alcohol **2d** (129 mg, 0.39 mmol) was dissolved in pyridine (1.5 ml) and tritylchloride (164 mg, 0.59 mmol) and DMAP (0.3% w/w) were added. The reaction was irradiated in the microwave for 30 min at 100 °C. Further tritylchloride (100 mg) was added and the reaction was irradiated in the microwave for further 30 min at 100 °C. The solvents were removed under vacuum. The reaction crude was purified by column chromatography on the Combiflash Companion using a Redisep 12 g disposable flash column and the following gradient: 5 min hold CH_2Cl_2, 20 min ramp to 10% MeOH in CH_2Cl_2, 5 min hold at 10% MeOH in CH_2Cl_2, 20 min ramp to 20% MeOH in CH_2Cl_2 and, finally, 5 min hold at 20% MeOH in CH_2Cl_2. After removing solvents the title compound was obtained as a white solid (49 mg, 22%). Purity by LC–MS (UV chromatogram, 190–450 nm) >99%; R_f = 0.4 (10% MeOH in CH_2Cl_2); ^{1}H NMR (500 MHz; $CDCl_3$): δ 9.91 (br s, 1H, NH), 7.42–7.21 (m, 15H, H-Ar), 7.19 (d, 1H, *J* = 7.9 Hz, C*H*N), 7.0 (br s, 1H, NH), 5.55 (d, 1H, *J* = 7.9 Hz, C*H*CO), 4.14–4.03 (m, 2H, CH_3CH_2O), 3.86 (dd, 1H, *J* = 13.4 Hz, *J* = 4.2 Hz, CHC*H*HN), 3.50–3.41 (m, 2H, CHC*H*HN and CH_2CHHN), 3.16–3.01 (m, 2H, CH_2CHHN and CH_2CHHO), 3.05–3.00 (m, 1H, CH_2CHHO), 2.95–2.89 (m, 1H, CH_2CHCH_2), 2.40–2.26 (m, 2H, CH_2CO), 1.92–1.85 (m, 1H, CH_2CHHCH), 1.57–1.56 (m, 1H, CH_2CHHCH), 1.23 (t, 1H, *J* = 7.1 Hz, CH_3); ^{13}C NMR (125 MHz; $CDCl_3$): δ 173.7 (C), 172.5 (C), 164.1 (C), 151.0 (C), 145.8 (CH), 143.9 (C), 128.6 (C), 127.8 (C), 127.0 (C), 101.4 (CH), 86.8 (C), 61.2 (CH_2), 61.0 (CH_2), 51.8 (CH_2), 43.1 (CH), 34.8 (CH_2), 33.8 (CH_2), 30.5 (CH_2), 14,1 (CH_3); LRMS (ES+) *m*/*z* 243.1 [$(Ph_3C)^+$, 100%]; 587.3 [$(M+NH_4)^+$, 1%]; HRMS (ES+) found 587.2865 [$M+NH_4]^+$, $C_{35}H_{40}N_5O_7^+$ requires 587.2864.

5.1.12. 1-[2-(*N*,*N*-Dimethylaminoethylamido)-4-trityloxybutyl]uracil (3e)

Alcohol **2e** (86 mg, 0.29 mmol) was dissolved in pyridine (1 ml) and tritylchloride (120 mg, 0.43 mmol) and DMAP (0.3% w/w) were added. The reaction was irradiated in the microwave for 2 × 30 min at 100 °C. The solvents were removed under vacuum. The reaction crude was purified by column chromatography on a Combiflash Companion using a Redisep 4 g disposable flash column and the following gradient: 1 min hold at 100% CH_2Cl_2, 15 min ramp to 20% MeOH–NH_3 in CH_2Cl_2, 5 min hold at 20% MeOH–NH_3 in CH_2Cl_2. After removing solvents a mixture containing product was further purified by semi preparative HPLC to obtain the title compound as a white solid (33 mg, 21%). Purity by LC–MS (UV chromatogram, 190–450 nm): 99%; R_f = 1.9 (10% MeOH in CH_2Cl_2); ^{1}H NMR (500 MHz; MeOD-d_3): δ 7.44–7.42 (m, 6H, H-Ar), 7.36 (d, 1H, *J* = 7.9 Hz, C*H*N), 7.32–7.22 (m, 9H, H-Ar), 5.59 (d, 1H, *J* = 7.9 Hz, C*H*CO), 3.99 (dd, 1H, *J* = 13.6 Hz, *J* = 4.9 Hz, CHC*H*HN), 3.66 (dd, 1H, *J* = 13.6 Hz, *J* = 10.1 Hz, CHC*H*HN), 3.25 (ddd, 1H, *J* = 13.9 Hz, *J* = 7.7 Hz, *J* = 6.4 Hz, CH_2CHHN), 3.18–3.03 (m, 3H, CH_2CHHN and CH_2CH_2O), 2.91 (dt, 1H, *J* = 9.8 Hz, *J* = 4.7 Hz, CH_2CHCH_2), 2.25–2.22 (m, 2H, $CH_2CH_2NMe_2$), 2.19 (s, 6H, CH_3), 1.97–1.91 (m, 1H, CH_2CHHCH), 1.76–1.69 (m, 1H, CH_2CHHCH); ^{13}C NMR (125 MHz; MeOD-d_3): δ 174.9 (C), 166.8 (C), 152.7 (C), 147.7 (CH)), 145.5 (C), 129.8 (C), 128.5 (C), 128.1 (C), 101.9 (CH), 88.2 (C), 62.6 (CH_2), 59.1 (CH_2), 52.5 (CH_2), 45.5 (CH_3), 44.6 (CH), 38.1 (CH_2), 31.5 (CH_2); LRMS (ES+) *m*/*z* 541.3 [$(M+H)^+$, 100%]; HRMS (ES+): found 541.2814 [$M+H]^+$, $C_{32}H_{37}N_4O_4^+$ requires 541.2809.

5.1.13. 1-(2-Morpholinamido-4-trityloxybutyl)uracil (3f)

