Rational Design of 5′-Thiourea-Substituted α-Thymidine Analogues as Thymidine Monophosphate Kinase Inhibitors Capable of Inhibiting Mycobacterial Growth

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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 of 0.6 μM and a selectivity index of 600 versus human TMPK. Moreover, it represents the first TMPK inhibitor showing good inhibitory activity on growing *M. bovis* (MIC_{99} = 20 μg/mL) and *M. tuberculosis* (MIC_{50} = 6.25 μg/mL) strains.

Introduction

At the beginning of the 21st century, tuberculosis (TB[a]) 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 1950s 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 multidrug-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] Besides 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.

HIV infection is especially 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 and 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.

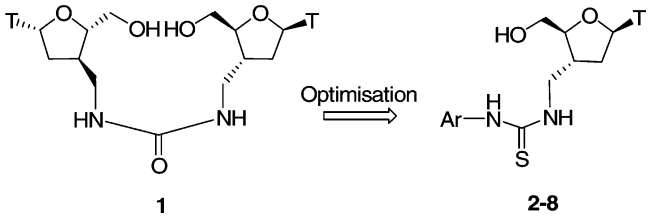

Figure 1. Dinucleoside **1** (K_i = 37 μM) and envisaged analogues.

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 structures of human[9,10] and mycobacterial[11] TMPK and their low (22%) sequence identity further promote TMPKmt as an attractive target for the development of selective inhibitors.

Thymidine is a moderately potent inhibitor of TMPKmt (K_i ≈ 27 μM). Both the sugar[12−14] 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. We only recently identified a TMPKmt inhibitor, derived from a 2′,3′-bicyclic thymidine analogue, that shows some activity against living mycobacteria cultures (MIC_{99} ≈ 100 μg/mL^{-1}).[17]

Lately, our group discovered an unusual dinucleoside **1** that produced significant inhibition of TMPKmt (K_i = 37 μM).[14] The second thymidine monomer attached to the 3′-position suggested the possibility of introducing large substituents at this

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[a] Abbreviations: TMPK, thymidine monophosphate kinase; TMPKh, human thymidine monophosphate kinase; TMPKmt, *Mycobacterium tuberculosis* thymidine monophosphate kinase; TB, tuberculosis; MDR, multidrug resistant; dTMP, thymidine monophosphate; AIBN, 2,2′-azabisisobutyronitrile.

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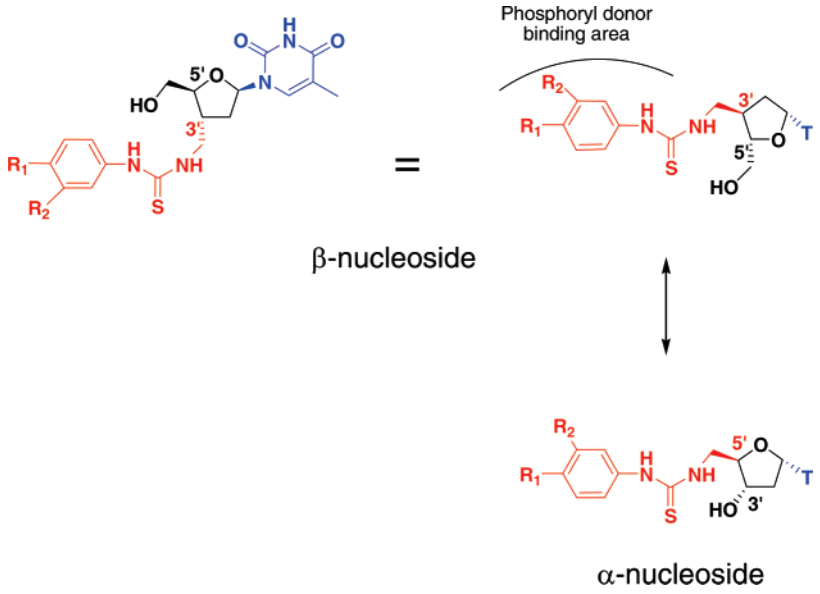


Figure 2. Inverse sugar binding of 3′-*C*-arylthiourea-modified *β*-thymidines and anticipated similar relative orientation of the colored moieties in 5′-deoxy-5′-arylthiourea modified α-thymidines.

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 an opportunity for further explorations of this type of analogue (Figure 1).

We investigated the possibility of replacing one thymidine monomer by different substituted phenyl groups. Concurrently, thiocarlide,[18] a drug that formerly proved to be 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 steric properties of the aryl substituents.

Modeling 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 to 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 to be 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 an 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′-azabisisobutyronitrile 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-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).

