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Rational design of 5'-thiourea-substituted α -
thymidine analogues as thymidine monophosphate
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Abbreviations: TMPK: thymidine monophosphate kinase; TMPKh: human TMPK; TMPKmt: Mycobacterium tuberculosis TMPK; TB: tuberculosis; MDR: multi-drug resistant; dTMP: thymidine monophosphate; AIBN: 2,2'-azobisisobutyronitrile.

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Abstract

Recently, thymidine monophosphate kinase (TMPK) emerged as an attractive target for developing inhibitors of *Mycobacterium tuberculosis* growth. The elucidation of the X-ray structure of TMPK of *M. tuberculosis* (TMPKmt), as well as the structure of an earlier serendipitously discovered dimeric thymidine inhibitor, laid the foundation for the design of potent and selective TMPKmt inhibitors reported here.

Several hits identified within a series of 3'-C-branched thiourea-substituted β -thymidine derivatives inspired us to construct a set of 5'-thiourea substituted α -thymidine derivatives, characterized by a similar relative orientation of the thymine and arylthiourea moieties.

α -Thymidine derivative **15**, featuring a (3-trifluoromethyl-4-chlorophenyl)thiourea moiety, has a K_i -value of 0.6 μ M and a selectivity index of 600 versus human TMPK. Moreover it represents the first TMPK inhibitor, showing good inhibitory activity on growing *M. bovis* (MIC₉₉ = 20 μ g/mL) and *M. tuberculosis* (MIC₅₀ = 6.25 μ g/mL) strains.

Keywords: thymidine monophosphate kinase; nucleoside-analogues; Mycobacterium tuberculosis.

Introduction

At the beginning of the 21st century, tuberculosis (TB) remains a major public health issue, killing over 1.6 million people annually. TB is one of the world's oldest infectious diseases and since the 1950's there has been an effective, affordable and accessible cure for this disease. However, TB is resurging as a serious threat, mainly as a result of the synergism with HIV and the emergence of multi-drug resistant strains (MDR-TB).¹

The current treatment of TB requires an exceedingly lengthy therapy of 6-9 months, often involving a cocktail of three or four different drugs.² Beside significant toxicity, the lengthy therapy also brings about poor patient compliance, which is a frequent source for selection of drug-resistant and often deadly MDR-TB bacteria.

Especially HIV infection is a major synergistic risk factor for TB: it increases the chance for reactivation of latent *Mycobacterium tuberculosis* infection,³ but also enhances rapid TB progression soon after (re)infection with TB.⁴ Consequently, TB is one of the most common causes of death in HIV-positive adults living in less-developed countries.

To face this expanding threat, extensive research for new antituberculosis agents has been performed, yet not yielding any new FDA-approved drug since the 1960s. The development of new drugs that shorten the long therapy as well as the discovery of new mycobacterial targets are necessary to eradicate strains being resistant to different types of drugs. An additional challenge in this search is the particularly impermeable nature of the lipid-rich mycobacterial cell wall,⁵ which causes the low uptake of active compounds in the mycobacteria and a very slow growth of the bacillus, resulting in long treatments required for complete sterilization.

However, some interesting compounds are currently being evaluated in clinical trials.⁶ One of the most promising early drug candidates is a diarylquinoline derivative developed by Johnson and Johnson, which is proposed to inhibit F1F0 proton ATP synthase, a new target in mycobacteria.⁷

Another recently discovered target is thymidine monophosphate kinase (TMPKmt), which plays an essential and unique role in the DNA synthesis of this bacillus.⁸ The elucidation of the X-ray structure of human¹⁰ and mycobacterial¹¹ TMPK and their low (22%) sequence identity further promotes TMPKmt as an attractive target for the development of selective inhibitors.

Thymidine is a moderately potent inhibitor of TMPKmt ($K_i \approx 27 \mu\text{M}$). Both the sugar^{12, 13, 14} and the base^{15, 16} moiety of thymidine have been the subject of different modifications to enhance affinity and selectivity for the bacterial enzyme. The most active TMPKmt inhibitors reported so far show K_i -values in the low micromolar range. Only recently we identified a TMPKmt inhibitor, derived from a 2',3'-bicyclic thymidine analogue, that shows some activity against living mycobacteria cultures ($\text{MIC}_{99} \approx 100 \mu\text{g mL}^{-1}$).¹⁷

Lately, our group discovered an unusual dinucleoside **1** that produced significant inhibition of TMPKmt ($K_i = 37 \mu\text{M}$).¹⁴ The second thymidine monomer attached to the 3'-position suggested the possibility to introduce large substituents at this position to fit in the thymidine monophosphate (dTMP)-pocket. Next to this surprising activity, compound **1** did not inhibit human TMPK (TMPKh) at 1 mM, creating opportunity for further explorations of this type of analogues (Figure 1).

We investigated the possibility to replace one thymidine monomer by different substituted phenyl groups. Concurrently, thiocarlide,¹⁸ a drug that formerly proved efficient in treating TB,^{19, 20} inspired us to replace the connecting urea group by a thiourea linker, resulting in 3'-branched thiourea-substituted β -thymidine derivatives.

By carefully selecting a limited set of compounds based on the Topliss' tree,²¹ we aimed to assess the relative importance of the lipophilic, electronic and sterical properties of the aryl substituents.

Modelling studies on the binding mode of dinucleoside **1** to its target suggested that the second monomer binds to the area where normally the phosphoryl donor binds, in this way forcing the sugar ring to tilt over 180° compared to the natural substrate dTMP.¹² Similarly, the envisaged β -thiourea derivatives are expected to bind the dTMP-pocket upside down, positioning the aromatic 3'-substituent into the phosphoryl donor binding area, and the nucleobase below the sugar plane.

As depicted in Figure 2, we hypothesized that the relative orientation of the nucleobase and the arylthiourea moiety in these β -thiourea derivatives might be imitated by 5'-substituted α -thymidine analogues. The fact that 5'-*O*-phosphorylated α -thymidine was accepted by TMPKmt as a substrate (see further) confirmed that α -nucleosides proved able to adopt the proposed binding mode, which incited us to synthesize the readily accessible 5'-deoxy-5'-*N*-arylthiourea α -thymidine derivatives.

After a detailed exploration of the 5'-thiourea pattern, we assessed the importance of the deoxyribofuranose moiety by the synthesis of 2',3'-dideoxy-, 2',3'-dideoxydidehydro- and acyclic nucleoside derivatives.

In an attempt to use adenosine to enhance the uptake in the mycobacterial cell,^{22, 23} a dinucleoside was designed, consisting of a α -thymidine monomer connected to adenosine by a thioureum linker.

Results and discussion

Chemistry

The synthesis of a series of 3'-*C*-branched- β -thiourea-derivatives started from β -D-thymidine. Different methods are described for the synthesis of 3'-*C*-branched nucleosides.^{24, 25} A radical introduction of a β -styrene residue appears to result in the shortest synthetic route (Scheme 1). Compound **34** was obtained following the procedure of Chu et al.²⁶ This compound was treated with β -tributylstannylstyrene and 2,2'-azabisobutyronitrile in benzene to give **35**. A

two-step one-pot reaction involving *cis*-dihydroxylation with osmium tetroxide and 4-methylmorpholine N-oxide as cooxidant, followed by sodium periodate cleavage of the diol, resulted in an unstable aldehyde, which was immediately reduced with sodium borohydride in aqueous ethanol to afford **36**. After mesylation of the primary alcohol, the azido group was introduced using sodium azide in DMF. Hydrogenation of **37** over Pd/C gave amine **38**. This precursor was reacted with different isothiocyanates in a parallel fashion to give target compounds **2-8** after final deprotection of the silyl group.

For the synthesis of α -D-thymidine 5'-*O*-monophosphate (**9**), 3',5'-*O*-diacetyl- β -thymidine was first anomerized to its α -anomer (**46**) according to the procedure of Ward et al.,²⁷ followed by deprotection and conversion to the 5'-monophosphate using classical phosphorylation conditions (Scheme 2).

The synthesis of 5'-substituted α -thymidine derivatives started from α -thymidine (**47**) which was converted to the 5'-deoxy-5'-(thio)urea-derivatives **10-27** as depicted in Scheme 3. Amide **30** (Table 2) was isolated as a side product upon treatment of **49** with benzoylisothiocyanate.

3'-Deoxygenation was performed by converting the 3'-hydroxyl group of **48** to its mesylate ester (Scheme 4). Upon treatment with base, elimination yielded the unsaturated sugar in nucleoside **51**. Hydrogenation over Pd/C gave the 2',3'-dideoxy-analogue **52**, while Staudinger reduction selectively converted the azide, affording the unsaturated amine **53**. Final treatment of these amines with 3-CF₃-4-Cl-isothiocyanate afforded thiourea derivatives **28** and **29**.

Acyclic derivatives were synthesized following a procedure as reported for uridine-derivatives by Danel et al.²⁸ (Scheme 5). The primary alcohol of **54** was converted to different thiourea-compounds as described above.

For the preparation of the adenosine-conjugate **33** (Scheme 6), 5'-amino-2',3'-*O*-isopropylidene adenosine (**57**) was treated with 1,1'-thiocarbonyldiimidazole to generate *in*

situ the isothiocyanate, which was further reacted with 5'-amino- α -thymidine (**49**)²⁹ to afford the envisioned dinucleoside after final deprotection of the acetonide.

Biological evaluation

All compounds have been evaluated for TMPKmt inhibition as described in the experimental section.

For the 3'-*C*-branched- β -thymidine derivatives, analogue **2** with an unsubstituted phenyl ring showed a K_i -value of 69.0 μ M (Table 1). This value indicates a weaker binding to the enzyme than dinucleoside **1** ($K_i = 37 \mu$ M) and the natural substrate ($K_m = 4.5 \mu$ M). However, this result suggests that this analogue can still be accommodated in the active pocket. In order to optimize the potency of this phenyl analogue, Topliss' scheme²¹ was used. This decision tree is based on the concept, pioneered by Hansch,³⁰ that principally the lipophilic, electronic and steric properties of the introduced substituent influence the biological activity. A 4-chloro-substitution of the phenyl ring (**3**) resulted in a 3-fold increased activity. According to Topliss' decision tree, this increase confirms the positive effect of lipophilic and electron withdrawing substituents on the inhibitory activity. This trend was validated by further improvement of the activity with a 3,4-dichloro-substitution pattern (**6**) which further increased activity by another 3-fold ($K_i = 7.2 \mu$ M). On the other hand, a 4-methoxy (**4**) or 4-methyl substituent (**5**) resulted in a decreased activity. To further validate electron withdrawing properties, compound **8** was synthesized, while **7** should indicate if sterically more demanding substituents were also allowed. Compound **8** with a 3-CF₃-4-Cl-substitution pattern was found to be the most potent inhibitor of this series with a K_i -value of 5.0 μ M, indicating that mainly the electronic features of the substituents positively influenced the inhibitory activity.

The good affinity observed amongst these β -thiourea derivatives suggests a binding mode analogous to that described for dinucleoside **1**¹¹ with the arylthiourea moiety occupying the phosphoryl donor binding site.

As illustrated in Figure 2, a newly designed family of 5'-thiourea substituted α -thymidine derivatives, was anticipated to bind in a similar way as the above described β -thiourea compounds. Before moving on to the synthesis of these compounds, α -thymidine monophosphate was synthesized to investigate if the anticipated binding mode of these compounds was practicable. α -Thymidine monophosphate was indeed accepted as a substrate by TMPKmt ($K_m = 450 \mu\text{M}$, $V_m = 0.77 \text{ U/mg}$ at 2 mM ATP), thereby competing with dTMP ($K_i = 15.0 \mu\text{M}$). Compared to the natural substrate however, the binding constant of this anomer was 10 times higher and its maximum velocity (V_m) only 7.5% of that of dTMP. The same broad stereoisomeric substrate specificity has been observed for mitochondrial thymidine kinase (TK2), deoxyguanosine kinase (dGK) and deoxycytosine kinase (dCK), which proved able to recognize β -D and β -L, as well as α -D and α -L nucleosides as a substrate.³¹

In a next step, the 5'-thiourea substituted α -nucleosides were synthesized. Their biological activities are represented in Table 2.