Alcohol **2f** (59 mg, 0.20 mmol) was dissolved in pyridine (643 μl) and tritylchloride (83 mg, 0.30 mmol) and DMAP (0.3% w/w) were added. The reaction was irradiated in the microwave for 2 × 30 min at 100 °C. The solvents were removed under vacuum. The reaction crude was purified by column chromatography using a Redisep 12 g disposable flash column with 2.5% MeOH in CH_2Cl_2 followed by 5% MeOH in CH_2Cl_2 as eluent. The product was further purified by semi preparative HPLC to obtain the title compound as a white solid (23 mg, 21%). Purity by LC–MS (UV chromatogram, 190–450 nm): 99%; R_f = 0.8 (10% MeOH in CH_2Cl_2); ^{1}H NMR (500 MHz; $CDCl_3$): δ 9.18 (s, 1H, NH), 7.42–7.24 (m, 16H, C*H*N and H-Ar), 5.63 (d, 1H, *J* = 7.9 Hz, C*H*CO), 4.06 (dd, 1H, *J* = 11.6 Hz, *J* = 3.2 Hz, CH_2CHHN), 3.69–3.61 (m, 3H, CH_2CHHN, CH_2CHCH_2, CH_2CHHO), 3.57–3.53 (m, 1H, CH_2CHHO), 3.48–3.42 (m, 3H, CH_2CH_2O and CHC*H*HN), 3.36–3.19 (m, 4H, CHC*H*HN and $CH_2CHHOCPh_3$), 3.07 (ddd, 1H, *J* = 9.5 Hz, *J* = 7.8 Hz, *J* = 4.6 Hz, $CH_2CHHOCPh_3$), 2.01–1.95 (m, 1H, CH_2CHHCH), 1.79–1.64 (m, 1H, CH_2CHHCH); ^{13}C NMR (125 MHz; $CDCl_3$): δ 171.5 (C), 163.6 (C), 150.8 (C), 146.2 (CH), 143.8 (C), 128.5 (C), 127.9 (C), 127.1 (C), 101.3 (CH), 87.1 (C), 68.8 (CH_2), 67.7 (CH_2), 61.0 (CH_2), 52.3 (CH_2), 46.3 (CH_2), 42.3 (CH_2), 36.7 (CH), 31.2 (CH_2); LRMS (ES+) *m*/*z* 243.2 [$(Ph_3C)^+$, 100%]; 562.4 [$(M+Na)^+$, 6%]; HRMS (ES+): found 562.2312 [$M+Na]^+$, $C_{32}H_{33}N_3O_5Na^+$ requires 562.2312.

5.1.14. 1-[2-(Methoxyethylamido)-4-trityloxybutyl]uracil (3g)

Alcohol **2g** (13 mg, 0.05 mmol) was dissolved in pyridine (500 μl) and tritylchloride (19 mg, 0.07 mmol) and DMAP (0.3% w/w) were added. The reaction was irradiated in the microwave for 2 × 30 min at 100 °C. The solvents were removed under vacuum. The reaction crude purified by preparative HPLC to obtain the title compound as a white solid (4.9 mg, 20%). Purity by LC–MS (UV chromatogram, 190–450 nm): 99%; R_f = 0.6 (10% MeOH in CH_2Cl_2); ^{1}H NMR (500 MHz; $CDCl_3$): δ 8.72 (s, 1H, NH), 7.44–7.23 (m, 16H, C*H*N and H-Ar), 6.59 (t, 1H, *J* = 5.3 Hz, NH), 5.60 (d, 1H, *J* = 7.9 Hz, C*H*CO), 4.01 (dd, 1H, *J* = 13.4 Hz, *J* = 4.2 Hz, CHC*H*HN), 3.58 (dd, 1H, *J* = 13.4 Hz, *J* = 10.9 Hz, CHC*H*HN), 3.49–3.42 (m, 1H, CH_2CHHN), 3.30–3.28 (m, 5H, $CH_2CH_2OCH_3$ and CH_3), 3.24–3.20 (m, 1H, CH_2CHHOCPh$_3$), 3.09–2.93 (m, 3H, CH_2CHCH_2, CH_2CHHOCPh$_3$ and CH_2CHHN), 1.90–1.83 (m, 1H, CH_2CHHCH), 1.74–1.67 (m, 1H, CH_2CHHCH); ^{13}C NMR (125 MHz; $CDCl_3$): δ 172.5 (C), 163.4 (C), 150.8 (C), 146.0 (CH)), 143.9 (C), 128.6 (C), 127.9 (C), 127.0 (C), 101.3 (CH), 86.9 (C), 71.1 (CH_2), 61.0 (CH_2), 58.6 (CH_3), 51.7 (CH_2), 43.3 (CH), 39.1 (CH_2), 30.7 (CH_2); LRMS (ES+) *m*/*z* 243.2 [(Ph$_3$C)$^+$, 100%]; 550.4 [(M+Na)$^+$, 2%]; HRMS (ES+) found 550.2308 [M+Na]$^+$, $C_{31}H_{33}N_3O_5Na^+$ requires 550.2312.

5.1.15. 1-[2-(*N*-Methylpiperazine)amido-4-trityloxybutyl]uracil (3h)

Lactone **1** (150 mg, 0.71 mmol) was dissolved in anhydrous ethanol (2.5 ml) and 1-methyl piperazine (792 μl, 7.14 mmol) was added. The reaction was heated at 90 °C for 48 h. Ethanol was removed under vacuum and the compound was purified by column chromatography on a Combiflash Companion using a Silicyle 12 g disposable flash column and the following gradient: 1 min 100% CH_2Cl_2, 20 min to 20% MeOH–NH_3 in CH_2Cl_2, 5 min 20% MeOH–NH_3 in CH_2Cl_2. After removing solvents a mixture of **2h** and 1-methyl piperazine was obtained (45 mg) that was used without further purification for the next step.

Alcohol **2h** (45 mg, 0.15 mmol) was dissolved in pyridine (0.5 ml) and tritylchloride (61 mg, 0.22 mmol) and DMAP (0.3% w/w) were added. The reaction was irradiated in the microwave for 30 min at 100 °C. Further tritylchloride (61 mg) was added and the reaction was irradiated in the microwave for further 30 min at 100 °C. The solvents were removed under vacuum. The reaction crude was dissolved in acetonitrile (8 ml) and purified by preparative HPLC. The product was further purified by column chromatography on a Combiflash Companion using a Silicyle 4 g disposable flash column and the following gradient: 1 min hold CH_2Cl_2, 20 min ramp to 20% MeOH–NH_3 in CH_2Cl_2, 5 min hold at 20% MeOH–NH_3 in CH_2Cl_2 to obtain the title compound as a white solid (20 mg, 25%). Purity by LC–MS (UV chromatogram, 190–450 nm): 99%; R_f = 0.6 (10% MeOH in CH_2Cl_2); ^{1}H NMR (500 MHz; $CDCl_3$): δ 8.65 (s, 1H, NH), 7.43–7.22 (m, 16H, C*H*N and *H*-Ar), 5.60 (d, 1H, *J* = 7.9 Hz, C*H*CO), 4.09–4.04 (m, 1H, CHC*H*HN), 3.81–3.78 (m, 1H, CH_2CHHN), 3.68–3.62 (m, 1H, CHC*H*HN), 3.54–3.50 (m, 1H, CH_2CHHN), 3.29–3.19 (m, 3H, CH_2CHHOCPh$_3$ and CH_2CHHN), 3.07 (ddd, 1H, *J* = 9.6 Hz, *J* = 7.7 Hz, *J* = 4.8 Hz, CH_2CHHOCPh$_3$), 2.40–2.31 (m, 2H, CH_2CHHN), 2.22 (s, 3H, CH_3), 2.07 (ddd, 1H, *J* = 11.4 Hz, *J* = 8.5 Hz, *J* = 2.9 Hz, CH_2CHHN), 2.00–1.93 (m, 2H, CH_2CHHCH and CH_2CHHN), 1.79–1.72 (m, 1H, CH_2CHHCH); ^{13}C NMR (125 MHz; $CDCl_3$): δ 171.2 (C), 163.3 (C), 150.6 (C), 146.2 (CH), 143.9 (C), 128.6 (C), 127.9 (C), 127.1 (C), 101.3 (CH), 87.1 (C), 61.1 (CH_2), 55.3 (CH_2), 54.8 (CH_2), 52.4 (CH_2), 45.84 (CH), 45.79 (CH_2), 41.9 (CH_2), 36.8 (CH_3), 31.2 (CH_2); LRMS (ES+) *m*/*z* 553.2 [M+H]$^+$; HRMS (ES+) found 553.2816 [M+H]$^+$, $C_{33}H_{37}N_4O_4^+$ requires 553.2809.