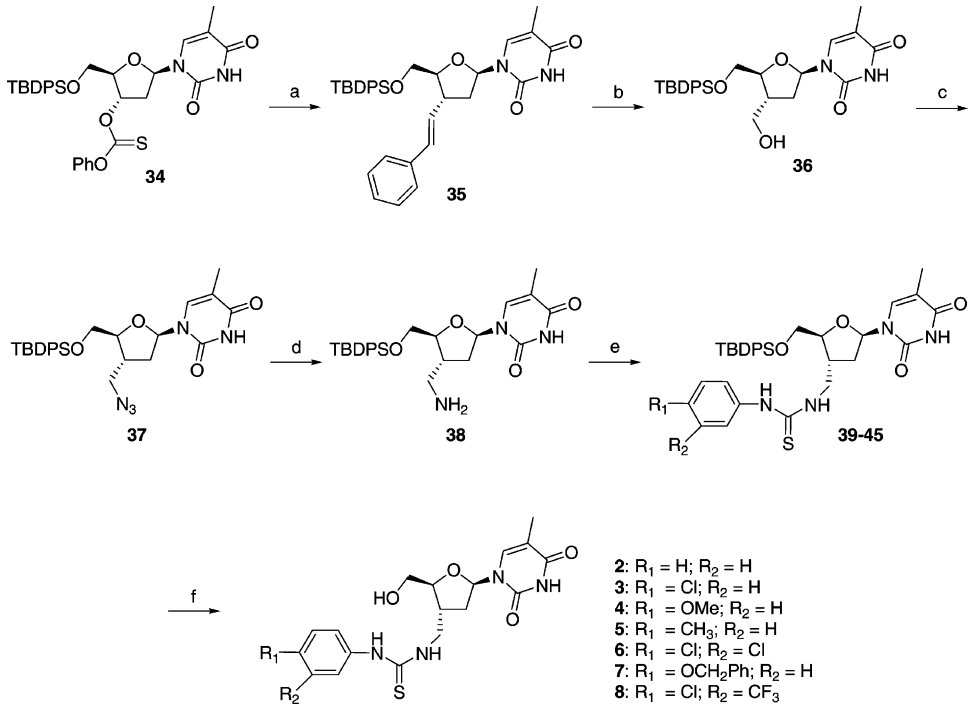
The synthesis of 5′-substituted α-thymidine derivatives started from α-thymidine (**47**), which was converted to the 5′-deoxy-5′-(thio)urea derivatives **10**−**27** as depicted in Scheme 3. Amide **30** (Table 2) was isolated as a side product upon treatment of **49** with benzoyl isothiocyanate.

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-CF_3-4-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.

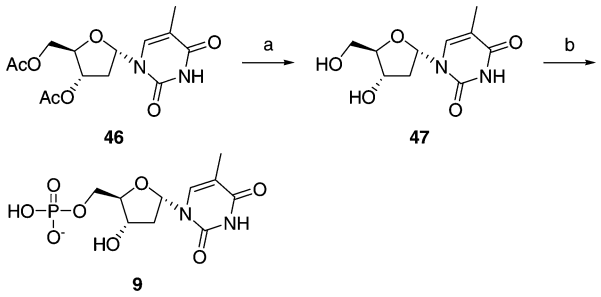
For the preparation of the adenosine conjugate **33** (Scheme 6), 5′-amino-2′,3′-*O*-isopropylidene adenosine (**57**) was treated with 1,1′-thiocarbonyldiimidazole to generate in situ the isothio-

Scheme 1. Synthesis of 3′-C-Branched β-Thioureaderivatives **2**−**8**[a]



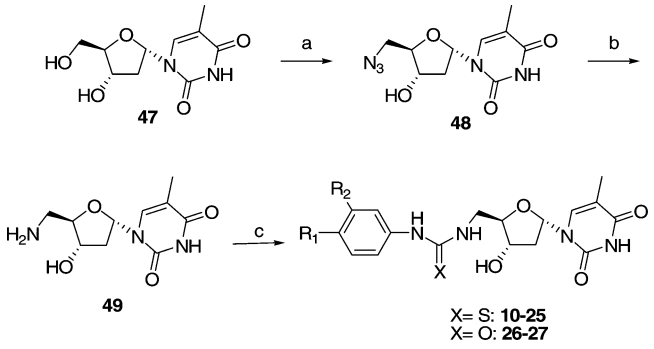
[a] Reagents and conditions: (a) $Bu_3SnCH{=}CHPh$, AIBN, benzene, reflux, 72 h, 24%; (b) (i) 4-NMO, OsO_4, dioxane, room temp, 16 h; (ii) $NaIO_4$, room temp, 2 h; (iii) $NaBH_4$, EtOH/H_2O, room temp, 2 h, 59%; (c) (i) MsCl, pyridine, 0 °C, 1 h; (ii) NaN_3, DMF, 60 °C, 7 h, 97%; (d) Pd/C, H_2, MeOH, room temp, 3 h, 99%; (e) 3-R_2-4-R_1-phenylisothiocyanate, DMF, 0 °C, 1 h, 70−88%; (f) TBAF, THF, room temp, 1 h, 87−92%.

Scheme 2. Synthesis of α-D-Thymidine 5′-*O*-Monophosphate **9**[a]



[a] Reagents and conditions: (a) NH_3, MeOH, room temp, 16 h, 98%; (b) $POCl_3$, $P(OMe)_3$, 0 °C, 4 h; then room temp, 0.5 h, 62%.