In analogy to our observations for the 3'-C-branched β -thymidine thiourea analogues, lipophilic and electronic withdrawing substituents favour high activity. With a K_i of 0.6 μM , analogue **15** ($R_1 = \text{Cl}$, $R_2 = \text{CF}_3$) proved to be the most potent TMPKmt inhibitor reported so far.

As the corresponding α - and β -thiourea derivatives were designed to bind in a similar fashion, a comparison between the corresponding activities is presented in Figure 3.

The graph shows that the α -derivatives are consistently (~ 4- to 5-fold) more active than the β -congeners, and clearly reveals a similar trend in both series, further supporting our design hypothesis.

To illustrate the binding mode of the two main series of inhibitors, docking of two representative compounds **2** (β) and **10** (α) was performed. This experiment resulted in 50 conformations, mainly containing two different orientations: one with the nucleobase stacking with Phe70 and one with the aromatic tail stacking to Phe70. As opposed to the former, the latter conformations preclude certain amino acids (*e.g.*, Arg74, Asn100) to form H-bonds and were therefore rejected. This rejection is justified as docking showed that the space close to Phe70 is too small to accommodate bulky substituents like a Cl or a CF₃ group. Moreover the docked conformations with the aromatic ring occupying the base site are shifted, reducing the stacking interaction and thus drastically limiting the number of meaningful conformations found.

When comparing corresponding analogues from both series such as **2** and **10** (Figure 4), both the nucleobase and the arylthiourea-moiety occupy the same areas. For the α -derivatives, the pyrimidine ring and Phe70 are arranged in a more parallel fashion compared to the β -derivatives, resulting in a stronger stacking. Moreover compound **10** is more favourably positioned to form an extra H-bond with Asp9 through a nitrogen of its thiourea function, which may contribute to the higher affinity of the α -analogues for TMPKmt.

Further structural modifications elucidated that small structural changes, such as introduction of an alkyl chain of 1 or 2 carbon atoms between the thiourea and phenyl, are well tolerated by TMPKmt, yet not enhancing the affinity for the enzyme.

It is found that the tail of molecules **10** and **24** points to the outside of the enzyme through a channel, in the same orientation as dinucleoside **1**.¹² This exit channel is surrounded by residues Arg14, Ala35, Phe36, Pro37, Tyr39, Arg160 (Figure 5). Adding functional groups in *para*- and *meta*-position on the phenyl ring seems to improve the activity, by increasing hydrophobic contact with these residues. However, since longer chains extend more out of the enzyme into the solvent (Figure 5), this indicates that no further improvement can be obtained by increasing the linker length.

Surprisingly, sterically demanding substituents as in **19** and **21** cause almost no change in inhibitory activity, so most likely a large pocket around the phosphoryl donor area is available for the inhibitor to interact with. The activity of fluoresceine-labelled compound **19** opens interesting perspectives for its use in the enzymatic activity determination or in mycobacterial uptake-studies. The lower activity of compounds **16** and **17** suggests a role for the aromatic moieties to stabilize the enzyme-inhibitor complex.

To check the importance of the hydrophobic sulphur of the thiourea linkage, urea derivatives **26** and **27** were synthesized. As those compounds retain their good affinity for the enzyme, a larger structural freedom for the linkage between the aromatic moiety and the nucleobase seems to be tolerated.

Only a small drop in affinity was observed upon removal of the 3'-hydroxyl (**28**) or upon additional introduction of a double bond between 2' and 3' (**29**). We therefore conclude that the most important feature for TMPKmt inhibition of this series is the relative orientation of the nucleobase and the 5'-substituent, combined with the ability of the 5'-substituent to interact with the enzyme active site pocket.

This led us to synthesize acyclic derivatives **31** and **32**, which exhibited only a very weak affinity for the enzyme. Despite the fact that the number of bonds between the thymine and the aromatic moiety is retained in these structures, the higher entropy changes required to form the complex are likely to be unfavourable for binding. Moreover, when comparing the acyclic derivatives with compound **10**, an important hydrogen bond at the 3'-position is lost, which may additionally cause the lack of activity.

The selectivity of the most potent TMPKmt vis-à-vis the human isozyme was investigated. Most tested compounds proved highly selective for the mycobacterial enzyme. Especially compound **15**, the most active compound of the series, showed a selectivity index of 600, indicating that the inverse binding mode of the sugar is not tolerated by the human enzyme.

Consequently, we assessed the potential of the most promising analogues to restrain mycobacterial growth. Whereas compound **15** proved to be the most potent TMPKmt inhibitor, it also proved superior in a *M. bovis* growth inhibition assay. Selected compounds were also tested for their capability to reduce *M. tuberculosis* H₃₇Rv growth at a concentration of 6.25 µg/mL, showing an inhibition of bacterial growth between 34 and 55% at this concentration.

For the first time, TMPKmt inhibitors proved capable to inhibit the growth of both *M. bovis* and *M. tuberculosis*, confirming that TMPKmt indeed represents a valuable target for designing antituberculosis drugs. Moreover, the compounds were found non-toxic in Vero cell cultures at 100 µM and also devoid of appreciable inhibitory activity against a broad variety of viruses including herpes simplex virus type 1 (KOS) and type 2 (G), vaccinia virus, vesicular stomatitis virus, Sindbis virus, reovirus-1, parainfluenza virus-3, Coxsackie B4, respiratory syncytial virus, Punta Toro virus, feline coronavirus, influenza virus A (H1N1 and H3N1) and influenza virus B. This lack of activity further points to the highly selective activity of several compounds against mycobacteria.

Very recently, a series of 5'-modified adenosine derivatives has been discovered as powerful siderophore biosynthesis inhibitors.^{22, 23} Some of these analogues showed MIC₉₉-values against *M. tuberculosis* H37Rv comparable to that of isoniazid, suggesting the presence of active adenosine-uptake mechanism. For this reason, conjugate **33** was synthesized to exploit this proposed adenosine uptake mechanism to enhance antimycobacterial activity. While still showing moderate TMPKmt inhibitor activity, dinucleoside **33** failed to inhibit mycobacterial growth.

Finally, all compounds tested in Vero cell cultures were devoid of cytotoxicity at 100 µM, while another assay proved **15** to have a minimum cytotoxic concentration of 300-400 µg/mL, more than 10 times the concentration needed to kill *M. bovis*. The urea analogues (**26** and **27**) of the most active compounds (**14** and **15**), which lack the thiourea moiety, earlier described

as the perpetrator of toxicity,³⁸ showed nearly equal inhibitory activity against TMPK_{mt}, while still retaining a sufficient selectivity versus the human enzyme (with SI= 90-105) and interesting inhibitory activity on the growing *M. bovis* and *M. tuberculosis* strains.

Conclusion

Based on the structure of the recently discovered dinucleoside **1** as a selective TMPK_{mt} inhibitor, this paper describes the synthesis and biological evaluation of a series of 3'-C-aryl thiourea derivatives of β -D-thymidine. Optimisation of the aryl thiourea led to analogue **8** that, with a K_i -value of 5.0 μ M, clearly surpassed **1** in inhibitory activity.

Modelling experiments suggested a distinct binding mode for these analogs, as compared to the natural substrate: the sugar moiety binds the active site upside down. The acceptance of α -thymidine-5'-phosphate as a substrate for TMPK_{mt} supported the synthesis of 5'-substituted α -thymidine thiourea derivatives, mainly characterized by a similar relative orientation of the 5'-aryl moiety and the nucleobase.

This led to the discovery of compound **15** as the most promising compound of this series with a K_i -value of 0.6 μ M, a selectivity index (versus TMPK_h) of 600, and a good inhibitory activity on the growing *M. bovis* (MIC₉₉ = 20 μ g/mL) and *M. tuberculosis* (39% inhibition at 6.25 μ g/mL) strains.

Next to the relative orientation between the aryl moiety and the nucleobase, structural exploration of the α -thymidine derivatives, revealed the positive impact of electronic withdrawing and lipophilic substituents on the 5'-aryl moiety and the need for aromatic residues at this 5'-position.

In conclusion, we have designed, synthesized, and evaluated a series of nucleoside inhibitors of *M. tuberculosis* TMPK, which resulted in the identification of 5'-arylthiourea α -thymidine analogues endowed with significant inhibitory activity against *M. tuberculosis* and *M. bovis*

growth and low cytotoxicity. This strategy represents a promising approach for the development of a new class of antibiotics effective for the treatment of TB.

In a broader sense, this study opens interesting perspectives for using sugar-modified α -nucleosides as readily accessible scaffolds for the rational design of biologically important tool compounds acting on other kinase targets or in other areas in chemical biology.

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Experimental section

Spectrophotometric binding assay

The *in vitro* tests were done on TMPK_{mt} and TMPK_h, recombinant enzymes overexpressed in *E. coli*. TMPK_{mt} and TMPK_h activity was determined using the coupled spectrophotometric assay described by Blondin et al.³⁹ using an Eppendorf ECOM 6122 photometer and a wavelength of 334 nm. The reaction medium (0.5 mL final volume) contained 50 mM Tris-HCl pH 7.4, 50 mM KCl, 2 mM MgCl₂, 0.2 mM NADH, 1 mM phosphoenol pyruvate kinase and 2 units each of lactate dehydrogenase, pyruvate kinase and nucleoside diphosphate kinase. The concentrations of ATP and dTMP were kept at a constant level of 0.5 mM and 0.05 mM respectively, whereas the concentrations of analogues varied between 0.01 and 2 mM. For the K_m determination of the 5'-phosphate derivatives, the ATP

concentration was kept constant at 2 mM and the compound concentration varied between 0.1 and 1 mM.

Biological assays on Mycobacterium bovis (BCG)

The different compounds were assayed for their inhibitory potency on *Mycobacterium bovis* var.BCG growth *in vitro*.⁴⁰ A micro-method of culture was performed in 7H9 Middlebrook broth medium containing 0.2% glycerol, 0.5% Tween 80 and supplemented with oleic acid, albumin, dextrose and catalase (Becton-Dickinson). Serial two-fold dilutions of each compound were prepared directly in 96-well plates. The bacterial inoculum was prepared previously at a concentration in the range of 10^7 bacteria (*M. bovis* BCG 1173P2) in 7H9 medium and stored at $-80\text{ }^{\circ}\text{C}$ until used. The bacteria, adjusted at 10^5 per ml, were delivered in 100 μl per well. The covered plates were sealed with Parafilm and incubated at $37\text{ }^{\circ}\text{C}$ in plastic boxes containing a humidified normal atmosphere. At day 8 of incubation, 30 μl of a resazurin (Sigma) solution at 0.01% (wt/vol) in water were added to each well. After an overnight incubation at $37\text{ }^{\circ}\text{C}$ the plates were assessed for colour development using the optical density difference at 570 nm and 630nm on a ELISA reader. The change from blue to pink indicates reduction of resazurin and therefore bacterial growth. The lowest compound concentration that prevented the colour change determined the MIC for the assayed compound.

Biological assays on Mycobacterium tuberculosis H₃₇Rv

The screening test against *M. tuberculosis* H37Rv was conducted at 6.25 mg/mL in BACTEC 12B medium using the Microplate Alamar Blue Assay (MABA).⁴¹

Cytotoxic activity of test compounds in Vero cell cultures

To monolayers of confluent Vero cell cultures in 96-well microtiter plates were added serial dilutions of the test compounds (total volume of compound-containing culture medium: 200 μ l). After 3 days of incubation at 37°C in a humidified CO₂-controlled atmosphere, the cell cultures were microscopically inspected for morphological alteration. The MCC was defined as the compound concentration required to cause a microscopical alteration of the mock-infected cell cultures at day 3 post compound administration.

Molecular Modelling

The published X-ray structure of the TMPKmt (PDB entry 1G3U)¹¹ was used in all docking experiments. The inhibitors **2**, **10** and **24** were drawn using JChemPaint⁴² and BUILD3D.⁴³ The molecular geometry was fed into gamess for geometry optimization using the AM1 force field.⁴⁴ The sugar conformation in **2** was modelled as C2'-endo with the base in an equatorial orientation by replacing the sugar and base fragments of thymidine monophosphate from the pdb file 1G3U.⁴⁵ The sugar-base in **10** and **24** was replaced by the sugar-base in CSD⁴⁶ entry LEDRIV⁴⁷ having the base in α -position and equatorial orientation. Polar hydrogen atoms were added to the enzyme and inhibitor structures using autodocktools.⁴⁸ The compounds were docked in the cavity close to Tyr-70 by means of the Autodock 3.05 software.⁴⁹ The top 50 of docked ligand conformations was examined and a manual selection procedure was used to validate the docked conformations.