5.1.16. 1-[2-(Hydroxyethylamido)-4-trityloxybutyl]uracil (4c)

To a solution of **3c** (196 mg, 0.26 mmol) in anhydrous THF (4.5 ml), TBAF in THF (1 M, 313 μl) was added. The reaction stirred at room temperature overnight. The solvents were removed under vacuum and the reaction crude was purified by column chromatography on a Redisep 12 g disposable flash column using CH_2Cl_2/MeOH 40:1 and then CH_2Cl_2/MeOH 20:1 as eluent. After removing solvents the title compound was obtained as a white solid (80 mg, 60%). Purity by LC–MS (UV chromatogram, 190–450 nm): 94%; R_f = 0.4 (10% MeOH in CH_2Cl_2); ^{1}H NMR (500 MHz; $CDCl_3$): δ 10.30 (br s, 1H, NH), 7.33–7.11 (m, 15H, H-Ar), 7.09 (d, *J* = 7.9 Hz, 1H, C*H*N), 5.46 (d, 1H, *J* = 7.9 Hz, C*H*CO), 3.85–3.82 (m, 2H, CH_2OH), 3.43–3.35 (m, 4H, CH_2CH_2N and $CHCH_2$N), 3.07–3.03 (m, 1H, CH_2CHHOCPh$_3$), 2.98–2.94 (m, 1H, CH_2CHHOCPh$_3$), 2.91–2.85 (m, 1H, CH_2CHCH_2), 2.78–2.74 (m, 1H, NH), 1.86–1.79 (m, 1H, CH_2CHHCH), 1.68 (br s, 1H, OH), 1.52–1.45 (m, 1H, CH_2CHHCH); ^{13}C NMR (125 MHz; $CDCl_3$): δ 173.1 (C), 1.65.0 (C), 151.2 (C), 146.1 (CH), 144.0 (C), 128.6 (C), 127.8 (C), 127.0 (C), 101.4 (CH), 86.8 (C), 61.6 (CH_2), 61.3 (CH_2), 52.1 (CH_2), 43.3 (CH), 41.8 (CH_2), 30.4 (CH_2); LRMS (ES+) *m*/*z* 243.1 [(Ph$_3$C)$^+$, 100%]; 536.3 [(M+Na)$^+$, 24%]; HRMS (ES+) found 536.2164 [M+Na]$^+$; $C_{30}H_{31}N_3O_4Na^+$ requires 536.2156.

5.1.17. 1-[2-(Carboxyethylamido)-4-trityloxybutyl]uracil (4d)

Ethyl ester **3d** (39 mg, 0.07 mmol) was dissolved in methanol/water 4:1 (4 ml) and lithium hydroxide (16 mg, 0.69 mmol) was added. The reaction was stirred at room temperature overnight. The solvents were removed under vacuum and the residue was dissolved in water (10 ml) and washed with ethyl acetate (2 × 10 ml). The aqueous layer was separated and acidified with 1 M HCl to pH 3. The product was extracted with ethyl acetate (2 × 25 ml). After removing solvents the title compound was obtained as a white solid (39 mg, 99%). Purity by LC–MS (UV chromatogram, 190–450 nm): 93%; R_f = baseline (10% MeOH in CH_2Cl_2); ^{1}H NMR (500 MHz; $CDCl_3$): δ 11.07 (br s, 1H, NH), 7.71 (dd, 1H, *J* = 7.7 Hz, *J* = 4.5 Hz, NH), 7.42–7.19 (m, 16H, H-Ar and C*H*N), 5.74 (d, 1H, *J* = 7.8 Hz, C*H*CO), 4.09 (dd, 1H, *J* = 13.0 Hz, *J* = 4.1 Hz, CHC*H*HN), 3.39–3.30 (m, 2H, CHC*H*HN and CH_2CHHN), 3.20–3.14 (m, 1H, CH_2CHCH_2), 3.07 (ddd, 1H, *J* = 9.3 Hz, *J* = 4.6 Hz, *J* = 4.6 Hz, CH_2CHHO), 2.80 (ddd, 1H, *J* = 9.3 Hz, *J* = 9.3 Hz, *J* = 3.2 Hz, CH_2CHHO), 2.59–2.53 (m, 1H, CH_2CHHN), 2.19 (ddd, 1H, *J* = 17.9 Hz, *J* = 12.0 Hz, *J* = 2.3 Hz, C*H*HCO), 1.94–189 (m, 1H, C*H*HCO), 1.82–1.75 (m. 1H, CH_2CHHCH), 1.64 (dddd, 1H, *J* = 8.6 Hz, *J* = 8.6 Hz, *J* = 8.5 Hz, *J* = 4.0 Hz, CH_2CHHCH); ^{13}C NMR (125 MHz; $CDCl_3$): δ 174.6 (C), 172.9 (C), 165.9 (C), 152.3 (C), 146.3 (CH), 144.0 (C), 128.6 (C), 127.8 (C), 126.9 (C), 102.4 (CH), 86.5 (C), 60.4 (CH_2), 52.7 (CH_2), 42.4 (CH), 34.2 (CH_2), 33.4 (CH_2), 30.3 (CH_2); LRMS (ES+): *m*/*z* 243.2 [(Ph$_3$C)$^+$, 100%]; 559.4 [(M+NH$_4$)$^+$, <1%]; 564.4 [(M+Na)$^+$, <1%]; HRMS (ES+): found 559.2544 [M+NH$_4$]$^+$; $C_{32}H_{36}N_5O_7^+$ requires 559.2551.