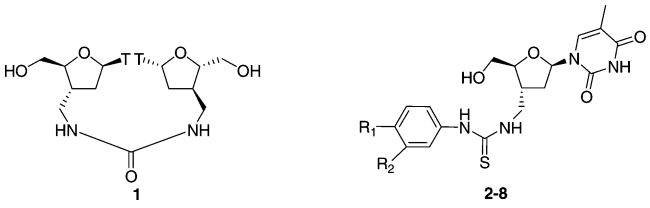
Scheme 3. Synthesis of 5′-Substituted α-Thymidine Derivatives **10**−**27** and **30**[a]



[a] Reagents and conditions: (a) (i) MsCl, pyridine, 0 °C, 1 h; (ii) NaN_3, DMF, 60 °C, 16 h, 68%; (b) H_2, Pd/C, MeOH, room temp, 6 h, 98%; (c) suitable iso(thio)cyanate, DMF, 71−91%; or suitable amine, 1,1-TCDI, DMF, room temp, 3 h, 75−82%.

cyanate, which was further reacted with 5′-amino-α-thymidine (**49**)[29] to afford the envisioned dinucleoside after final deprotection of the acetonide.

Table 1. Kinetic Parameters of TMPKmt with Compounds **1**−**8**



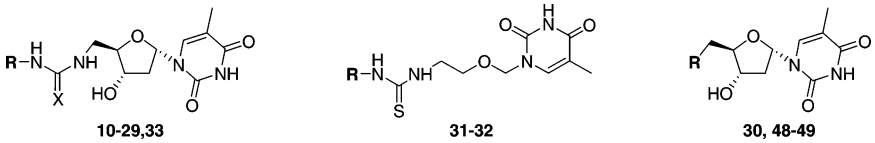
compd	R_1	R_2	K_i (μM) TMPKmt	K_i (μM) TMPKh	MCC (μM) Vero cell cultures[a]
dTMP			4.5[b]	5[b]	
1			37.0	>1000	
2	H	H	69.0		
3	Cl	H	21.0		>100
4	OCH_3	H	46.0		>100
5	CH_3	H	36.0		>100
6	Cl	Cl	7.2		>100
7	OCH_2Ph	H	12.0		>100
8	Cl	CF_3	5.0	>1000	>100

[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.

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 of 69.0 μM (Table 1). This value indicates a weaker binding to the enzyme than dinucleoside **1** (K_i = 37 μM) and the natural substrate (K_m = 4.5 μM). 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, the Topliss scheme[21] 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

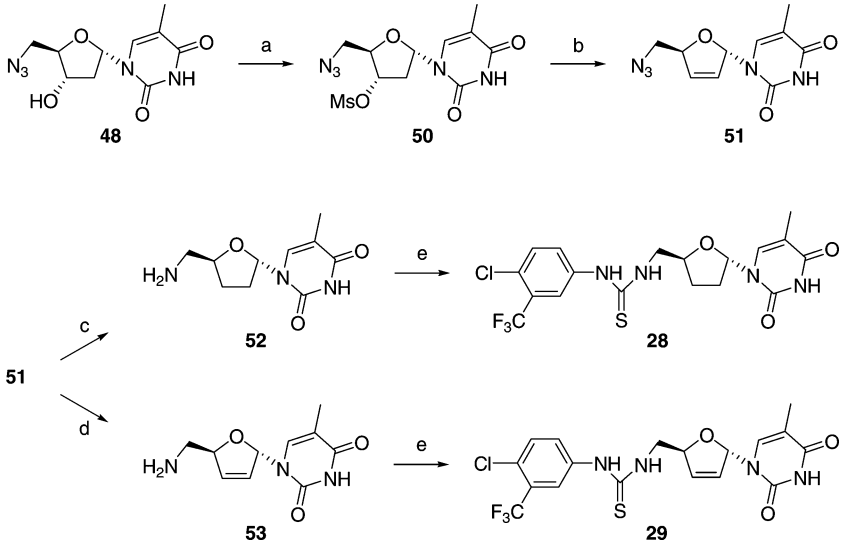
Table 2. Kinetic Parameters of TMPKmt with Compounds **9**−**33** and **48**−**49**



compd	R	X	K_i (μM) TMPKmt	K_i (μM) TMPKh	SI	MIC_{99} (μg/mL) *M. bovis*	inhibition (%) *M. tuberculosis* H37Rv[d]	MCC (μM) 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-di-Cl-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	fluoresceinyl	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-di-Cl-phenylethyl	S	2.2	79	40	50		>100
26	3,4-di-Cl-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		

[a] K_m value. [b] 3′-deoxyribonucleoside. [c] 3′-Deoxy-2′,3′-didehydronucleoside. [d] Inhibition at 6.25 μg/mL. [e] Minimum cytotoxic concentration in Vero cell cultures, i.e., the concentration required to cause microscopically detectable alteration of normal cell morphology.

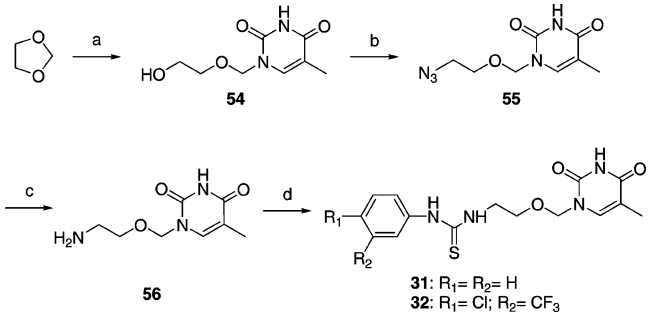
Scheme 4. Synthesis of 3′-Deoxy- and 3′-Deoxy-2′,3′didehydro-α-thymidinederivatives **28** and **29**[a]



[a] Reagents and conditions: (a) MsCl, pyridine, 0 °C, 2 h, 81%; (b) DBU, THF, 80 °C, 16 h, 95%; (c) H_2, Pd/C, MeOH, room temp, 5 h, 95%; (d) PPh_3, pyridine, room temp, 3 h, 92%; (e) 3-CF_3-4-Cl-phenylisothiocyanate, DMF, room temp, 3 h, 78%.