Synthesis

General

NMR spectra were obtained with a Varian Mercury 300 spectrometer. Chemical shifts are given in ppm (δ) relative to residual solvent peak, in the case of DMSO-d₆ 2.54 ppm for ¹H and 40.5 ppm for ¹³C, in the case of CDCl₃ 7.26 ppm for ¹H and 77.4 ppm for ¹³C. All signals assigned to hydroxyl groups were exchangeable with D₂O. Mass spectra and exact mass

measurements were performed on a quadrupole/orthogonal-acceleration time-of-flight (Q/oaTOF) tandem mass spectrometer (qT of 2, Micromass, Manchester, U.K.) equipped with a standard electrospray ionization (ESI) interface. Samples were infused in a 2-propanol/water (1:1) mixture at 3 $\mu\text{L}/\text{min}$. Precoated Merck silica gel F254 plates were used for TLC, and spots were examined under UV light at 254 nm and further visualized using a sulphuric acid-anisaldehyde spraying reagent. Column chromatography was performed on ICN silica gel (63-200 μm , ICN, Asse Relegem, Belgium).

5'-*O*-*tert*-Butyldiphenylsilyl-3'-deoxy-3'-O-((*E*)-2-phenylethenyl)thymidine (35)

To a solution of carbonothioate **34** (7.3 g, 11.85 mmol) in benzene (40 mL) was added β -tributylstannylstyrene (11.64 g, 29.62 mmol) in 20 mL benzene. The resulted solution was degassed with nitrogen during 30 minutes at room temperature and during the same time at 45 $^{\circ}\text{C}$. 2,2'-Azobisisobutyronitrile (AIBN) (583 mg, 3.56 mmol) was added and the solution was refluxed for 2 h. A second portion of AIBN (583 mg, 3.56 mmol) was added after cooling the reaction mixture to 40 $^{\circ}\text{C}$. The reaction mixture was refluxed again for 2 h. This procedure was repeated during 72 hours. After evaporation of the solvent, the residue was purified by column chromatography (hexane/ethyl acetate 85:15) to give **35** as an oil (1.8 g, 24%).

^1H NMR (300 MHz, CDCl_3): δ 1.05 (9H, s, *tert*-butyl), 1.54 (3H, s, 5- CH_3), 2.22-2.40 (2H, m, H-2' and H-2''), 3.26 (1H, m, H-3'), 3.85 (1H, m, H-4'), 4.10 (2H, m, H-5' and H-5''), 5.98 (1H, dd, J = 8.4 and 15.9 Hz, CH-6'), 6.19 (1H, dd, J = 3.3 and 7.2 Hz, H-1'), 6.50 (1H, d, J = 15.6 Hz, CH-Ph), 7.25-7.41 (11H, m, 11 arom H), 7.55 (1H, d, J = 1.2 Hz, H-6), 7.67 (4H, m, arom H), 8.21 (1H, br s, N(3)H); HRMS (ESI-MS) for $\text{C}_{34}\text{H}_{38}\text{N}_2\text{O}_4\text{SiNa}$ $[\text{M}+\text{Na}]^+$ found, 589.2492; calcd, 589.2499.

5'-*O*-*tert*-Butyldiphenylsilyl-3'-deoxy-3'-(hydroxymethyl)thymidine (36)

To a mixture of **35** (1.8 g, 3.11 mmol) and 4-methylmorpholine N-oxide (546 mg, 4.66 mmol) in dioxane (50 mL) was added an aqueous solution of OsO₄ (1 mL, 0.16 mmol, 1% in water). After stirring overnight at room temperature under light protection, the reaction was completed. Sodium periodate (1.33 g, 6.22 mmol) was added and after 2 h the reaction was completed. The mixture was diluted with ethyl acetate, filtered through a Celite path and solids were washed with ethyl acetate. The combined filtrates were washed with brine, dried over MgSO₄ and evaporated under reduced pressure. To the resulting crude aldehyde dissolved in ethanol/water (4:1; 45 mL) at 0 °C was added NaBH₄ (540.8 mg, 14.3 mmol) in small portions. After 2 h at room temperature, the reaction mixture was diluted with ethyl acetate and washed with water. The organic layer was dried over MgSO₄ and concentrated to dryness. After purification by column chromatography (CH₂Cl₂/MeOH 97:3), the title compound (900 mg, 59% from **35**) was isolated as a white foam.

¹H NMR (300 MHz, DMSO-d₆): δ 1.00 (9H, s, *tert*-but), 1.49 (3H, s, 5-CH₃), 2.03-2.19 (2H, m, H-2' and H-2''), 2.48 (under DMSO-signal, H-3'), 3.43 (2H, d, *J*= 5.1 Hz, H-6' and H-6''), 3.77 (1H, dd, *J*= 4.2 and 10.5 Hz, H-5'), 3.86-3.95 (2H, m, H-4' and H-5''), 4.77 (1H, br s, 6'-OH), 6.03 (1H, dd, *J*= 5.4 and 6.6 Hz, H-1'), 7.37-7.44 (7H, m, 6 arom H and H-6), 7.61-7.64 (4H, m, 4 arom H), 11.23 (1H, br s, N(3)H); HRMS (ESI-MS) for C₂₇H₃₄N₂O₅SiNa [M+Na]⁺ found, 517.2139; calcd, 517.2134.

3'-(Azidomethyl)-5'-O-*tert*-butyldiphenylsilyl-3'-deoxythymidine (37)

Methanesulfonyl chloride (0.366 mL, 4.73 mmol) was added to a solution of **36** (900 mg, 1.82 mmol) in pyridine (10 mL) at 0 °C. The reaction was stirred at 0 °C during 1 h. The mixture was diluted with CH₂Cl₂ (25 mL), washed with saturated aqueous NaHCO₃ and dried over MgSO₄. The solvent was evaporated *in vacuo* to give the crude mesylate. The obtained residue was dissolved in DMF (50 mL) and treated with NaN₃ (1.18 g, 18.2 mmol) at 60 °C. After 7 h the reaction was completed. The reaction mixture was evaporated to dryness and the

residue was dissolved in CH₂Cl₂ (25 mL). The organic layer was washed with water, dried over MgSO₄ and evaporated to give a syrup which was purified by column chromatography (CH₂Cl₂/MeOH 99:1) yielding **37** (915 mg, 97%) as a white foam.

¹H NMR (300 MHz, DMSO-d₆): δ 1.00 (9H, s, *tert*-but), 1.50 (3H, d, *J* = 0.6 Hz, 5-CH₃), 2.15 (2H, m, H-2' and H-2''), 2.62 (1H, m, H-3'), 3.42-3.57 (2H, m, H-6' and H-6''), 3.77-3.85 (2H, m, H-4' and H-5'), 3.93 (1H, m, H-5''), 6.06 (1H, t, *J* = 6.6 Hz, H-1'), 7.36-7.45 (7H, m, 6 arom H and H-6), 7.61-7.65 (4H, m, 4 arom H), 11.28 (1H, br s, N(3)H); HRMS (ESI-MS) for C₂₇H₃₃N₅O₄SiNa [M+Na]⁺ found, 542.2197; calcd, 542.2199.

3'-(Aminomethyl)-5'-*O*-*tert*-butyldiphenylsilyl-3'-deoxythymidine (38)

A solution of **37** (915 mg, 1.76 mmol) in methanol (50 mL) was hydrogenated under atmospheric pressure for 5 hours in the presence of 10 % Pd/C (90 mg). The catalyst was removed by filtration through a Celite path and the filtrate was evaporated to give pure compound **38** (860 mg, 99%).

¹H NMR (300 MHz, DMSO-d₆): δ 1.00 (9H, s, *tert*-but), 1.48 (3H, d, *J* = 0.9 Hz, 5-CH₃), 2.03-2.19 (2H, m, H-2' and H-2''), 2.32 (1H, m, H-3'), 2.57 (2H, dd, *J* = 3.9 and 5.7 Hz, H-6' and H-6''), 3.74-3.85 (2H, m, H-4' and H-5'), 3.93 (1H, dd, *J* = 2.4 and 10.8 Hz, H-5''), 6.02 (1H, dd, *J* = 5.1 and 6.9 Hz, H-1'), 7.37-7.43 (7H, m, 6 arom H and H-6), 7.61-7.65 (4H, m, 4 arom H); HRMS (ESI-MS) for C₂₇H₃₆N₂O₄Si [M+H]⁺ found, 494.2478; calcd, 494.2474.

***N*-[(5'-*O*-*tert*-Butyldiphenylsilyl-3'-deoxythymidin-3'-yl)methyl]-*N'*-phenylthiourea (39)**

To a solution of **38** (55 mg, 0.11 mmol) in DMF (1 mL), phenylisothiocyanate (15 mg, 0.11 mmol) in 1 mL DMF was added at 0 °C. The reaction mixture was stirred during 1 h. The solvent was evaporated to dryness and the residue was purified by column chromatography (CH₂Cl₂/MeOH 98:2) affording thiourea **39** (48 mg) in 70% yield.

¹H NMR (300 MHz, DMSO-d₆): δ 0.99 (9H, s, *tert*-but), 1.45 (3H, s, 5-CH₃), 2.17 (2H, m, H-2' and H-2''), 2.83 (1H, m, H-3'), 3.51-3.67 (2H, m, H-6' and H-6''), 3.80-3.93 (3H, m, H-5', H-4' and H-5''), 6.08 (1H, t, *J*= 5.4, H-1'), 7.10 (1H, t, *J*= 7.2 Hz, 1 arom H), 7.26-7.43 (11H, m, 10 arom H and H-6), 7.61-7.66 (4H, m, 4 arom H), 7.93 (1H, br s, N(6')H), 9.55 (1H, br s, N(ar)H), 11.27 (1H, br s, N(3)H); HRMS (ESI-MS) for C₃₄H₄₁N₄O₄SSi [M+H]⁺ found, 629.2618; calcd, 629.2617.

***N*-[(3'-Deoxythymidin-3'-yl)methyl]-*N'*-phenylthiourea (2)**

Compound **39** (48 mg, 0.08 mmol) was dissolved in THF (4 mL). A solution of 1 M *n*-butylammonium fluoride in THF (4 mL) was added. After 1 h at room temperature the reaction was completed. The solvent was evaporated, and the dry residue was purified by column chromatography (CH₂Cl₂/MeOH 97:3) to give pure compound **2** (27 mg) in 87% yield.

¹H NMR (300 MHz, DMSO-d₆): δ 1.75 (3H, s, 5-CH₃), 2.11 (2H, m, H-2' and H-2''), 2.57 (1H, m, H-3'), 3.51-3.78 (5H, m, H-4', H-5', H-5'', H-6' and H-6''), 5.10 (1H, t, *J*= 5.1 Hz, 5'-OH), 5.99 (1H, t, *J*= 4.8, H-1'), 7.09 (1H, t, *J*= 7.2 Hz, ar-H), 7.30 (2H, t, *J*= 7.5 Hz, 2 arom H), 7.38 (2H, d, *J*= 7.5 Hz, 2 arom H), 7.85 (1H, s, H-6), 7.95 (1H, br s, N(6')H), 9.60 (1H, br s, N(ar)H), 11.22 (1H, br s, N(3)H); ¹³C NMR (75 MHz, DMSO-d₆): δ 12.99 (5-CH₃), 36.73 (C-2'), 38.10 (C-3'), 45.85 (C-6'), 62.09 (C-5'), 84.57 and 84.63 (C-4' and C-1'), 109.38 (C-5), 124.02 (arom C), 125.03 (arom C), 129.34 (arom C), 137.03 (C-6), 139.76 (arom C), 151.07 (C-4), 164.54 (C-2), 181.39 (C=S); HRMS (ESI-MS) for C₁₈H₂₂N₄O₄SNa [M+Na]⁺ found, 413.1246; calcd, 413.1259. Anal. (C₁₈H₂₂N₄O₄S) C, H, N.

α-D-Thymidine (47)

3',5'-Di-*O*-acetyl-α-thymidine²⁵ (4.54 g, 13.91 mmol) was dissolved in 150 mL NH₃ in MeOH (7N solution). The reaction mixture was stirred at room temperature overnight. The

solvent was evaporated, the residue was dissolved in ethyl acetate and extracted with water three times, yielding 3.30 g of pure compound **47** (98%).