5.1.18. 1-[2-(Carboxy)-4-(*tert*-butyldiphenylsilyloxy)butyl] uracil (6)

The TBDPS protected intermediate **5** (1.5 g, 3.31 mmol), BAIB (2.4 g, 7.29 mmol) and TEMPO (104 mg, 0.66 mmol) were dissolved in ACN/H_2O 1:1 (10 ml). The yellow solution was stirred at room temperature for 3 h. The solvent was removed under reduced pressure and the title compound was triturated from diethyl ether as a white solid (1.31 g, 85%). R_f = baseline (10% MeOH in CH_2Cl_2); mp = 65–66 °C; ^{1}H NMR (500 MHz; CD_3CN) δ 7.76 (d, 1H, *J* = 7.7 Hz, C*H*N), 7.43–7.68 (m, 10H, H-Ar), 5.58 (d, 1H, *J* = 7.7 Hz, C*H*CO), 3.94–3.85 (m, 2H, CH_2CH_2OSi), 3.74 (m, 2H, $CHCH_2$N), 3.06 (m, 1H, CH_2CHCH_2), 1.88–1.75 (m, 2H, CH_2CH_2CH), 1.01 (s, 9H, CH_3); ^{13}C NMR (125 MHz; CD_3CN) δ 176.6 (C), 164.2 C), 150.8 (C), 146.1 (CH), 133.0 (C), 135.0 (C), 129.5 (C), 127.5 (C), 100.4 (CH), 61.0 (CH_2), 49.8 (CH_2), 40.8 (CH), 31.7 (CH_2), 25.8 (CH_3), 18.2 (C); LRMS (ES$^+$) *m*/*z* 467.0 [M+H]$^+$; HRMS (ES$^-$) found 465.1834 [M−H]$^-$, $C_{25}H_{28}N_2O_5Si^+$ requires 465.1840.

5.1.19. 1-[2-(Acetamide)-4-(*tert*-butyldiphenylsilyloxy)butyl] uracil (7a)

Compound **6** (300 mg, 0.64 mmol), NH_4Cl (42 mg, 0.77 mmol) and DhbtOH (121 mg, 0.74 mmol) were dissolved in DMF (5 ml) under argon at 0 °C. NEt_3 (96 mg, 0.96 mmol) was added, followed by DCC (159 mg, 0.77 mmol) in DMF (2 ml). The reaction was stirred at 0 °C for 1 h and then at room temperature overnight. A precipitate which was identified by ^1H NMR as DCU was filtered off. Following removal of solvent, further DCU side product was removed by precipitation from acetone. The crude was purified by column chromatography on a Flasmaster II using an Isolute propylene disposable flash column and the following gradient: EtOAc/hexane 0:100→50:50 and then, $CHCl_3$/MeOH 100:0→90:10 to yield the title compound as a yellow solid (240 mg, 80%). R_f = 0.4 (10% MeOH in CH_2Cl_2); mp 90–93 °C; ^1H NMR (500 MHz; $(CD_3)_2CO$) δ 11.30 (s, 1H, N*H*), 7.64–7.44 (m, 11H, H-Ar and C*H*N), 6.89 (s, 1H, NH_2), 6.34 (s, 1H, NH_2), 5.52 (d, 1H, J = 7.7 Hz, C*H*CO), 3.64–3.85 (m, 4H, CH_2CH_2OSi and $CHCH_2N$), 2.78 (m, 1H, CH_2CHCH_2), 1.61–1.79 (m, 2H, CH_2CH_2CH), 1.01 (s, 9H, $(CH_3)_3C$); ^{13}C NMR (125 MHz; $(CD_3)_2CO$) δ 175.7 (C), 164.1 (C), 151.8 (C), 147.0 (CH), 136.2 (C), 134.4 (C), 130.6 (C), 128.8 (C), 101.5 (CH), 62.6 (CH_2), 51.8 (CH_2), 42.0 (CH), 34.1 (CH_2), 27.5 (CH_3), 19.7 (C); LRMS (ES^+) m/z 466.0 $[M+H]^+$; HRMS (ES^+) found 488.1970 $[M+Na]^+$, $C_{25}H_{30}N_3O_4Si^+$ required 488.1976.

5.1.20. 1-[2-(Methylamido)-4-(*tert*-butyldiphenylsilyloxy)butyl] uracil (7b)

A solution of compound **6** (200 mg, 0.43 mmol) and EDC (89 mg, 0.46 mmol) in DMF (1 ml) was added to a stirred solution of methyl amine hydrochloride (24 mg, 0.36 mmol) and Et_3N (43 mg, 0.43 mmol). The reaction was stirred at room temperature overnight. The crude was purified by flash chromatography on a Flasmaster II using an Isolute propylene disposable flash column and the following gradient: EtOAc/hexane 0:100→50:50 and then $CHCl_3$/MeOH 100:0→90:10 to yield the title compound as a clear wax (12 mg, 7%). R_f = 0.4 (5% MeOH in CH_2Cl_2); ^1H NMR (500 MHz; $CDCl_3$) δ 8.87 (s, 1H, NH), 7.57–7.19 (m, 11H, H-Ar and C*H*N), 5.67 (s, 1H, NH), 5.50 (d, 1H, J = 7.8 Hz, C*H*CO), 3.95–3.55 (m, 4H, CH_2CH_2OSi and $CHCH_2N$), 2.91 (m, 1H, CH_2CHCH_2), 2.55 (s, 3H, CH_3N), 1.70–1.66 (m, 2H, CH_2CH_2CH), 1.00 (s, 9H, $(CH_3)_3C$); ^{13}C NMR (125 MHz; $CDCl_3$) δ 172.0 (C), 162.7 (C), 149.7 (C), 145.6 (CH), 134.5 (C), 132.2 (C), 128.9 (C), 126.8 (C), 100.3 (CH), 60.2 (CH_2), 50.5 (CH_2), 41.4 (CH), 32.0 (CH_2), 25.9 (CH_3), 25.3 (CH_3), 18.1 (C); LRMS (ES^+) m/z 480.1 $[M+H]^+$; HRMS (ES^+) found 502.2139 $[M+Na]^+$, $C_{26}H_{32}N_3O_4SiNa^+$ requires 502.2133.

5.1.21. 1-[2-(Ethylamido)-4-(*tert*-butyldiphenylsilyloxy) butyl] uracil (7c)

Compound **6** (200 mg, 0.43 mmol), ethyl amine hydrochloride (42 mg, 0.52 mmol), DhbtOH (81 mg, 0.49 mmol) were dissolved in DMF (3 ml) under argon at 0 °C. NEt_3 (65 mg, 0.64 mmol) was added, followed by DCC (106 mg, 0.52 mmol) in DMF (0.5 ml). The reaction was stirred at 0 °C for 1 h and then at room temperature overnight. The DCU precipitate was removed by filtration. The crude mixture was purified by flash chromatography on a Flasmaster II using an Isolute propylene disposable flash column and the following gradient: EtOAc/hexane 0:100→50:50 and then $CHCl_3$/MeOH 100:0→90:10 to yield the title compound as a yellow wax (34 mg, 16%). R_f = 0.6 (10% MeOH in CH_2Cl_2); ^1H NMR (500 MHz; $(CD_3)_2CO$) δ 9.90 (s, 1H, NH), 7.58–7.21 (m, 12H, H-Ar, C*H*N and NH), 5.35 (d, 1H, J = 7.9 Hz, C*H*CO), 3.90–3.51 (m, 4H, CH_2CH_2OSi and $CHCH_2N$), 3.08–2.94 (m, 2H, CH_3CH_2NH); 2.80 (m, 1H, CH_2CHCH_2), 1.80–1.57 (m, 2H, CH_2CH_2CH), 0.90 (s, 9H, $(CH_3)_3C$), 0.86 (t, 3H, J = 7.3 Hz, CH_3CH_2); ^{13}C NMR (125 MHz; $(CD_3)_2CO$) δ 173.1 (C), 164.6 (C), 152.0 (C), 146.6 (CH), 136.3 (C), 134.5 (C), 130.7 (C), 128.8 (C), 101.0 (CH), 61.8 (CH_2), 51.4 (CH_2), 43.7 (CH), 34.8 (CH_2), 33.7 (CH_2), 27.1 (CH_3), 19.4 (C), 15.2 (CH_3); LRMS (ES^+) m/z 494.2 $[M+H]^+$; HRMS (ES^+) found 494.2482 $[M+H]^+$, $C_{27}H_{32}N_3O_4Si^+$ requires 494.2470.