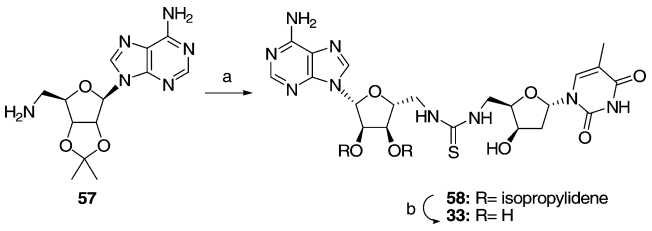
(**3**) resulted in a 3-fold increased activity. According to the Topliss decision tree, this increase confirms the positive effect of lipophilic and electron-withdrawing substituents on the inhibitory activity. This trend was validated by further improvement of the activity with a 3,4-dichloro substitution pattern (**6**), which further increased activity by another 3-fold (K_i = 7.2 μM). 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-CF_3-4-Cl substitution pattern was found to be the most potent inhibitor of this series with a

Scheme 5. Synthesis of Acyclic Derivatives **31** and **32**[a]



[a] Reagents and conditions: (a) silylated thymine, TMSOTf, acetonitrile, −45 °C for 2 h, then room temp, 16 h, 76%; (b) (i) MsCl, pyridine, 0 °C, 2 h; (ii) NaN_3, DMF, 60 °C, 16 h, 96%; (c) Pd/C, H_2, MeOH, room temp, 16 h, 99%; (d) 3-R_2-4-R_1-phenylisothiocyanate, DMF, room temp, 3 h, 83−86%.

Scheme 6. Synthesis of Adenosine Conjugate **33**[a]



[a] Reagents and conditions: (a) 5′-deoxy-5′-NH_2-α-thymidine (**49**), TCDI, DMF, room temp, 2 h; (b) 50% TFA, room temp, 2 h, 73%.

K_i of 5.0 μM, indicating that mainly the electronic features of the substituents positively influenced the inhibitory activity.

The good affinity observed among 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 the synthesis of these compounds, α-thymidine monophosphate was synthesized to investigate if the anticapted binding mode of these compounds was practicable. α-Thymidine monophosphate was indeed accepted as a substrate by TMPKmt (K_m = 450 μM, V_m = 0.77 U/mg at 2 mM ATP), thereby competing with dTMP (K_i= 15.0 μM). 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 substrates.[31]

In the 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 favor high activity. With a K_i of 0.6 μM, analogue **15** (R_1 = Cl, R_2= CF_3) proved to be the most potent TMPKmt inhibitor reported so far.

Because 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.

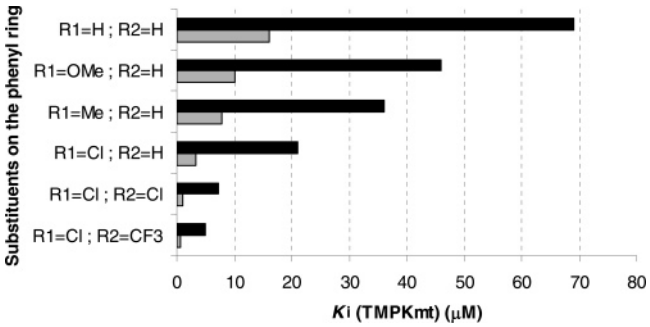


Figure 3. Comparison of the parallel synthesized α- and β- thiourea derivatives: (black bars) β-derivatives; (gray bars) α-derivatives.

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) from forming H-bonds and were therefore rejected. This rejection is justified because docking showed that the space close to Phe70 is too small to accommodate bulky substituents like a Cl or a CF_3 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 corresponding analogues from both series such as **2** and **10** (Figure 4) are compared, 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 favorably 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 one or two carbon atoms between the thioureum 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 the para and meta positions 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 fluorescein-labeled 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 sulfur of the thiourea linkage, urea derivatives **26** and **27** were synthesized. Because those compounds retain their good affinity for the