^1H NMR (300 MHz, DMSO): δ 1.78 (3H, d, J = 1.2 Hz, 5-CH₃), 1.84-1.91 (1H, ddd, J = 3.0 and 14.4 Hz, H-2'), 2.48-2.60 (1H, m, H-2''), 3.39 (2H, t, J = 5.2 Hz, H-5' and H-5''), 4.13 (1H, m, H-3'), 4.23 (1H, m, H-4'), 4.81 (1H, t, J = 5.7 Hz, 5'-OH), 5.30 (1H, d, J = 3.0 Hz, 3'-OH), 6.12 (1H, dd, J = 3.3 and 7.5 Hz, H-1'), 7.75 (1H, d, J = 1.2 Hz), 11.20 (1H, br s, N(3)H); HRMS (ESI-MS) for C₁₀H₁₅N₂O₅ [M+H]⁺ found, 243.0975; calcd, 243.0981.

α -D-Thymidine 5'-monophosphate (9**)**

A solution of α -D-thymidine (150 mg, 0.62 mmol) in trimethyl phosphate (6.2 mL) was cooled to 0 °C, POCl₃ (369 μ L, 4.03 mmol) was added dropwise and the mixture was stirred for 4 hours at 0 °C and for 30 minutes at room temperature. The mixture was poured into ice-water (12 mL), neutralised with concentrated NH₄OH and evaporated to dryness. The resulting residue was purified by column chromatography (iPrOH/NH₄OH/H₂O 77.5:15:2.5). Further purification was performed by HPLC (C-18, CH₃CN/MeOH/0.05% HCOOH in H₂O 45:45:10, 3 mL/min). After lyophilisation of the collected pure fractions compound **9** was obtained (123 mg, 62%) as a white powder.

^1H NMR (300 MHz, D₂O-d₆): δ 1.81 (3H, d, J = 0.9 Hz, 5-CH₃), 2.02-2.09 (2H, ddd, J = 3.0 and 14.7 Hz, H-2'), 2.66-2.76 (1H, m, H-2''), 3.78 (2H, app t, J = 5.1 Hz, H-5' and H-5''), 4.43 (2H, m, H-3' and H-4'), 6.12 (1H, dd, J = 3.0 and 7.2 Hz, H-1'), 7.68 (1H, d, J = 0.9 Hz, H-6); ^{31}P -NMR (500 MHz, D₂O): δ 2.97; ^{13}C NMR (75 MHz, D₂O-d₆): δ 11.73 (5-CH₃), 39.49 (C-2'), 64.67 (C-5'), 71.20 (C-3'), 87.38 and 87.81 (C-4' and C-1'), 110.75 (C-5), 138.39 (C-6), 151.76 (C-4), 166.97; HRMS (ESI-MS) for C₁₀H₁₄N₂O₈PNa [M+Na]⁺ found, 345.0477; calcd, 345.0464; Anal. (C₁₀H₁₄N₂O₈P) C, H, N.

5'-Azido-5'-deoxy- α -D-thymidine (48**)**

To a solution of α -D-thymidine **47** (886 mg, 3.66 mmol) in pyridine (13.5 mL) at -78 °C, methanesulfonyl chloride (256 μ L, 0.41 mmol) was added. The reaction mixture was stirred for 1 h at 0 °C. The reaction was quenched by adding saturated aqueous NaHCO₃-solution and extracted with CH₂Cl₂ three times, dried over MgSO₄ and evaporated. The residue was purified by column chromatography (CH₂Cl₂/MeOH 98:2) to give the mesylated compound (916 mg, 78%).

¹H NMR (300 MHz, DMSO-d₆): 1.75 (3H, s, 5-CH₃), 1.95 (1H, m, H-2'), 2.55 (1H, m, H-2''), 3.19 (3H, s, CH₃SO₂), 4.12-4.26 (3H, m, H-5', H-5'' and H-3'), 4.33 (1H, m, H-4'), 5.58 (1H, br s, 3'-OH), 6.12 (1H, dd, *J*= 4.4 and 7.6 Hz, H-1'), 7.73 (1H, s, H-6), 11.27 (1H, br s, N(3)H); HRMS (ESI-MS) for C₁₁H₁₇N₂O₇S [M+H]⁺ found, 321.0759; calcd, 321.0756.

A solution of 5'-mesylated α -D-thymidine (916 mg, 2.88 mmol) and NaN₃ (1.87 g, 29 mmol) in DMF (50 mL) was heated to 60 °C overnight. The reaction mixture was evaporated *in vacuo*. The residue was resolved in CH₂Cl₂ and washed with H₂O. The organic layer was dried over MgSO₄, evaporated and purified by column chromatography (CH₂Cl₂/MeOH 98:2) to afford compound **48** (672 mg, 87%).

¹H NMR (300 MHz, DMSO-d₆): 1.76 (3H, s, 5-CH₃), 1.93 (1H, m, H-2'), 2.51 (1H, m, H-2''), 3.49 (2H, m, H-5' and H-5''), 4.12 (1H, dd, H-3'), 4.24 (1H, m, H-4'), 5.50 (1H, br s, 3'-OH), 6.13 (1H, dd, *J*= 4.5 and 7.5 Hz, H-1'), 7.72 (1H, s, H-6), 11.26 (1H, br s, N(3)H). HRMS (ESI-MS) for C₁₀H₁₄N₅O₄ [M+H]⁺ found, 268.1045; calcd, 268.1046. Anal. (C₁₀H₁₃N₅O₄) C, H, N.

5'-Amino-5'-deoxy- α -D-thymidine (49)

A solution of azide **48** (531 mg, 1.99 mmol) in methanol (30 mL) was hydrogenated under atmospheric pressure for 6 hours in the presence of 10% Pd/C (53.1 mg). The catalyst was

removed by filtration through Celite and the filtrate was evaporated to yield pure amine **49** (471 mg, 98%).

¹H NMR (300 MHz, DMSO-d₆): 1.74 (3H, s, 5-CH₃), 1.85 (1H, m, H-2'), 2.49 (1H, m, H-2''), 3.43 (2H, m, H-5' and H-5''), 4.02 (1H, m, H-3'), 4.14 (1H, m, H-4'), 5.27 (1H, br s, 3'-OH), 6.05 (1H, dd, *J* = 3.3 and 7.5 Hz, H-1'), 7.69 (1H, s, H-6), 11.24 (1H, br s, N(3)H). HRMS (ESI-MS) for C₁₀H₁₆N₃O₄ [M+H]⁺ found, 242.1134; calcd, 242.1141. Anal. (C₁₀H₁₅N₃O₄·1/2H₂O) C, H, N; C: calcd, 47.99; found, 48.46.

***N*-(5'-Deoxy- α -D-thymidin-5'-yl)-*N*'-phenylthiourea (10)**

For the synthesis of compound **10**, compound **49** (54 mg, 0.22 mmol) was dissolved in DMF (2 mL). At 0 °C, phenyl isothiocyanate (36 mg, 0.26 mmol) was added and the reaction mixture was allowed to stir at room temperature during 3 h. After completion of the reaction, the reaction mixture was evaporated to dryness and the residue was purified by column chromatography (CH₂Cl₂/MeOH 97:3) to obtain the pure final compound **10** (69 mg, 83%).

¹H NMR (300 MHz, DMSO-d₆): δ 1.77 (3H, d, *J* = 1.2 Hz, 5-CH₃), 1.89-1.98 (1H, ddd, *J* = 3.3 and 14.1 Hz, H-2'), 2.53-2.63 (1H, m, H-2''), 3.49 (1H, m, H-5'), 3.64 (1H, m, H-5''), 4.22 (1H, m, H-3'), 4.34 (1H, m, H-4'), 5.47 (1H, d, *J* = 3.3 Hz, 3'-OH), 6.18 (1H, dd, *J* = 3.6 and 8.1, H-1'), 7.10 (1H, t, *J* = 7.2 Hz, arom H), 7.32 (2H, t, *J* = 7.5 Hz, 2 arom H), 7.77 (2H, d, *J* = 1.2 Hz, H-6 + N(5')H), 9.63 (1H, br s, N(ar)H), 11.26 (1H, br s, N(3)H); ¹³C NMR (75 MHz, DMSO-d₆): δ 13.03 (5-CH₃), under DMSO (C-2'), 46.18 (C-5'), 71.58 (C-3'), 85.40 (C-1'), 86.57 (C-4'), 109.51 (C-5), 123.83 (arom C), 124.97 (arom C), 129.29 (arom C), 137.68 (C-6), 139.84 (arom C), 151.21 (C-4), 164.55 (C-2), 181.41 (C=S); HRMS (ESI-MS) for C₁₇H₂₁N₄O₄S [M+H]⁺ found, 377.1279; calcd, 377.1283; Anal. (C₁₇H₂₀N₄O₄S) C, H, N.

***N*-(5'-Deoxy- α -D-thymidin-5'-yl)-*N*'-(3-trifluoromethyl-4-chlorobenzyl)thiourea (23)**

4-Chloro-3-trifluoromethylbenzylamine (65 mg, 0.31 mmol) was added at 0°C to a stirred solution of 1,1'-thiocarbonyldiimidazole (61 mg, 0.34 mmol) and imidazole (6.3 mg, 0.09 mmol) in 4 mL acetonitril. After 10 minutes at 0°C, the reaction was allowed to stir for 3 hours at room temperature. A solution of **49** (75 mg, 0.31 mmol) in 2 mL DMF was added and the reaction mixture was stirred at room temperature during overnight. The reaction mixture was evaporated to dryness and purified by column chromatography (CH₂Cl₂/MeOH 99:1) to obtain compound **23** (114 mg, 75%).

¹H NMR (300 MHz, DMSO-d₆): δ 1.77 (3H, d, *J* = 0.9 Hz, 5-CH₃), 1.87-1.95 (1H, ddd, *J* = 2.9 and 14.4 Hz, H-2'), 2.50-2.60 (1H, m, H-2''), 3.47 (1H, m, H-5'), 3.55 (1H, m, H-5''), 4.18 (1H, m, H-3'), 4.26 (1H, m, H-4'), 4.71 (2H, d, *J* = 4.8 Hz, CH₂NH), 5.41 (1H, d, *J* = 3.0 Hz, 3'-OH), 6.14 (1H, dd, *J* = 3.2 and 7.6 Hz, H-1'), 7.56 (1H, dd, *J* = 2.0 and 8.8 Hz, arom H), 7.65 (1H, d, *J* = 8.7 Hz, arom H), 7.73 (2H, app s, arom H and H-6), 7.78 (1H, t, *J* = 4.7 Hz, N(5')H), 8.14 (1H, d, *J* = 4.9 Hz, CH₂NH), 11.20 (1H, br s, N(3)H); ¹³C NMR (75 MHz, DMSO-d₆): δ 13.00 (5-CH₃), 31.47 (CH₂NH), 46.48 (C-2'), 46.58 (C-5'), 71.58 (C-3'), 85.46 (C-1'), 86.98 (C-4'), 109.44 (C-5), 127.10 (arom C), 127.17 (arom C), 129.49 (arom C), 132.14 (arom C), 133.57 (arom C), 137.58 (C-6), 140.54 (arom C), 151.19 (C-4), 164.52 (C-2), 182.28 (C=S); HRMS (ESI-MS) for C₁₉H₂₁N₄O₄SClF₃ [M+H]⁺ found,; calcd, 493.0924; Anal. (C₁₉H₂₀N₄O₄SClF₃) C, H, N.

***N*-(3,4-Dichlorophenyl)-*N'*-(5'-deoxy- α -D-thymidin-5'-yl)urea (**26**)**

Urea **26** was synthesized from **49** (85 mg, 0.35 mmol) and 3,4-dichlorophenyl isocyanate (79 mg, 0.42 mmol) using the same procedure as described for the synthesis of **10**. After purification by column chromatography (CH₂Cl₂/MeOH 97:3), compound **26** (113 mg, 75%) was obtained.