5.1.22. 1-[2-(Diethylamido)-4-(*tert*-butyldiphenylsilyloxy) butyl] uracil (7d)

Compound **6** (200 mg, 0.43 mmol), diethyl amine hydrochloride (56 mg, 0.52 mmol), DhbtOH (81 mg, 0.49 mmol) were dissolved in DMF (3 ml) under argon at 0 °C. NEt_3 (65 mg, 0.64 mmol) was added, followed by DCC (106 mg, 0.52 mmol) in DMF (0.5 ml). The reaction was stirred at 0 °C for 1 h and then at room temperature overnight. The DCU precipitate was removed by filtration. The crude mixture was purified by flash chromatography on a Flasmaster II using an Isolute propylene disposable flash column and the following gradient: EtOAc/hexane 0:100→50:50 and then $CHCl_3$/MeOH 100:0→90:10 to yield the title compound as a yellow wax (85 mg, 38%). R_f = 0.5 (5% MeOH in CH_2Cl_2); ^1H NMR (500 MHz; $CDCl_3$) δ 9.06 (s, 1H, NH), 7.61–7.19 (m, 11H, H-Ar and C*H*N), 5.50 (d, 1H, J = 7.9 Hz, C*H*CO), 4.03–3.16 (m, 8H, CH_2CH_2OSi, $CHCH_2N$, CH_3CH_2N), 3.06 (m, 1H, CH_2CHCH_2), 1.76–1.60 (m, 2H, CH_2CH_2CH), 1.00–0.94 (m, 15H, $(CH_3)_3C$ and CH_3CH_2); ^{13}C NMR (125 MHz; $CDCl_3$) δ 172.6 (C), 163.9 (C), 150.6 (C), 146.6 (CH), 135.9 (C), 135.5 (C), 130.3 (C), 128.4 (C), 101.3 (CH), 60.8 (CH_2), 51.9 (CH_2), 42.3 (CH_2), 41.2 (CH_2), 37.2 (CH), 34.1 (CH_2), 26.9 (CH_3), 19.2 (C), 14.9 (CH_3), 13.3 (CH_3); LRMS (ES^+) m/z 522.2 $[M+H]^+$; HRMS (ES^+) found 522.2795$[M+H]^+$, $C_{29}H_{40}N_3O_4Si^+C_{29}$-$H_{40}N_3O_4Si^+$ requires 522.2783.

5.1.23. 1-[2-(*N-Z*-Piperazinamido)-4-(*tert*-butyldiphenyl silyloxy) butyl] uracil (7e)

Compound **6** (200 mg, 0.43 mmol), *N*-Cbz-piperazine (113 mg, 0.52 mmol), DhbtOH (81 mg, 0.49 mmol) were dissolved in DMF (3 ml) under argon at 0 °C. DCC (106 mg, 0.52 mmol) in DMF (0.5 ml) was added. The reaction was stirred at 0 °C for 1 h and then at room temperature overnight. The DCU precipitate was removed by filtration. The crude mixture was purified by flash chromatography on a Flasmaster II using an Isolute propylene disposable flash column and the following gradient: EtOAc/hexane 0:100→50:50 and then $CHCl_3$/MeOH 100:0→90:10 to yield the title compound as a yellow wax (233 mg, 81%). R_f = 0.2 (5% MeOH in CH_2Cl_2); ^1H NMR (500 MHz; $CDCl_3$) δ 9.82 (s, 1H, NH), 7.66–7.29 (m, 16H, H-Ar and C*H*N), 5.60 (d, 1H, J = 7.7 Hz, C*H*CO), 3.77–3.25 (m, 13H, CH_2CH_2OSi, $CHCH_2N$, CH_2CHCH, 4 x CH_2CH_2N), 1.85–1.67 (m, 2H, CH_2CH_2CH), 1.04–0.94 (s, 9H, $(CH_3)_3C$); ^{13}C NMR (125 MHz; $CDCl_3$) δ 171.2 (C), 164.1 (C), 154.0 (C), 150.8 (C), 146.2 (CH), 136.3 (C), 135.5 (C), 133.0 (C), 130.3 (C), 128.3 (C), 128.1 (C), 127.8 (C), 101.3 (CH), 68.1 (CH_2), 61.5 (CH_2), 51.8 (CH_2), 45.4 (CH_2), 41.8 (CH_2), 36.6 (CH), 33.4 (CH_2), 26.9 (CH_3), 19.1 (C); LRMS (ES^+) m/z 669.3 $[M+H]^+$; HRMS (ES^+) found 669.3128 $[M+H]^+$, $C_{37}H_{45}N_4O_6Si^+$ requires 669.3157.

5.1.24. 1-[2-(Carbobenzoxymethyl)-4-aminobutyl] uracil (9)

Intermediate **8** (3 g, 8.04 mmol) was dissolved in pyridine (40 ml) under argon and triphenylphosphine (3.4 g, 12.87 mmol) was added. The solution was stirred at room temperature for 2.5 h and then, NH_4OH conc. (117 ml) was added. The solution was stirred at 50 °C for 2 days. The solvent was removed under reduced pressure and reaction crude was dissolved in $CHCl_3$ (30 ml). A precipitate formed which was identified by ^1H NMR as the phosphoimine intermediate. The filtrate was purified by flash chromatography on a Flasmaster II using an Isolute propylene disposable flash column and the following gradient NH_3/MeOH/$CHCl_3$ 0:0:100→1.2:6:94 to afford the title compound as a white solid (1.02 g, 27%). R_f = 0.4 (10% MeOH in CH_2Cl_2); ^1H NMR (500 MHz; pyridine) δ 13.28 (s, 1H, NH), 8.07 (s, 1H, NH), 7.53–7.11 (m, 6H, H-Ar and C*H*N), 5.70 (d, 1H, J = 7.7 Hz, C*H*CO), 5.24 (m. 2H,

PhCH_2O), 3.95–3.49 (m, 6H, $CH_2CH_2NH_2$, CHCH_2N, OCH_2CH), 2.27 (m, 1H, CH_2CHCH_2), 1.87–1.73 (m, 2H, CH_2CH_2CH); ^{13}C NMR (125 MHz; pyridine) δ 165.3 (C), 157.6 (C), 153.1 (C), 146.6 (CH), 138.7 (C), 129.4 (C), 128.6 (C), 102.0 (CH), 66.6 (CH_2), 61.5 (CH_2), 49.9 (CH_2), 39.7 (CH_2), 39.0 (CH), 30.4 (CH_2); LRMS (ES^+) m/z 348.1 $[M+H]^+$; HRMS (ES^+) found 370.1357 $[M+Na]^+$, $C_{37}H_{45}N_4O_6Si^+$ requires 370.1373.