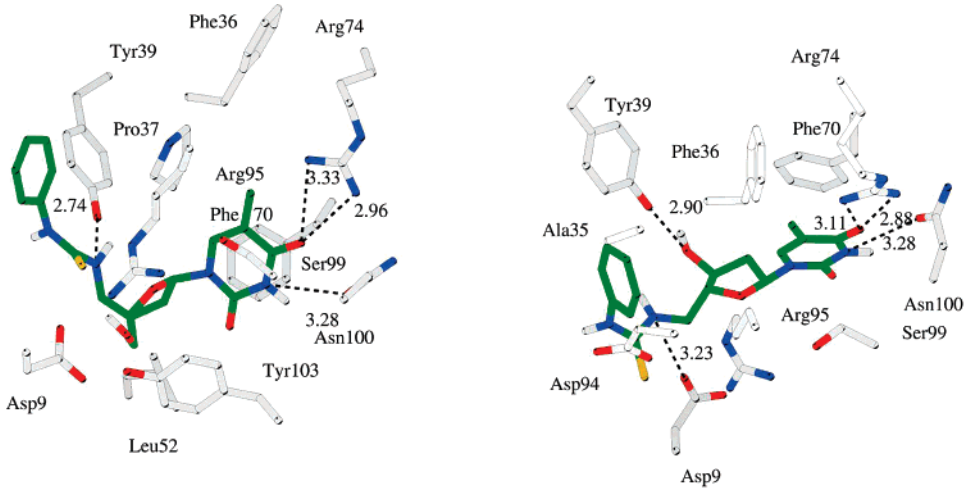


Figure 4. Compound **2** (left) and compound **10** (right) docked in the active site of TMPKmt. All residues interacting with the inhibitors (hydrophobic contact) are shown as well as the hydrogen-bonding parttern (calculated using Ligplot and HBPlus).[32,33] Drawings were created using Molscript.[34]

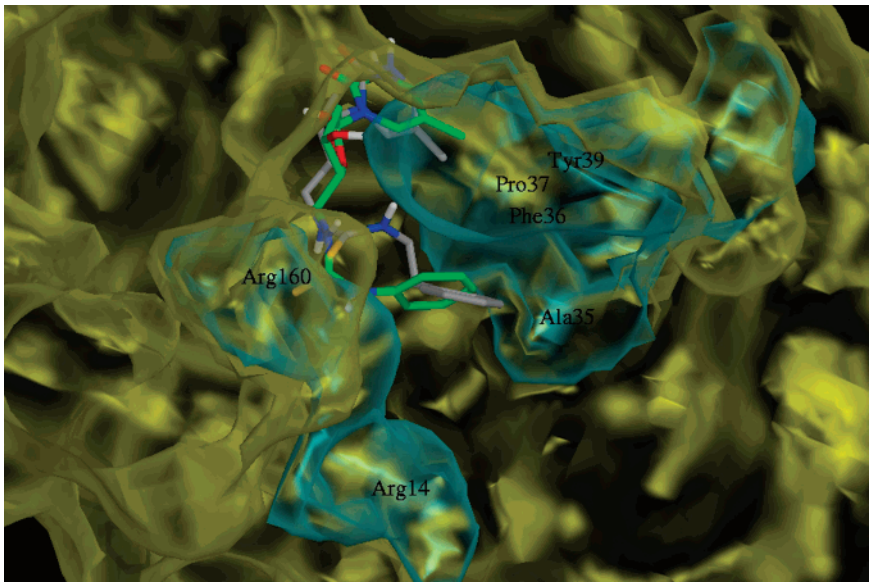


Figure 5. Docking and superposition of α-derivatives **10** (green carbons) and **24** (gray carbons) in the active site of TMPKmt, with **24** having a longer linker ($n = 2$). The phenyl tails of the inhibitors point to the front and seem to exit the enzyme through a channel lined up by residues Arg14,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

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 unfavorable for binding. Moreover, when the acyclic derivatives are compared with compound **10**, it is found that 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 inhibitors vis-à-vis the human isozyme was investigated. Most tested compounds proved to be highly selective for the mycobacterial enzyme. In particular, 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 to be superior in a *M. bovis* growth inhibition assay. Selected compounds were also tested for their capability to reduce *M. tuberculosis* $H_{37}Rv$ growth at 6.25 μg/mL, showing an inhibition of bacterial growth between 34% and 55% at this concentration.

For the first time, TMPKmt inhibitors proved to be capable of inhibiting 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 to be nontoxic 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, some 5′-modified adenosine derivatives have been discovered as powerful siderophore biosynthesis inhibitors.[22,23] Some of these analogues showed MIC_{99} values against *M. tuberculosis* H37Rv comparable to that of isoniazid, suggesting the presence of an 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 and earlier described as the perpetrator of toxicity,[38] showed nearly equal inhibitory activity against TMPKmt 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

On the basis of the structure of the recently discovered dinucleoside **1** as a selective TMPKmt inhibitor, this paper describes the synthesis and biological evaluation of a series of 3′-*C*-arylthiourea derivatives of β-D-thymidine. Optimization of the arylthiourea led to analogue **8** that, with a K_i of 5.0 μM, clearly surpassed **1** in inhibitory activity.

Modeling experiments suggested a distinct binding mode for these analogues, 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 TMPKmt 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 of 0.6 μM, a selectivity index (versus TMPKh) of 600, and a good inhibitory activity on the growing *M. bovis* (MIC_{99} = 20 μg/mL) and *M. tuberculosis* (39% inhibition at 6.25 μg/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′-arylthiorea 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.