¹H NMR (300 MHz, DMSO-d₆): δ 1.78 (3H, s, 5-CH₃), 1.89-1.97 (1H, ddd, *J* = 3.2 and 14.3 Hz, H-2'), 2.51-2.61 (1H, m, H-2''), 3.09 (1H, m, H-5'), 3.21 (1H, m, H-5''), 4.16 (2H, m, H-

3' and H-4'), 5.42 (1H, d, $J= 3.3$ Hz, 3'-OH), 6.15 (1H, dd, $J= 3.6$ and 7.8, H-1'), 6.44 (1H, t, $J= 5.1$ Hz, N(5')H), 7.22 (1H, dd, $J= 2.4$ and 9.0 Hz, arom H), 7.43 (1H, d, $J= 9.0$ Hz, arom H), 7.75 (1H, d, $J= 0.9$ Hz, H-6), 7.82 (1H, d, $J= 2.4$ Hz, arom H), 8.92 (1H, br s, N(ar)H), 11.24 (1H, br s, N(3)H); ^{13}C NMR (75 MHz, DMSO- d_6): δ 12.99 (5-CH $_3$), 41.77 (C-2'), 49.28 (C-5'), 71.57 (C-3'), 85.35 (C-1'), 87.32 (C-4'), 109.54 (C-5), 118.40 (arom C), 119.36 (arom C), 122.97 (arom C), 131.12 (arom C), 131.61 (arom C), 137.60 (C-6), 141.33 (arom C), 151.21 (C-4), 155.56 (C=O), 164.51 (C-2); HRMS (ESI-MS) for C $_{17}$ H $_{18}$ N $_4$ O $_5$ Cl $_2$ Na [M+Na] $^+$ found, 451.0548; calcd, 451.0552; Anal. (C $_{17}$ H $_{18}$ Cl $_2$ N $_4$ O $_5$) C, H, N.

5'-Azido-5'-deoxy- α -D-thymidine 3'-methanesulfonate (50)

Compound **48** (400 mg, 1.5 mmol) was dissolved in pyridine (5 mL) and methanesulphonylchloride (150 μL , 1.95 mmol) was added at 0 $^\circ\text{C}$. After 2h, the reaction was quenched by saturated NaHCO $_3$ -solution (5 mL) and extracted with CH $_2$ Cl $_2$ (3x 10 mL). The combined organic layers were dried with MgSO $_4$ and evaporated to dryness, to obtain pure compound **50** (420 mg, 81%).

^1H NMR (300 MHz, DMSO- d_6): δ 1.77 (3H, d, $J= 1.0$ Hz, 5-CH $_3$), 2.32-2.40 (1H, ddd, $J= 3.6$ and 15.0 Hz, H-2'), 2.81-2.91 (1H, m, H-2''), 3.27 (3H, s, SO $_2$ CH $_3$), 3.52 (2H, d, $J= 5.1$ Hz, H-5' and H-5''), 4.70 (1H, m, H-4'), 5.17 (1H, m, H-3'), 6.16 (1H, dd, $J= 3.6$ and 6.9, H-1'), 7.52 (1H, d, $J= 1.1$ Hz, H-6), 11.31 (1H, br s, N(3)H); HRMS (ESI-MS) for C $_{11}$ H $_{15}$ N $_5$ O $_6$ S [M+H] $^+$ found, 346.0818; calcd, 346.0821.

5'-Azido-3',5'-dideoxy-2',3'-didehydro- α -D-thymidine (51)

To a solution of mesylate ester **50** (420 mg, 1.21 mmol) in THF (15 mL) was added DBU (906 μL , 6.07 mmol), and the reaction was refluxed at 80 $^\circ\text{C}$ during overnight. After cooling down, the reaction mixture was poured into NH $_4$ Cl (15 mL) solution, and extracted with

CH₂Cl₂ (3 x 25 mL). The organic layers were dried over MgSO₄, evaporated and purified by column chromatography (CH₂Cl₂/MeOH 99:1) to yield 290 mg (95%) pure title compound.

¹H NMR (300 MHz, DMSO-d₆): δ 1.77 (3H, d, *J* = 1.0 Hz, 5-CH₃), 3.36 (1H, dd, *J* = 4.8 and 13.2 Hz, H-5'), 3.62 (1H, dd, *J* = 3.3 and 13.2 Hz, H-5''), 5.32 (1H, m, H-4'), 6.03 (1H, dd, *J* = 1.5 and 5.7, H-1'), 6.40 (1H, dd, *J* = 1.5 and 6.0 Hz, H-2''), 6.89 (1H, dd, *J* = 1.5 and 5.4 Hz, H-3'), 7.52 (1H, d, *J* = 1.1 Hz, H-6), 11.31 (1H, br s, N(3)H); HRMS (ESI-MS) for C₁₀H₁₁N₅O₃Na [M+Na]⁺ found, 272.0761; calcd, 272.0759.

5'-Amino-3',5'-dideoxy-α-D-thymidine (52)

To a solution of azide **51** (150 mg, 0.6 mmol) in methanol (4 mL) was added 10% Pd/C (15 mg) and the reaction placed under a H₂ atmosphere. After 5 hours, the reaction mixture was filtered through a plug of Celite, which was further washed with MeOH and the combined filtrates were evaporated to yield pure **52** as a white solid (128 mg, 95%).

¹H NMR (300 MHz, DMSO-d₆): δ 1.70 (1H, m, H-3'), 1.77 (3H, d, *J* = 1.2 Hz, 5-CH₃), 1.89-2.06 (2H, m, H-2' and H-3''), 2.27 (1H, m, H-2''), 2.56 (2H, d, *J* = 5.1 Hz, H-5' and H-5''), 4.27 (1H, m, H-4'), 5.98 (1H, dd, *J* = 5.1 and 6.6, H-1'), 7.41 (1H, d, *J* = 1.1 Hz, H-6); HRMS (ESI-MS) for C₁₀H₁₆N₃O₃ [M+H]⁺ found, 226.1188; calcd, 226.1191.

5'-Amino-3',5'-dideoxy-2',3'-didehydro-α-D-thymidine (53)

To a solution of azide **51** (80 mg, 0.32 mmol) in dry pyridine (4 mL) triphenylphosphine (135 mg, 1.6 mmol) was added. The reaction was stirred for 3 hours at room temperature, evaporated to dryness and purified by column chromatography (CH₂Cl₂/MeOH 90:10), to afford 65 mg of amine **53** (92%), which was used in the next step without purification.

¹H NMR (300 MHz, DMSO-d₆): δ 1.69 (3H, d, *J* = 1.2 Hz, 5-CH₃), 2.59 (1H, dd, *J* = 4.7 and 13.5 Hz, H-5'), 2.70 (1H, dd, *J* = 4.7 and 13.7 Hz, H-5''), 4.99 (1H, m, H-4'), 5.83 (1H, app dt, *J* = 2.4 and 5.7, H-1'), 6.33 (1H, app dt, *J* = 1.5 and 6.0 Hz, H-2'), 6.81 (1H, app dt, *J* =

1.5 and 4.9 Hz, H-3'), 7.10 (1H, d, $J=1.2$, H-6); ^{13}C NMR (75 MHz, DMSO- d_6): δ 11.96 (5- CH_3), 45.68 (C-5'), 88.49 (C-1'), 89.41 (C-4'), 109.75 (C-5), 125.37 (C-2'), 135.72 (C-3'), 137.42 (C-6), 150.53 (C-4), 163.75 (C-2); HRMS (ESI-MS) for $\text{C}_{10}\text{H}_{14}\text{N}_3\text{O}_3$ $[\text{M}+\text{H}]^+$ found, 224.1039; calcd, 224.1035.

1-[(2-Hydroxyethoxy)methyl]thymine (54)

A mixture of thymine (400 mg, 3.17 mmol), HMDS (16 mL) and ammonium sulphate (16 mg) was refluxed during 2 hours under nitrogen atmosphere. After cooling to room temperature, the mixture was evaporated to dryness and redissolved in acetonitril (32 mL). After cooling the mixture to $-45\text{ }^\circ\text{C}$, trimethylsilyl triflate (602 μL , 3.33 mmol) was added dropwise, followed by dropwise addition of dioxolane (470 mg, 6.34 mmol). After 2 hours the reaction was allowed to warm to room temperature and stirred overnight. To quench the reaction, a saturated aqueous NaHCO_3 -solution (20 mL) was added at $-45\text{ }^\circ\text{C}$. The resulting mixture was extracted three times with diethylether (25 mL). The combined organic layers were dried over MgSO_4 and evaporated to dryness. The crude residue was purified by column chromatography ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 95:5) to obtain 480 mg (76%) of pure compound **54**.

^1H NMR (300 MHz, DMSO- d_6): δ 1.75 (3H, d, $J=1.2$ Hz, 5- CH_3), 3.45 (4H, m, CH_2CH_2), 4.61 (1H, t, $J=5.1$ Hz, OH), 5.03 (2H, s, OCH_2N), 7.54 (1H, d, $J=1.2$, H-6), 11.26 (1H, br s, N(3)H); HRMS (ESI-MS) for $\text{C}_8\text{H}_{12}\text{N}_2\text{O}_4$ $[\text{M}+\text{Na}]^+$ found, 223.0721; calcd, 223.0694.

1-[(2-Azidoethoxy)methyl]thymine (55)

Alcohol **54** (480 mg, 1.82 mmol) was dissolved in anhydrous pyridine (14 mL) and methanesulfonyl chloride (183 μL , 2.36 mmol) was slowly added at $0\text{ }^\circ\text{C}$. After 2 hours, the reaction was quenched with aqueous NaHCO_3 -solution (10 mL) and the mixture was extracted three times with ethyl acetate (20 mL). The combined organic layers were dried over MgSO_4 and evaporated to dryness. The obtained residue was dissolved in DMF (40 mL)

and 1.46 g (22.5 mmol) of NaN₃ was added. The reaction mixture was heated at 60 °C during the night, evaporated and purified by column chromatography (CH₂Cl₂/MeOH 97:3), yielding 393 mg (96%) of **55**.

¹H NMR (300 MHz, DMSO-d₆): δ 1.75 (3H, d, *J*= 1.2 Hz, 5-CH₃), 3.39 (2H, t, *J*= 4.8 Hz, CH₂O), 4.64 (2H, t, *J*= 4.8 Hz, CH₂N₃), 5.07 (2H, s, OCH₂N), 7.56 (1H, d, *J*= 1.2 Hz, H-6), 11.30 (1H, br s, N(3)H); HRMS (ESI-MS) for C₈H₁₂N₅O₃ [M+H]⁺ found, 226.0948; calcd, 226.0940.

1-[(2-Aminoethoxy)methyl]thymine (56)

To a solution of azide **55** (393 mg, 1.75 mmol) in methanol (15 mL) was slowly added 50 mg of 10% Pd/C. The reaction mixture was submitted to a hydrogen atmosphere overnight. After filtration of the catalyst over a plug of Celite and evaporation of the solvent in vacuo, resulting compound **56** (345 mg, 99%) was obtained and used without further purification.

¹H NMR (300 MHz, DMSO-d₆): δ 1.77 (3H, s, 5-CH₃), 2.65 (2H, t, *J*= 5.7 Hz, CH₂NH₂), 3.43 (2H, t, *J*= 4.8 Hz, CH₂O), 5.05 (2H, s, OCH₂N), 7.48 (1H, s, H-6); HRMS (ESI-MS) for C₈H₁₄N₃O₃ [M+H]⁺ found, 200.1034; calcd, 200.1035.

***N*-[(Thymin-1-yl)methoxyethyl]-*N'*-phenylthiourea (31)**

The title compound was synthesized from **56** (65 mg, 0.33 mmol) and phenyl isothiocyanate (57 mg, 0.42 mmol) in 2 mL DMF using the same procedure as described for the synthesis of **10**. After purification by column chromatography (CH₂Cl₂/MeOH 99:1), thiourea **31** (94 mg, 86%) was obtained as a white powder.