5.1.25. 1-[2-(Carbobenzoxymethyl)-4-(triphenylmethyl)amino butyl] uracil (10)

Amine **9** (1 g, 2.88 mmol) and trityl chloride (1.2 g, 4.32 mmol) were dissolved in pyridine (12 ml). The solution was divided into three equal batches in 5 ml reaction vials and DMAP (1 mg, 0.3%w/w) was added to each batch. The solutions were exposed to microwave radiation for 15 min at 160 °C twice. The crude was purified by flash chromatography on a Flashmaster II using an Isolute propylene disposable flash column and the following gradient EtOAc/hexane 50:50→100:0, then MeOH/$CHCl_3$ 0:100→8:92 to yield the title compound as a sticky gum (1.69 g, >99%) R_f = 0.5 (EtOAc/hexane 50:50); ^{1}H NMR (500 MHz; CD_2Cl_2) δ 9.47 (s, 1H, NH), 7.32–7.44 (m, 21H, H-Ar and C*H*N), 6.75 (m, 1H, NH), 5.30 (d, 1H, J = 7.7 Hz, C*H*CO), 5.2410 (m. 2H, PhCH_2O), 3.89–3.72 (m, 2H, OCH_2CH), 3.27–3.11 (m, 4H, CH_2CH_2NH, CHCH_2N), 2.06 (m, 1H, CH_2CHCH_2), 1.71–1.59 (m, 2H, CH_2CH_2CH); ^{13}C NMR (125 MHz; CD_2Cl_2) δ 163.9 (C), 156.7 (C), 151.7 (C), 145.6 (CH), 144.0 (C), 137.5 (C), 127.7 (C), 129.0 (C), 101.7 (CH), 87.1 (C), 66.7 (CH_2), 62.7 (CH_2), 49.4 (CH_2), 39.0 (CH_2), 37.0 (CH), 30.1 (CH_2); LRMS (ES^+) m/z 612.3 $[M+H]^+$; HRMS (ES^+) found 612.2475 $[M+Na]^+$, $C_{36}H_{35}N_3O_5Na^+$ requires 612.2469.

5.1.26. 1-[2-(Hydroxymethyl)-4-(triphenylmethyl)aminobutyl] uracil (11)

Five percentage of Pd/C (28 mg, 0.26 mmol) was preactivated with hydrogen in a round bottomed flask using a hydrogen balloon. Carbonate **10** (1.5 g, 2.55 mmol) dissolved in 1:1 mixture of MeOH/EtOH was added. The mixture was stirred under hydrogen until the disappearance of the starting material was observed by TLC. The catalyst was removed by filtration and the crude was purified by flash chromatography on a Flasmaster II using an Isolute propylene disposable flash column and the following gradient EtOAc/$CHCl_3$ 100:0→50:50, then MeOH/$CHCl_3$ 0:100→10:90 to give the title compound as a sticky gum (616 mg, 53%). R_f = 0.4 (5% MeOH in CH_2Cl_2); ^{1}H NMR (500 MHz; DMSO-d_6) δ 7.35–7.26 (m, 18H, H-Ar, C*H*N and 2xN*H*), 5.42 (d, 1H, J = 7.8 Hz, C*H*CO), 3.76–3.69 (m, 2H, CH_2OH), 2.94–3.02 (m, 4H, CH_2CH_2NH, CH_2CH_2N), 2.14 (m, 1H, CH_2CHCH_2), 1.67–1.57 (m, 2H, CH_2CH_2CH); ^{13}C NMR (125 MHz; DMSO-d_6) δ 163.4 (C), 150.9 (C), 145.8 (CH), 143.6 (C), 128.2 (C), 127.8 (C), 126.9 (C), 100.8 (CH), 85.9 (C), 78.6 (CH_2), 64.2 (CH_2), 47.7 (CH_2), 36.4 (CH), 30.1 (CH_2); LRMS (ES^+) m/z 456.2 $[M+H]^+$; HRMS (ES^+) found 456.2277$[M+H]^+$, $C_{28}H_{30}N_3O_3^+$ requires 456.2282.

5.1.27. 1-[2-(Carboxy)-4-(triphenylmethyl)aminobutyl] uracil (12)

Alcohol **11** (600 mg, 1.30 mmol), BAIB (921 mg, 2.86 mmol) and TEMPO (41 mg, 0.26 mmol) were stirred in a mixture of acetonitrile/water 1:1 overnight at room temperature. The crude was purified by flash chromatography on a Flasmaster II using an Isolute propylene disposable flash column and 7% MeOH in $CHCl_3$ to yield the title compound as a white solid (350 mg, 57%). R_f = 0.4 (10% MeOH in CH_2Cl_2); mp 71–75 °C; ^{1}H NMR (500 MHz; $CDCl_3$) δ 9.39 (br, 1H, CO_2H), 7.31–7.14 (m, 17H, H-Ar, C*H*N and N*H*), 6.65 (s, 1H, N*H*); 5.32 (d, 1H, J = 7.7 Hz, C*H*CO), 3.80–3.14 (m, 4H, CH_2CH_2NH, CH_2CH_2N), 2.34 (m, 1H, CH_2CHCH_2), 1.24 (m, 2H, CH_2CH_2CH); ^{13}C NMR (125 MHz; DMSO-d_6) δ 172.1 (C), 163.5 (C), 151.7 (C), 145.5 (CH), 143.8 (C), 128.5 (C), 127.2 (C), 100.6 (CH), 86.3 (C), 62.7 (CH_2), 47.9 (CH_2), 35.1 (CH), 30.8 (CH_2); LRMS (ES^-) m/z 469.2 $[M-H^+]$.