Experimental Section

Spectrophotometric Binding Assay. The in vitro tests were done on TMPKmt and TMPKh, recombinant enzymes overexpressed in *E. coli*. TMPKmt and TMPKh 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 $MgCl_2$, 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 constant at 0.5 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 micromethod of culture was performed in 7H9 Middlebrook broth medium containing 0.2% glycerol and 0.5% Tween-80 and supplemented with oleic acid, albumin, dextrose, and catalase (Becton-Dickinson). Serial 2-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 °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 °C in plastic boxes containing a humidified normal atmosphere. At day 8 of incubation, an amount of 30 μL of a resazurin (Sigma) solution at 0.01% (wt/vol) in water was added to each well. After an overnight incubation at 37 °C, the plates were assessed for color development using the optical density difference at 570 and 630 nm on an ELISA reader. The change from blue to pink indicates reduction of resazurin and therefore bacterial growth. The lowest compound concentration that prevented the color change determined the MIC for the assayed compound.

Biological Assays on *Mycobacterium tuberculosis* H_{37}Rv. 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 μL). After 3 days of incubation at 37 °C in a humidified CO_2-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 after compound administration.

Molecular Modeling. 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 JChemPaint[42] 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 modeled 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 CSD[46] entry LEDRIV[47] 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 docked ligand conformations were 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 the residual solvent peak: in the case of DMSO-d_6, it is 2.54 ppm for ^{1}H and 40.5 ppm for ^{13}C; in the case of $CDCl_3$, it is

7.26 ppm for ^{1}H and 77.4 ppm for ^{13}C. All signals assigned to hydroxyl groups were exchangeable with D_2O. 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 μL/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 sulfuric 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 of benzene. The resulting solution was degassed with nitrogen during 30 min at room temperature and during the same time at 45 °C. 2,2′-Azabisisobutyronitrile (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 °C. The reaction mixture was refluxed again for 2 h. This procedure was repeated during 72 h. 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%). ^{1}H 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 $C_{34}H_{38}N_2O_4SiNa$ [M + 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 OsO_4 (1 mL, 0.16 mmol, 1% in water). After the mixture was stirred 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 and filtered through a Celite path, and solids were washed with ethyl acetate. The combined filtrates were washed with brine, dried over $MgSO_4$, and evaporated under reduced pressure. To the resulting crude aldehyde dissolved in ethanol/water (4:1, 45 mL) at 0 °C was added $NaBH_4$ (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 $MgSO_4$ and concentrated to dryness. After purification by column chromatography (CH_2Cl_2/MeOH, 97:3), the title compound (900 mg, 59% from **35**) was isolated as a white foam. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.00 (9H, s, *tert*-butyl), 1.49 (3H, s, 5-CH_3), 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 $C_{27}H_{34}N_2O_5SiNa$ [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 mixture was stirred at 0 °C during 1 h. The mixture was diluted with CH_2Cl_2 (25 mL), washed with saturated aqueous $NaHCO_3$, and dried over $MgSO_4$. The solvent was evaporated in vacuo to give the crude mesylate. The obtained residue was dissolved in DMF (50 mL) and treated with NaN_3 (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 CH_2Cl_2 (25 mL). The organic layer was washed with water, dried over $MgSO_4$, and evaporated to give a syrup, which was purified by column chromatography (CH_2Cl_2/MeOH, 99:1), yielding **37** (915 mg, 97%) as a white foam. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.00 (9H, s, *tert*-butyl), 1.50 (3H, d, J = 0.6 Hz, 5-CH_3), 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 $C_{27}H_{33}N_5O_4SiNa$ [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 h 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%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.00 (9H, s, *tert*-butyl), 1.48 (3H, d, J = 0.9 Hz, 5-CH_3), 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 $C_{27}H_{36}N_2O_4Si$ [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), phenyl isothiocyanate (15 mg, 0.11 mmol) in 1 mL of 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 (CH_2Cl_2/MeOH, 98:2), affording thiourea **39** (48 mg) in 70% yield. ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.99 (9H, s, *tert*-butyl), 1.45 (3H, s, 5-CH_3), 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 $C_{34}H_{41}N_4O_4SSi$ [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 tetra-*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 (CH_2Cl_2/MeOH, 97:3) to give pure compound **2** (27 mg) in 87% yield. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.75 (3H, s, 5-CH_3), 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). ^{13}C NMR (75 MHz, DMSO-d_6): δ 12.99 (5-CH_3), 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 $C_{18}H_{22}N_4O_4SNa$ [M + Na]$^+$ found, 413.1246; calcd, 413.1259. Anal. ($C_{18}H_{22}N_4O_4S$) C, H, N.