¹H NMR (300 MHz, DMSO-d₆): δ 1.74 (3H, d, *J*= 0.9 Hz, 5-CH₃), 3.62 (4H, app s, CH₂CH₂), 5.06 (2H, s, OCH₂N), 7.07 (1H, m, arom H), 7.28 (2H, m, 2 arom H), 7.37 (2H, d, *J*= 7.5 Hz, 2 arom H), 7.56 (1H, d, *J*= 1.2 Hz, H-6), 7.76 (1H, br s, N(5')H), 9.61 (1H, br s, N(ar)H), 11.28 (1H, br s, N(3)H); ¹³C NMR (75 MHz, DMSO-d₆): δ 12.56 (5-CH₃), 44.10 (CH₂NH'),

67.35 (CH₂O), 76.84 (OCH₂N), 109.92 (C-5), 123.79 (arom C), 124.93 (arom C), 129.33 (arom C), 139.77 (C-6), 141.23 (arom C), 151.78 (C-4), 164.98 (C-2), 181.18 (C=S); HRMS (ESI-MS) for C₁₅H₁₉N₄O₃S [M+H]⁺ found, 335.1175; calcd, 335.1177. Anal. (C₁₅H₁₈N₄O₃S) C, H, N.

***N*-(5'-Deoxy-2',3'-O-isopropylideneadenosin-5'-yl)-N'-(α -D-thymidin-5'-yl)thiourea (58)**

5'-Amino-2',3'-O-isopropylidene adenosine (**57**) (130 mg, 0.43 mmol), imidazole (6mg, 0.08 mmol) and 1,1-thiocarbonyldiimidazole (83 mg, 0.47 mmol) were dissolved in DMF (7 mL) at 0 °C and after 10 minutes the reaction was allowed to warm up to room temperature. After 2 hours, **49** (112 mg, 0.43 mmol) in 2 mL of DMF was added. After 3 hours, the reaction was finished and evaporated to dryness. The obtained crude compound was purified by column chromatography (CH₂Cl₂/MeOH 95:5) to obtain 205 mg (82%) **58**.

¹H NMR (300 MHz, DMSO-d₆): δ 1.32 (3H, s, CCH₃ (A)), 1.53 (3H, s, CCH₃ (A)), 1.76 (3H, d, J = 0.9 Hz, 5-CH₃ (T)), 1.87-1.94 (2H, ddd, J = 2.6 and 14.2 Hz, H-2' (T)), 2.49-2.59 (1H, m, H-2'' (T)), 3.41-3.67 (3H, m, H-5'(T), H-5'' (T) and H-5' (A)), 3.78-3.90 (1H, m, H-5'' (A)), 4.13-4.34 (3H, m, H-3' (T), H-4' (T) and H-4' (A)), 5.01 (1H, dd, J = 3.4 and 6.2 Hz, H-3' (A)), 5.41 (1H, d, J = 3.4 Hz, 3'-OH (T)), 5.46 (1H, dd, J = 2.8 and 6.3 Hz, H-2' (A)), 6.15 (2H, m, H-1' (T) and H-1' (A)), 7.34 (2H, s, NH₂), 7.55 and 7.64 (2H, 2 x br s, 2 x N(5')H), 7.73 (1H, d, J = 1.2 Hz, H-6), 8.18 and 8.33 (2H, 2 x s, H-2 and H-8 (A)), 11.24 (1H, br s, N(3)H); HRMS (ESI-MS) for C₂₄H₃₂N₉O₇S [M+H]⁺ found, 390.2148; calcd, 390.2145.

***N*-(5'-Deoxyadenosin-5'-yl)-N'-(α -D-thymidin-5'-yl)thiourea (33)**

Compound **58** (120 mg, 0.20 mmol) was dissolved in 50% trifluoroacetic acid in H₂O (10 mL) and stirred for 2 hours at room temperature. The reaction mixture was evaporated to dryness and purified by column chromatography (CH₂Cl₂/MeOH 93:7) to obtain 98 mg of pure title compound (89%).

¹H NMR (300 MHz, DMSO-d₆): δ 1.76 (3H, d, *J*= 1.2 Hz, 5-CH₃ (T)), 1.86-1.94 (2H, ddd, *J*= 2.6 and 14.6 Hz, H-2' (T)), 2.49-2.59 (1H, m, H-2'' (T)), 3.41-3.67 (3H, m, H-5'(T), H-5'' (T) and H-5' (A)), 3.82-3.93 (1H, m, H-5''' (A)), 4.01-4.29 (4H, m, H-3' (T), H-4' (T), H-4' (A) and H-3' (A)), 4.70 (1H, t, *J*= 5.4 Hz, H-2' (A)), 5.29 (1H, br s, 3'-OH (A), 5.43 (1H, br s, 3'-OH (T)), 5.49 (1H, br s, 2'-OH (A)), 5.88 (1H, d, *J*= 6.0 Hz, H-1' (A)), 6.14 (1H, dd, *J*= 3.0 and 7.5 Hz, H-1' (T)), 7.43 (2H, s, NH₂), 7.59 (1H, br s, N(5')H), 7.74 (2H, app d, *J*= 1.2 Hz, H-6 and N(5'(A)H)), 8.20 and 8.38 (2H, 2 x s, H-2 and H-8 (A)), 11.25 (1H, br s, N(3)H); HRMS (ESI-MS) for C₂₁H₂₈N₉O₇S [M+H]⁺ found, 550.1833; calcd, 550.1832. Anal. (C₂₁H₂₇N₉O₇S) C, H, N.

Supporting Information Available: Experimental section and analytical data for intermediates (**40-45**) and final products (**3-8, 11-22, 24-25, 27-30, 32**) and elemental analysis of final products. Also provided is a more detailed explanation of the computational methodology. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Figure and Scheme Legends

Figure 1. Dinucleoside **1** ($K_i = 37 \mu\text{M}$) and envisaged analogues

Figure 2. Inverse sugar binding of 3'-C-arylthiourea-modified β -thymidines and anticipated similar relative orientation of the coloured moieties in 5'-deoxy-5'-arylthiourea modified α -thymidines.

Figure 3. Comparison of the parallel synthesized α - and β - thiourea derivatives;(■ β -derivatives, ■ α -derivatives).

Figure 4. Compound **2** (left) and compound **10** (right) docked in the active site of TMPKmt. All residues interacting with the inhibitors (hydrophobic contact) are shown as well as the hydrogen bonding pattern (calculated using Ligplot and HBPlus^{32, 33}). Drawings created using Molscript.³⁴

Figure 5. Docking and superposition of α -derivatives **10** (green carbons) and **24** (grey carbons) in the active site of TMPKmt, **24** having a longer linker ($n=2$). The phenyl tails of the inhibitors point to the front and seem to exit the enzyme through a channel lined up by residues Arg14, Ala35, Phe36, Pro37, Tyr39, Arg160. The "channel" residues are coloured blue. Picture created using Molscript,³⁵ Bobsript,³⁶ Raster3D.³⁷

Scheme 1. Synthesis of 3'-C-branched- β -thiourea derivatives **2-8**. Reagents and conditions: (a) $\text{Bu}_3\text{SnCH}=\text{CHPh}$, AIBN, benzene, reflux, 72h, 24%; (b) (i) 4-MNO, OsO_4 , dioxane, rt, 16h; (ii) NaIO_4 , rt, 2h; (iii) NaBH_4 , $\text{EtOH}/\text{H}_2\text{O}$, rt, 2h, 59%; (c) (i) MsCl , pyridine, 0 °C, 1h; (ii) NaN_3 , DMF, 60 °C, 7h, 97%; (d) Pd/C , H_2 , MeOH, rt, 3h, 99%; (e) 3-R₂-4-R₁-phenylisothiocyanate, DMF, 0 °C, 1h, 70-88%; (f) TBAF, THF, rt, 1h, 87-92% .

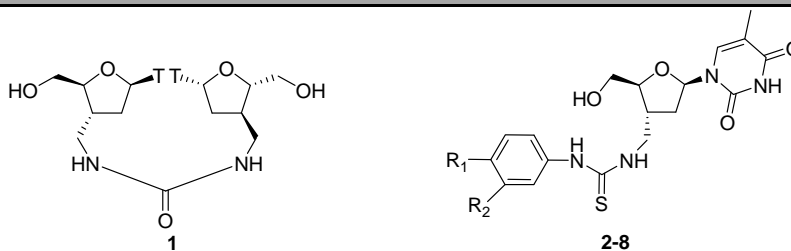
Scheme 2. Synthesis of α -D-thymidine 5'-*O*-monophosphate **9**. Reagents and conditions: (a) NH_3 , MeOH, rt, 16h, 98%; (b) POCl_3 , $\text{P}(\text{OMe})_3$, 0 °C, 4h; then rt, 0.5h, 62%.

Scheme 3. Synthesis of 5'-substituted α -thymidine derivatives **10-27**, **30**. Reagents and conditions: (a) (i) MsCl , pyridine, 0 °C, 1h (ii) NaN_3 , DMF, 60 °C, 16h, 68%; (b) H_2 , Pd/C, MeOH, rt, 6h, 98%; (c) suitable iso(thio)cyanate, DMF, 71-91%; or: suitable amine, 1,1'-TCDI, DMF, rt, 3h, 75-82%.

Scheme 4. Synthesis of 3'-deoxy- and 3'-deoxy-2',3'didehydro- α -thymidine derivatives **28** and **29**. Reagents and conditions: (a) MsCl , Pyridine, 0 °C, 2h, 81%; (b) DBU, THF, 80 °C, 16h, 95%; (c) H_2 , Pd/C, MeOH, rt, 5h, 95%; (d) PPh_3 , Pyridine, rt, 3h, 92% (e) 3- CF_3 -4-Cl-phenylisothiocyanate, DMF, rt, 3h, 78%.

Scheme 5. Synthesis of acyclic derivatives **31** and **32**. Reagents and conditions: (a) silylated thymine, TMSOTf, acetonitrile, -45 °C for 2h then rt, 16h, 76 %; (b) (i) MsCl , pyridine, 0 °C, 2h (ii) NaN_3 , DMF, 60 °C, 16h, 96%; (c) Pd/C, H_2 , MeOH, rt, 16h, 99%; (d) 3- R_2 -4- R_1 -phenylisothiocyanate, DMF, rt, 3h, 83-86%.

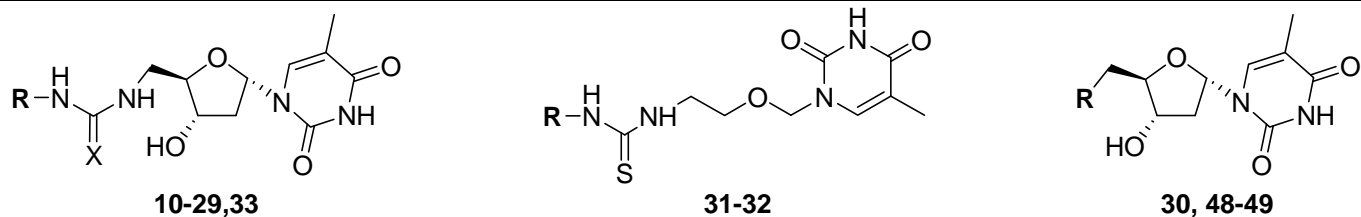
Scheme 6. Synthesis of adenosine conjugate **33**. Reagents and conditions: (a) 5'-deoxy-5'- NH_2 - α -thymidine (**49**), TCDI, DMF, rt, 2h; (b) 50% TFA, rt, 2h, 73%.

Table 1: Kinetic parameters of TMPK_{mt} with compounds 1-8

Compound	R ₁	R ₂	K _i (μM) TMPK _{mt}	K _i (μM) TMPK _h	MCC Vero cell cultures ^a
dTMP			4.5 ^b	5 ^b	
1			37.0	> 1000	
2	H	H	69.0		
3	Cl	H	21.0		> 100
4	OCH ₃	H	46.0		> 100
5	CH ₃	H	36.0		> 100
6	Cl	Cl	7.2		> 100
7	OCH ₂ Ph	H	12.0		> 100
8	Cl	CF ₃	5.0	> 1000	> 100

^a Minimum cytotoxic concentration in Vero cell cultures, i.e. the concentration required to cause microscopically detectable alteration of normal cell morphology.