5.1.28. 1-[2-(Acetamide)-4-(triphenylmethyl)aminobutyl] uracil (13)

Carboxylic acid **12** (50 mg, 0.11 mmol), NH_4Cl (7 mg, 0.13 mmol) and DhbtOH (20 mg, 0.12 mmol) were dissolved in DMF (5 ml) under argon at 0 °C. NEt_3 (16 mg, 0.16 mmol) was added, followed by DCC (26 mg, 0.13 mmol) in DMF (3 ml). The reaction was stirred at 0 °C for 1 h and then at room temperature overnight. A precipitate which was identified by ^{1}H NMR as DCU was filtered off. Following removal of solvent, further DCU side product was removed by precipitation from acetone. The crude was purified by column chromatography on a Flasmaster II using an Isolute propylene disposable flash column and the following gradient: EtOAc/hexane 50:50→100:0 and then, MeOH/$CHCl_3$ 0:100→10:90 to yield the title compound as a yellow solid (5 mg, 10%). R_f = 0.8 (10% MeOH in CH_2Cl_2); ^{1}H NMR (500 MHz; $(CD_3)_2CO$) δ 7.46–7.24 (m, 17H, H-Ar, C*H*N and NH), 6.89 (s, 1H, NH_2); 6.25 (s, 1H, NH_2); 5.37 (d, 1H, J = 7.7 Hz, C*H*CO), 3.87–3.14 (m, 4H, CH_2CH_2NH, CH_2CH_2N), 2.61 (m, 1H, CH_2CHCH_2), 2.43–2.37 (m, 2H, CH_2CH_2CH); ^{13}C NMR (125 MHz; $(CD_3)_2CO$) δ 173.3 (C), 164.2 (C), 151.8 (C), 146.2 (CH), 144.8 (C), 129.5 (C), 127.5 (C), 102.0 (CH), 87.5 (C), 63.8 (CH_2), 50.0 (CH_2), 40.0 (CH), 35.8 (CH_2).

5.2. Biological assays. Enzyme purification and inhibition assays

5.2.1. In vitro assays

Both recombinant *P. falciparum* and human dUTPases were expressed in *Escherichia coli* BL21 (DE3) cells which had been transformed with the pET11Pfdut and pET3Hudut (kindly provided by P.O. Nyman, Lund University, Sweden) expression vectors, respectively. For dUTPase purification, the same procedure was used for both the human and the *Plasmodium* enzymes. Cell pellets from a 2.8 L IPTG-induced culture were resuspended in 70 ml of buffer A (20 mM MES, 50 mM NaCl, 1 mM DTT, pH 5.5) containing protease inhibitor cocktail. The cells were lysed by sonication, and the cell extract was cleared by centrifugation at 14,000 rpm for 30 min. The supernatant was loaded onto a 40 ml phosphocellulose (Whatman P-11) column at 4 °C and was eluted with a 50 mM to 1 M NaCl gradient in buffer A. Protein was further purified by means of gel filtration chromatography on a Superdex 200 HA 10/30 column at 4 °C. Pooled fractions are concentrated by centrifugation at 4 °C and desalted using a PD-10 column. The enzyme was eluted with 10 mM bicine and 5 mM $MgCl_2$, pH 8 and stored at −80 °C. Purified fractions contained dUTPase of $\geqslant$96% purity.

Nucleotide hydrolysis was monitored by mixing 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 previously.[19] Protons, released through the hydrolysis of nucleotides, were neutralized by a pH indicator in weak buffered medium with similar pK_a and 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 (M), and the absorbance changes were kept within 0.1 U. The indicator/buffer pair used was red cresol/bicine (pH 8, 573 nm). Assay mixes contained 30 nM of *Pf*dUTPase, 50 μM dUTP, 5 mM $MgCl_2$, 1 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 1) were obtained according to Eq. 1.

$$K_{Mapp} = K_M/K_i[I] + K_M$$

5.2.1.1. *Plasmodium falciparum*. Activity against *P. falciparum* was determined as reported previously. Toxicity against L6 cells was also determined.[11]

5.3. Protein crystallization and X-ray data collection

The pET11Pfdut plasmid construct was used as a template for the production of a new plasmid for the overexpression and purification of *Pf*dUTPase for crystallisation trials. DNA encoding full length *Pf*dUTPase (522 bps) was amplified by PCR using the following primers: CCAGGGACCAGCAATGCATTTAAAAATTGTATGTCT GAGT-GATGAGGTC and GAGGAGAAGGCGCGTTAATATTTATTATTC GATGTCGATC-CAAAACCTC. The PCR product was cloned by Ligation Independent Cloning (LIC)[20] into the plasmid vector pET-YSB-LIC3C,[21] appending a His_6-affinity purification tag and a 3C protease cleavage site to the N-terminus of the *Pf*dUTPase peptide sequence. The recombinant plasmid was used to transform the *E. coli* expression strain BL21 (DE3), and *Pf*dUTPase protein overexpressed in auto-induction media ZYM-5052[22] for a period of 20 h at 20 °C. Pelleted cells were resuspended in buffer A (50 mM Tris–HCl pH 8.5, 500 mM NaCl, 20 m M imidazole) containing protease inhibitor (Pefabloc SC, Roche Diagnostics GmbH) to a final concentration of 1 mM. Following cell disruption by sonication, cleared lysate was loaded onto a 5 ml HiTrap Chelating HP affinity column (Amersham Biosciences), washed with buffer A containing 70 mM imidazole and the protein eluted with buffer A containing 500 mM imidazole directly onto a HiLoad 16/60 Superdex 75 prep-grade gel filtration column (Amersham), pre-equilibrated with a buffer containing 50 mM Tris–HCl pH 8.5 and 500 mM NaCl. Purified protein was concentrated to approximately 30 mg/ml and stored at −80 °C.

Prior to crystallisation trials, the N-terminal His_6-tag was removed from the protein according to manufacturer's instructions using 3C protease in a 1:50 weight ratio in the presence of 10 mM dithiothreitol. The **4c** ligand was dissolved in minimal volume of DMSO and mixed with the protein solution prior to crystallisation. This was performed with a Mosquito nanolitre-dispensing robot (sitting drop vapour diffusion) and led to crystals of approximately 200 μM using a protein solution with 10 mg/ml protein in 50 mM Tris–HCl pH 8.5, 50 mM NaCl, 10 mM TCEP and 1.5 mM **4c** inhibitor, and a reservoir solution containing 3.3 M ammonium sulphate, 0.1 M Bis–Tris pH 6.0. X-ray data extending to 2.1 Å resolution were collected from a single crystal at 120 K on station ID23eh2 at the ESRF, Grenoble, France using an ADSC CCD detector. Data were processed using MOSFLM (Table 2). The space group is $P4_1$ with cell dimensions $a = b = 77.16$ Å and $c = 110.980$ Å, with a trimer of the complex in the asymmetric unit. The data statistics are shown in Table 2.

Table 2
X-ray data collection statistics for the **4c** complex

Data collection	
Space group	$P4_1$
Cell dimensions	
a,*b*,*c* (Å)	77.16,77.16,111.98
Resolution (Å)	38.9–2.08 (2.20–2.08)
Total observations	198,174 (28,013)
Unique observations	38,815 (5641)
R_{merge}	0.072 (0.563)
$I/\sigma(I)$	13.2 (2.9)
Completeness (%)	100.0 (100.0)
Multiplicity	5.1 (5.0)
Refinement	
Resolution (Å)	2.1
No. reflections	35,984
R_{work}/R_{free} (%)	20.3/24.4
No. amino acid residues	
No. atoms	3402
Protein	3154
Water	124
4c	114
Sulfate	10
B-factors (Å^2)	37
Protein	37
Water	40
4c	37
Rms deviations	
Bond lengths (Å)	0.015 (0.022)
Bond angles (°)	1.61 (2.02)
Ramachandran plot (%)	
Most favoured	91.7
Additionally allowed	6.5
Generously allowed	0.9
Disallowed	0.9

5.3.1. Structure solution and refinement

The structure was solved by molecular replacement using the known structure of the enzyme reported earlier[10] (PDB code 1vyq) as a search model. The model was refined using REFMAC[23] alternating with rebuilding using COOT,[24] and the final *R* factor and R_{free} were 20.3% and 24.4%, respectively. The coordinates and refinement dictionary for the **4c** ligand were generated using SKETCHER from the CCP4 suite.[25] The refinement statistics are shown in Table 2. The X-ray data and coordinates have been deposited in the PDB with the accessions code 2y8c.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.02.012.