α-D-Thymidine (47). 3′,5′-Di-*O*-acetyl-α-thymidine[25] (4.54 g, 13.91 mmol) was dissolved in 150 mL of NH_3 in MeOH (7 N 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%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.78 (3H, d, J = 1.2 Hz, 5-CH_3), 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 $C_{10}H_{15}N_2O_5$ [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. $POCl_3$ (369 μL, 4.03 mmol) was added dropwise, and the mixture was stirred for 4 h at 0 °C and for 30 min at room temperature. The mixture was poured into ice−water (12 mL), neutralized with concentrated NH_4OH, and evaporated to dryness. The resulting residue was purified by column chromatography (^{i}PrOH/NH_4OH/H_2O, 77.5:15:2.5). Further purification was performed by HPLC (C-18, CH_3CN/MeOH/0.05% HCOOH in H_2O, 45:45:10, 3 mL/min). After lyophilization of the collected pure fractions, compound **9** was obtained (123 mg, 62%) as a white powder. ^{1}H NMR (300 MHz, D_2O): δ 1.81 (3H, d, J = 0.9 Hz, 5-CH_3), 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, D_2O): δ 2.97. ^{13}C NMR (75 MHz, D_2O): δ 11.73 (5-CH_3), 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 $C_{10}H_{14}N_2O_8PNa$ $[M + Na]^+$ found, 345.0477; calcd, 345.0464. Anal. ($C_{10}H_{14}N_2O_8P$) 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 μL, 0.41 mmol) was added. The reaction mixture was stirred for 1 h at 0 °C. The reaction was quenched by adding saturated aqueous $NaHCO_3$ solution and extracted with CH_2Cl_2 three times, dried over $MgSO_4$, and evaporated. The residue was purified by column chromatography (CH_2Cl_2/MeOH, 98:2) to give the mesylated compound (916 mg, 78%). ^{1}H NMR (300 MHz, DMSO-d_6): 1.75 (3H, s, 5-CH_3), 1.95 (1H, m, H-2′), 2.55 (1H, m, H-2″), 3.19 (3H, s, CH_3SO_2), 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 $C_{11}H_{17}N_2O_7S$ $[M + H]^+$ found, 321.0759; calcd, 321.0756.