^b K_m-Value

Table 2: Kinetic parameters of TMPKmt with compounds 9-33 and 48-49


Compound	R	X	K_i (μ M) TMPK mt	K_i (μ M) TMPK h	SI	MIC ₉₉ (μ g/ml) (<i>M. bovis</i>)	% Inh (<i>M. tub</i> H37Rv) ^d	MCC μ M Vero cell cultures ^e
dTMP			4.5 ^a	5.0 ^a				
α -dTMP			15.0					
10	Phenyl	S	16.0					> 100
11	4-Cl-phenyl	S	3.2	> 1000				
12	4-MeO-phenyl	S	10.0					
13	4-Me-phenyl	S	7.8					
14	3,4-diCl-phenyl	S	1.0	274	270	25	38%	> 100
15	3-CF ₃ -4-Cl-phenyl	S	0.6	362	600	20	39%	> 100
16	4-morpholinophenyl	S	19.2					
17	1-adamantyl	S	15.0					> 100
18	3-pyridyl	S	18.0					
19	Flouresceinyl	S	5.4			> 150		
20	Phenylmethyl	S	5.4					
21	Benzhydryl	S	4.9	> 500				> 100
22	Benzoyl	S	7.2					
23	3-CF ₃ -4-Cl-phenylmethyl	S	2.6	50	20	40		> 100
24	Phenylethyl	S	3.8	> 800				> 100
25	3,4-diCl-phenylethyl	S	2.2	79	40	50		> 100
26	3,4-diCl-phenyl	O	1.1	116	105	40 (MIC ₅₀)	41%	
27	3-CF ₃ -4-Cl-phenyl	O	1.9	166	90	40 (MIC ₅₀)	49%	
28 ^b	3-CF ₃ -4-Cl-phenyl	S	2.3	> 500		40	34%	
29 ^c	3-CF ₃ -4-Cl-phenyl	S	3.8	190	50	100	55%	
30	Benzamido		35					
31	Phenyl	S	260					> 100
32	3-CF ₃ -4-Cl-phenyl	S	37.0				34%	100
33	5'-deoxy- β -D-adenosin-5'-yl	S	26	280	10			
48	N ₃		26.5					
49	NH ₂		16.0			> 40 μ g/ml		

^a K_m -Value; ^b 3'-deoxy-ribonucleoside; ^c 3'-deoxy-2',3'-didehydronucleoside; ^d inhibition at 6.25 μ g/mL; ^e Minimum cytotoxic concentration in Vero cell cultures, i.e. the concentration required to cause microscopically detectable alteration of normal cell morphology.