References and notes

1. World Health Organization World Malaria Report 2010; World Health Organization: Geneva, Switzerland, ISBN 978 92 4 156410 6, 2010.
2. Miller, L. H.; Baruch, D. I.; Marsh, K.; Doumbo, O. K. *Nature* **2002**, *415*, 673.
3. World Health Organization Global Report on Antimalarial Efficacy and Drug Resistance: 2000–2010; World Health Organization: Geneva, Switzerland, ISBN 978 92 4 150047 0, 2010.
4. Wells, T. N. C.; Alonso, P. L.; Gutteridge, W. E. *Nat. Rev. Drug Disc.* **2009**, *8*, 879.
5. Tye, B. K.; Lehman, I. R. *J. Biol. Chem.* **1977**, *117*, 293.
6. Gadsden, M. H.; McIntosh, E. M.; Game, J. C.; Wilson, P. J.; Haynes, R. H. *EMBO J.* **1993**, *12*, 4425.
7. el-Hajj, H. H.; Zhang, H.; Weiss, B. *J. Bacteriol.* **1988**, *170*, 1069.
8. Wilson, P. M.; Fazzone, W.; LaBonte, M. L.; Deng, J.; Neamati, N.; Ladner, R. D. *Mol. Cancer Ther.* **2008**, *7*, 3029.
9. Nguyen, C.; Kasinathan, G.; Leal-Cortijo, I.; Musso-Buendia, A.; Kaiser, M.; Brun, R.; Ruiz-Pérez, L. M.; Johansson, N. G.; González-Pacanowska, D.; Gilbert, I. H. *J. Med. Chem.* **2005**, *48*, 5942.
10. Whittingham, J. L.; Leal, I.; Nguyen, C.; Kasinathan, G.; Bell, E.; Jones, A. F.; Berry, C.; Benito, A.; Turkenburg, J. P.; Dodson, E. J.; Ruiz-Pérez, L. M.; Wilkinson, A. J.; Johansson, N. G.; Brun, R.; Gilbert, I. H.; González-Pacanowska, D.; Wilson, K. S. *Structure* **2005**, *13*, 329.
11. Nguyen, C.; Ruda, G. F.; Schipani, A.; Kasinathan, G.; Leal, I.; Musso-Buendia, A.; Kaiser, M.; Brun, R.; Ruiz-Pérez, L. M.; Sahlberg, B.; Johansson, N. G.; González-Pacanowska, D.; Gilbert, I. H. *J. Med. Chem.* **2006**, *49*, 4183.
12. McCarthy, O.; Musso-Buendia, A.; Kaiser, M.; Burn, R.; Ruiz-Pérez, L. M.; Johansson, N. G.; González-Pacanowska, D.; Gilbert, I. H. *Eur. J. Med. Chem.* **2009**, *44*, 678.
13. (a) Goodford, P. J. *J. Med. Chem.* **1985**, *28*, 849; (b) von Itzstein, M.; Wu, W. Y.; Kok, G. B.; Pegg, M. S.; Dyason, J. C.; Jin, B.; Phan, T. V.; Smythe, M. L.; White, H. F.; Oliver, S. W.; Colman, P. M.; Varghese, J. N.; Ryan, D. M.; Woods, J. M.; Bethell, R. C.; Hotham, V. J.; Cameron, J. M.; Penn, C. R. *Nature* **1993**, *363*, 418; (c) Kastenholz, M. A.; Pastor, M.; Cruciani, G.; Haaksma, E. E. J.; Fox, T. *J. Med. Chem.* **2000**, *43*, 3033.
14. Jean, M.; Le Roch, M.; Renault, J.; Uriac, P. *Org. Lett.* **2005**, *7*, 2663.
15. Epp, J. B.; Widlanski, T. S. *J. Org. Chem.* **1999**, *64*, 293.

16. Wenninger, D.; Sliger, H. *Nucleosides Nucleotides* **1997**, *16*, 997.
17. Vanheusden, V.; Munier-Lehmann, H.; Froeyen, M.; Dugue, L.; Heyerick, A.; De Keukeleire, D.; Pochet, S.; Busson, R.; Herdewijn, P. *J. Med. Chem.* **2003**, *46*, 3811.
18. Potterton, L.; McNicholas, S.; Krissinel, E.; Gruber, J.; Cowtan, K.; Emsley, P.; Murshudov, G. N.; Cohen, S.; Perrakis, A.; Noble, M. *Acta Crystallogr., Sect. D. Biol. Crystallogr.* **2004**, *60*, 2288.
19. Hidalgo-Zarco, F.; Camacho, A. G.; Bernier-Villamor, V.; Nord, J.; Ruiz-Perez, L. M.; Gonzalez-Pacanowska, D. *Protein Sci.* **2001**, *10*, 1426.
20. Aslandis, C.; De Jong, P. J. *Nucleic Acids Res.* **1990**, *18*, 6069.
21. (a) Alzari, P. M.; Berglund, H.; Berrow, N. S.; Blagova, E.; Busso, D.; Cambillau, C.; Campanacci, V.; Christodoulou, E.; Eiler, S.; Fogg, M. J.; Folkers, G.; Geerlof, A.; Hart, D.; Haouz, A.; Herman, M. D.; Macieira, S.; Nordlund, P.; Perrakis, A.; Quevillon-Cheruel, S.; Tarandeau, F.; van Tilbeurgh, H.; Unger, T.; Luna-Vargas, M. P.; Velarde, M.; Willmanns, M.; Owens, R. J. *Acta Crystallogr., Sect. D. Biol. Crystallogr.* **2006**, *62*, 1103; (b) Fogg, M. J.; Wilkinson, A. J. *Biochem. Soc. Trans.* **2009**, *36*, 771.
22. Studier, F. W. *Protein Expr. Purif.* **2005**, *41*, 207.
23. Murshudov, G. N.; Vagin, A. A.; Dodson, E. J. *Acta Crystallogr., Sect. D. Biol. Crystallogr.* **1997**, *53*, 240.
24. Emsley, P.; Cowtan, K. *Acta Crystallogr., Sect D. Biol. Crystallogr.* **2004**, *60*, 2126.
25. Collaborative Computational Project Number 4. The CCP4 suite: programs for protein crystallography. *Acta Crystallogr., Sect. D. Biol. Crystallogr.* **1994**, *50*, 760.