A solution of 5′-mesylated α-D-thymidine (916 mg, 2.88 mmol) and NaN_3 (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 CH_2Cl_2 and washed with H_2O. The organic layer was dried over $MgSO_4$, evaporated, and purified by column chromatography (CH_2Cl_2/MeOH, 98:2) to afford compound **48** (672 mg, 87%). ^{1}H NMR (300 MHz, DMSO-d_6): 1.76 (3H, s, 5-CH_3), 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 $C_{10}H_{14}N_5O_4$ $[M + H]^+$ found, 268.1045; calcd, 268.1046. Anal. ($C_{10}H_{13}N_5O_4$) 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 h 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%). ^{1}H NMR (300 MHz, DMSO-d_6): 1.74 (3H, s, 5-CH_3), 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 $C_{10}H_{16}N_3O_4$ $[M + H]^+$ found, 242.1134; calcd, 242.1141. Anal. ($C_{10}H_{15}N_3O_4 \cdot {}^1/_2H_2O$) 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 (CH_2Cl_2/MeOH, 97:3) to obtain the pure final compound **10** (69 mg, 83%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.77 (3H, d, J = 1.2 Hz, 5-CH_3), 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). ^{13}C NMR (75 MHz, DMSO-d_6): δ 13.03 (5-CH_3), 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 $C_{17}H_{21}N_4O_4S$ $[M + H]^+$ found, 377.1279; calcd, 377.1283. Anal. ($C_{17}H_{20}N_4O_4S$) 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 of acetonitrile. After 10 min at 0 °C, the mixture was allowed to stir for 3 h at room temperature. A solution of **49** (75 mg, 0.31 mmol) in 2 mL of DMF was added, and the reaction mixture was stirred at room temperature overnight. The reaction mixture was evaporated to dryness and purified by column chromatography (CH_2Cl_2/MeOH, 99:1) to obtain compound **23** (114 mg, 75%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.77 (3H, d, J = 0.9 Hz, 5-CH_3), 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, CH_2NH), 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, CH_2NH), 11.20 (1H, br s, N(3)H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 13.00 (5-CH_3), 31.47 (CH_2NH), 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 $C_{19}H_{21}N_4O_4SClF_3$ $[M + H]^+$ found; calcd, 493.0924. Anal. ($C_{19}H_{20}N_4O_4SClF_3$) 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 (CH_2Cl_2/MeOH, 97:3), compound **26** (113 mg, 75%) was obtained. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.78 (3H, s, 5-CH_3), 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 $C_{17}H_{18}N_4O_5Cl_2Na$ $[M + Na]^+$ found, 451.0548; calcd, 451.0552. Anal. ($C_{17}H_{18}Cl_2N_4O_5$) 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 methanesulfonyl chloride (150 μL, 1.95 mmol) was added at 0 °C. After 2 h, the reaction was quenched by saturated $NaHCO_3$ solution (5 mL) and extracted with CH_2Cl_2 (3 × 10 mL). The combined organic layers were dried with $MgSO_4$ and evaporated to dryness to obtain pure compound **50** (420 mg, 81%). ^{1}H 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_2CH_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 $C_{11}H_{15}N_5O_6S$ $[M + H]^+$ 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 °C overnight. After cooling down, the reaction mixture was poured into NH_4Cl (15 mL) solution and extracted with CH_2Cl_2 (3 × 25 mL). The organic layers were dried over $MgSO_4$, evaporated, and purified by column chromatography (CH_2Cl_2/MeOH, 99:1) to yield 290 mg (95%) of the pure title compound. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.77 (3H, d, J = 1.0 Hz, 5-CH_3), 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 $C_{10}H_{11}N_5O_3Na$ [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 mixture was placed under a H_2 atmosphere. After 5 h, the reaction mixture was filtered through a plug of Celite, which was further washed with MeOH. The combined filtrates were evaporated to yield pure **52** as a white solid (128 mg, 95%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.70 (1H, m, H-3′), 1.77 (3H, d, J = 1.2 Hz, 5-CH_3), 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 $C_{10}H_{16}N_3O_3$ [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) was added triphenylphospine (135 mg, 1.6 mmol). The mixture was stirred for 3 h at room temperature, evaporated to dryness, and purified by column chromatography (CH_2Cl_2/MeOH, 90:10) to afford 65 mg of amine **53** (92%), which was used in the next step without purification. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.69 (3H, d, J = 1.2 Hz, 5-CH_3), 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 $C_{10}H_{14}N_3O_3$ [M + 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 sulfate (16 mg) was refluxed during 2 h under nitrogen atmosphere. After cooling to room temperature, the mixture was evaporated to dryness and redissolved in acetonitrile (32 mL). After the mixture was cooled to −45 °C, trimethylsilyl triflate (602 μL, 3.33 mmol) was added dropwise, followed by dropwise addition of dioxolane (470 mg, 6.34 mmol). After 2 h, the mixture 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 °C. The resulting mixture was extracted three times with diethyl ether (25 mL). The combined organic layers were dried over $MgSO_4$ and evaporated to dryness. The crude residue was purified by column chromatography (CH_2Cl_2/MeOH, 95:5) to obtain 480 mg (76%) of pure compound **54**. ^{1}H 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 $C_8H_{12}N_2O_4$ [M + 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 °C. After 2 h, 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 NaN_3 was added. The reaction mixture was heated at 60 °C during the night, evaporated, and purified by column chromatography (CH_2Cl_2/MeOH, 97:3), yielding 393 mg (96%) of **55**. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.75 (3H, d, J = 1.2 Hz, 5-CH_3), 3.39 (2H, t, J = 4.8 Hz, CH_2O), 4.64 (2H, t, J = 4.8 Hz, CH_2N_3), 5.07 (2H, s, OCH_2N), 7.56 (1H, d, J = 1.2 Hz, H-6), 11.30 (1H, br s, N(3)H). HRMS (ESI-MS) for $C_8H_{12}N_5O_3$ [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. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.77 (3H, s, 5-CH_3), 2.65 (2H, t, J = 5.7 Hz, CH_2NH_2), 3.43 (2H, t, J = 4.8 Hz, CH_2O), 5.05 (2H, s, OCH_2N), 7.48 (1H, s, H-6). HRMS (ESI-MS) for $C_8H_{14}N_3O_3$ [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 of DMF using the same procedure as described for the synthesis of **10**. After purification by column chromatography (CH_2Cl_2/MeOH, 99:1), thiourea **31** (94 mg, 86%) was obtained as a white powder. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.74 (3H, d, J = 0.9 Hz, 5-CH_3), 3.62 (4H, app s, CH_2CH_2), 5.06 (2H, s, OCH_2N), 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). ^{13}C NMR (75 MHz, DMSO-d_6): δ 12.56 (5-CH_3), 44.10 (*C*H_2NH′), 67.35 (*C*H_2O), 76.84 (O*C*H_2N), 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 $C_{15}H_{19}N_4O_3S$ [M + H]$^+$ found, 335.1175; calcd, 335.1177. Anal. ($C_{15}H_{18}N_4O_3S$) 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 (6 mg, 0.08 mmol), and 1,1-thiocarbonyldiimidazole (83 mg, 0.47 mmol) were dissolved in DMF (7 mL) at 0 °C, and after 10 min the mixture was allowed to warm up to room temperature. After 2 h, **49** (112 mg, 0.43 mmol) in 2 mL of DMF was added. After 3 h, the reaction was finished and the mixture was evaporated to dryness. The obtained crude compound was purified by column chromatography (CH_2Cl_2/MeOH, 95:5) to obtain 205 mg (82%) **58**. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.32 (3H, s, CCH_3 (A)), 1.53 (3H, s, CCH_3 (A)), 1.76 (3H, d, J = 0.9 Hz, 5-CH_3 (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, NH_2), 7.55 and 7.64 (2H, 2 × br s, 2 × N(5′)H), 7.73 (1H, d, J = 1.2 Hz, H-6), 8.18 and 8.33 (2H, 2 × s, H-2 and H-8 (A)), 11.24 (1H, br s, N(3)H). HRMS (ESI-MS) for $C_{24}H_{32}N_9O_7S$ [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 H_2O (10 mL) and stirred for 2 h at room temperature. The reaction mixture was evaporated to dryness and purified by column chromatography (CH_2Cl_2/MeOH 93:7) to obtain 98 mg of pure title compound (89%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.76 (3H, d, J = 1.2 Hz, 5-CH_3 (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, NH_2), 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 × s, H-2 and H-8 (A)), 11.25 (1H, br s, N(3)H). HRMS (ESI-MS) for $C_{21}H_{28}N_9O_7S$ $[M + H]^+$ found, 550.1833; calcd, 550.1832. Anal. ($C_{21}H_{27}N_9O_7S$) C, H, N.

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Supporting Information Available: Experimental section and analytical data for intermediates **40**−**45** and final products **3**−**8**, **11**−**22**, **24**, **25**, **27**−**30**, **32**; elemental analysis results of final products; and 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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