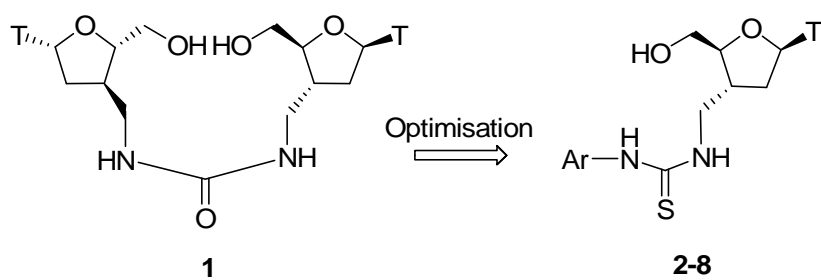


Figure 1

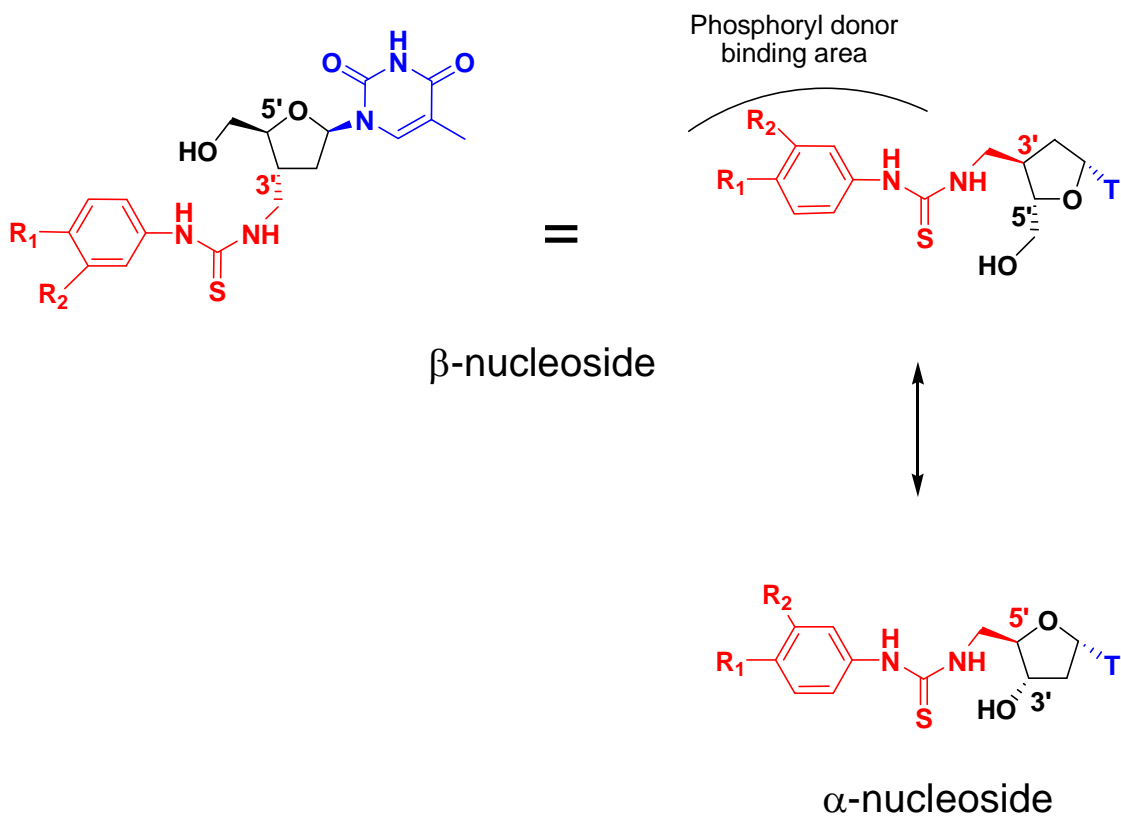


Figure 2

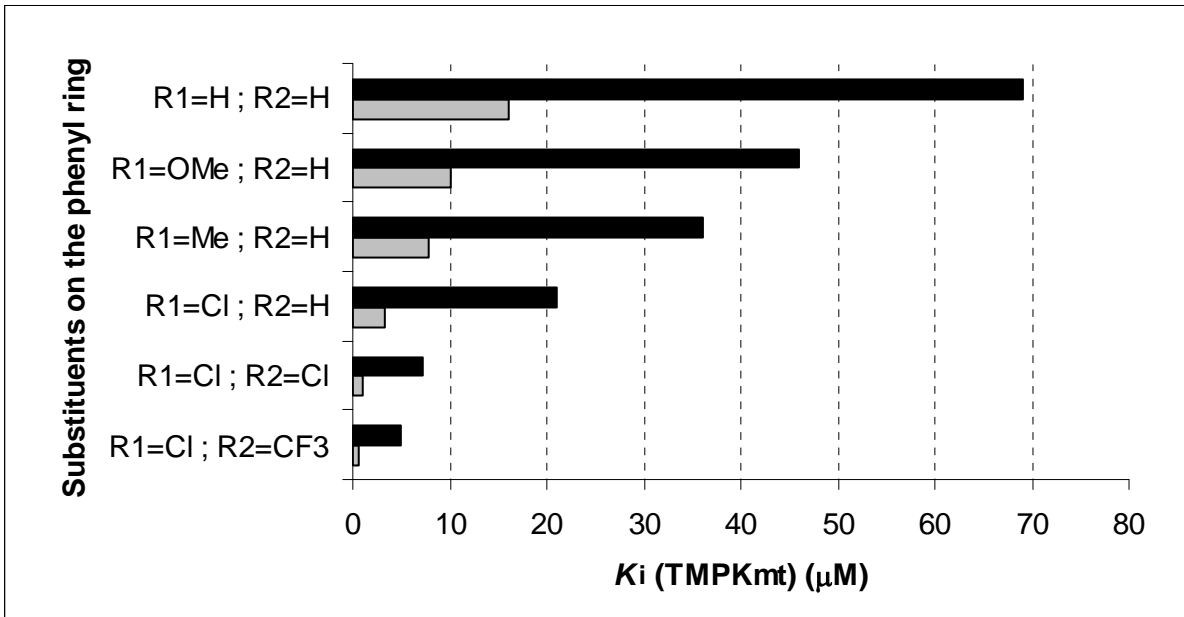


Figure 3

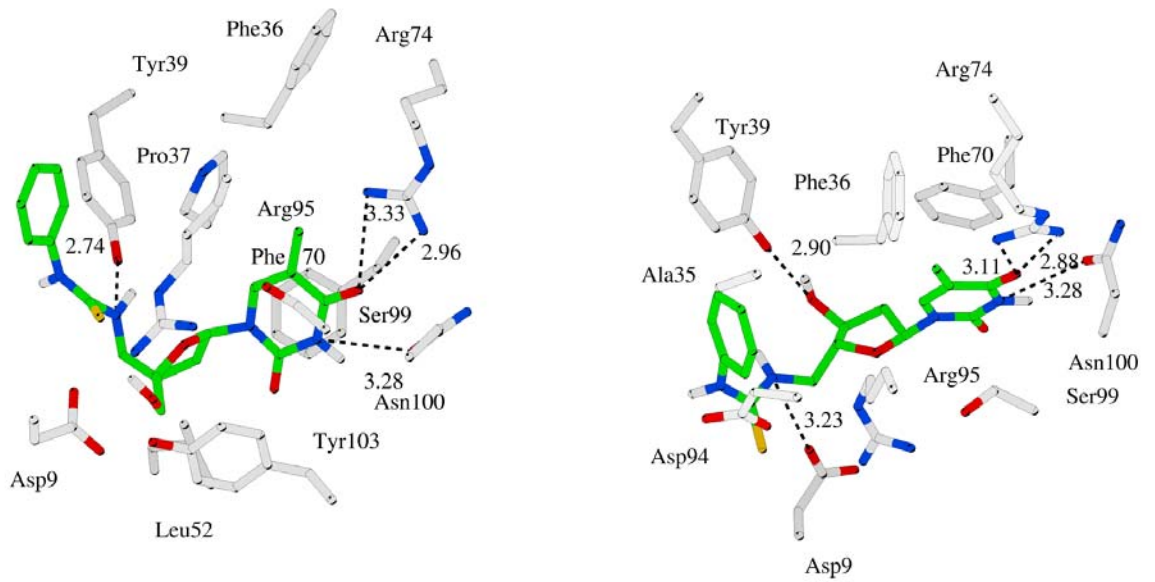


Figure 4

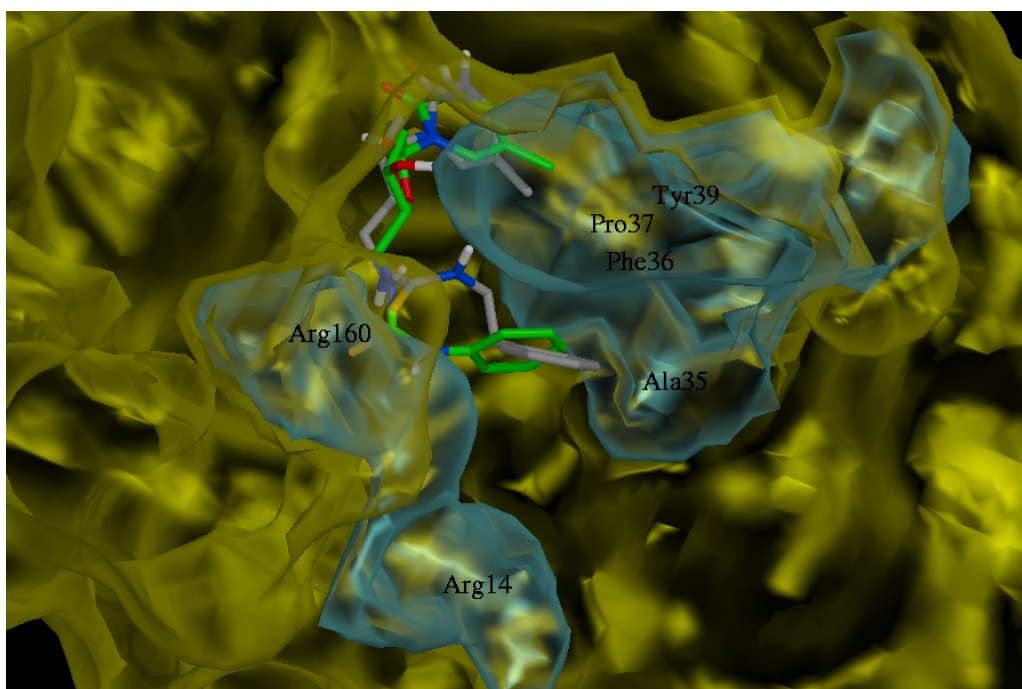
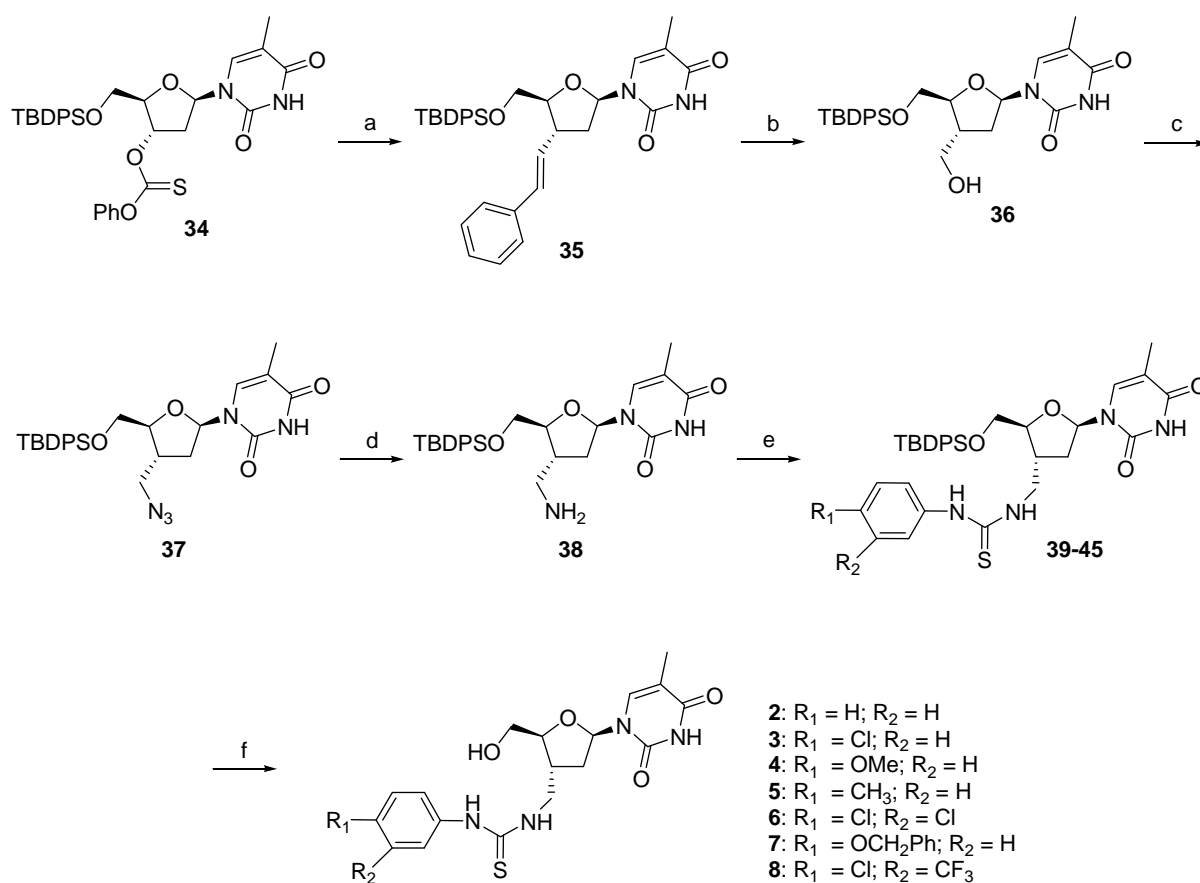
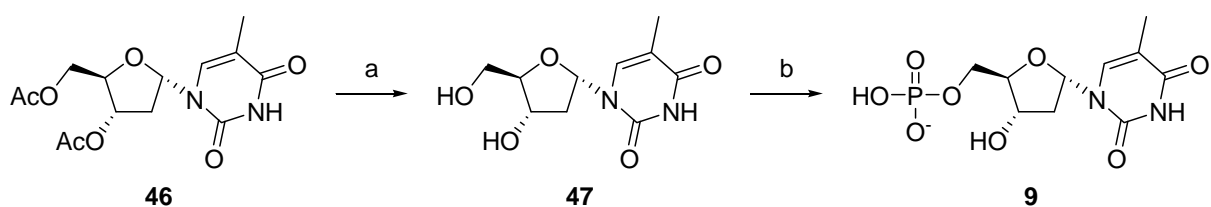


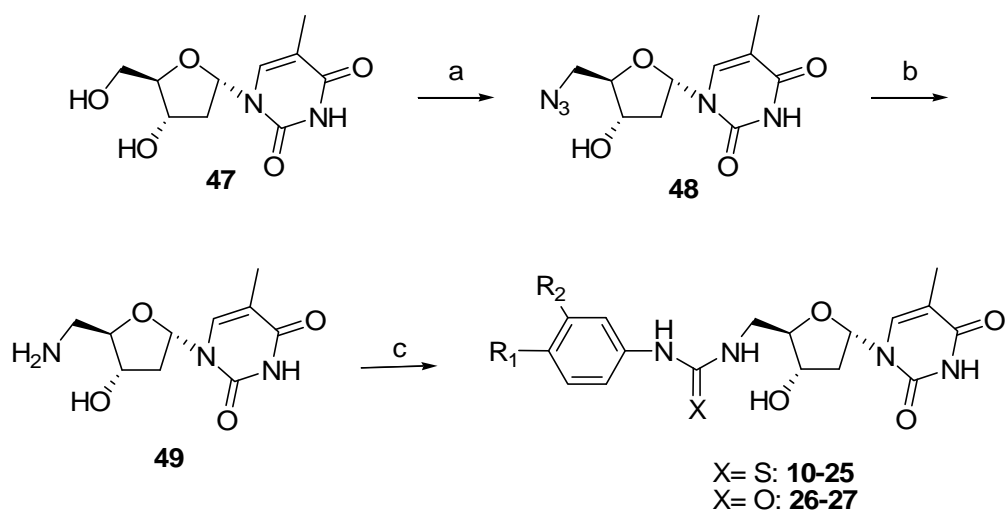
Figure 5



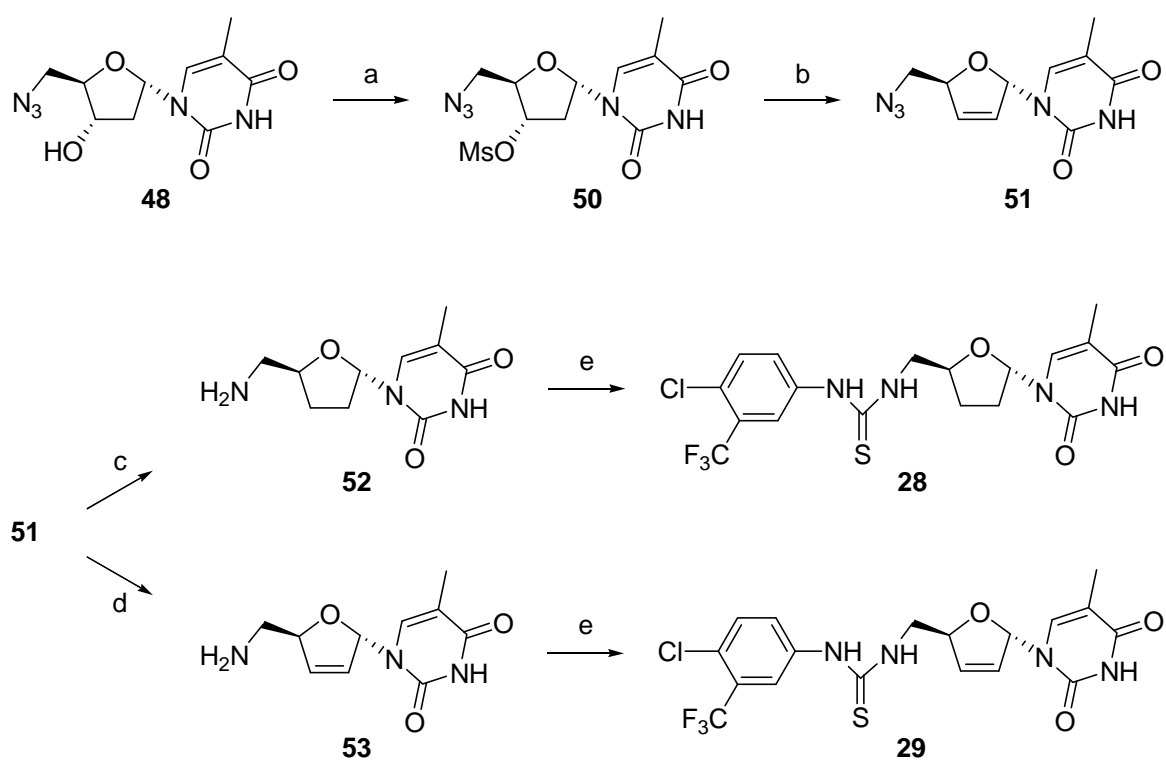
Scheme 1



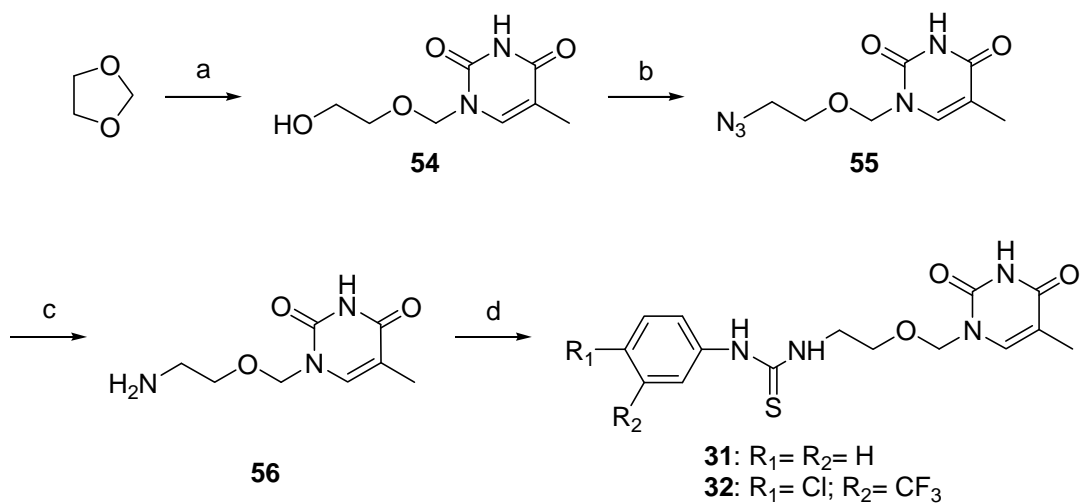
Scheme 2



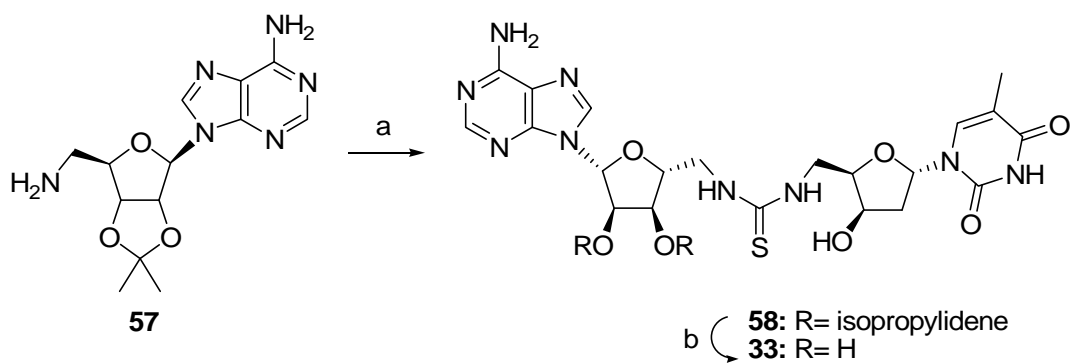
Scheme 3



Scheme 4



Scheme 5



Scheme 6

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