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Exploring Acyclic Nucleoside Analogues as Inhibitors of *Mycobacterium tuberculosis* Thymidylate Kinase

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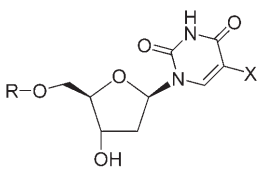
In the search for novel inhibitors of the enzyme thymidine monophosphate kinase of Mycobacterium tuberculosis *(TMPKmt), an attractive target for novel antituberculosis agents, we report herein the discovery of the first acyclic nucleoside analogues that potently and selectively inhibit TMPKmt. The most potent compounds in this series are (Z)-butenylthymines carrying a naphtholactam or naphthosultam moiety at position 4, which display K_i values of 0.42 and 0.27 μM, respectively. Docking studies followed by molecular dynamics simulations performed to rationalize the interaction of this new family of inhibitors with the target enzyme revealed a key interaction between the distal substituent and Arg 95 in the target enzyme. The fact that these inhibitors are more easily synthesizable than previously identified TMPKmt inhibitors, together with their potency against the target enzyme, makes them attractive lead compounds for further optimization.*

Introduction

Tuberculosis (TB) remains a major cause of morbidity and mortality worldwide. *Mycobacterium tuberculosis*, the causative agent of TB, is responsible each year for about eight million new cases and two million deaths. Today the treatment against TB involves three to four different drugs in combination over six to nine months, which leads to poor patient compliance and selection of drug resistant bacteria.[1–3] The increasing number of multidrug resistant strains and the lengthy therapy makes the discovery of novel TB drugs imperative. After 40 years with very few new chemical entities against TB, many efforts are being devoted to the discovery of new targets and the development of new drug candidates.[4–6]

In the search for novel targets, *M. tuberculosis* thymidine monophosphate kinase (TMPKmt) is an attractive candidate to interfere with the replication of the pathogen.[7–10] TMPK catalyzes the γ–phosphate transfer from ATP to thymidine monophosphate (**1**, dTMP) in the presence of magnesium ion yielding thymidine diphosphate (dTDP) and ADP. TMPK is crucial for maintaining the thymidine triphosphate (dTTP) pools that are required for DNA synthesis in replicating organisms. Subtle differences in the active site and in the biochemical properties between the mycobacterial (TMPKmt) and the human (TMPKh) isoenzyme make the former a potential target for selective inhibition of mycobacteria.[7,8]



1: X = CH_3; R = PO_3H
2: X = CH_3; R = H
3: X = Br; R = H

Initially it was considered that the potential inhibitors of TMPKmt should resemble the natural substrate dTMP (**1**) and therefore should incorporate a monophosphate moiety. However, such compounds suffer from several drawbacks as a consequence of the presence of the phosphate, such as degradation by phosphatases, low membrane permeability, etc. It was therefore fortunate to find that the nonphosphorylated nucleoside thymidine (**2**), with a free 5′-OH, has good affinity for TMPKmt.[9] Interestingly, a search of the *M. tuberculosis* genome[11] did not identify a gene coding for a thymidine kinase.[9] The observation that nucleosides such as 5-bromo-2′deoxyuridine (**3**), with a free 5′-OH, were able to efficiently inhibit the enzyme[9] has opened new possibilities in the search for novel chemical entities able to inhibit the target enzyme, TMPKmt. Moreover, it has also expanded the potential of this target for selective inhibition of mycobacteria replication and in fact several papers have reported nucleoside analogues that inhibit TMPKmt.[12–18]

Based on a structure-based drug-design program, thymidine analogues were synthesized where the ribose moiety was replaced by substituted benzyl groups. Some of these benzylic

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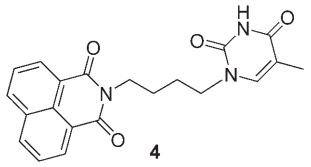
derivatives were inhibitory to TMPKmt with K_i values of approximately 12 μM for the most potent compound.[19] These results prompted us to expand the screening for novel TMPKmt inhibitors towards acyclic analogues of nucleosides that incorporate a thymine base. Acyclic nucleoside analogues have been extensively studied in the antiviral field,[20–22] and are now also being investigated as antimalarial drugs.[23] Because of the relaxed substrate specificity of some viral enzymes, it has been found that acyclic nucleoside analogues may afford an increase in the selectivity index (SI) because these compounds, in general, are better recognized by the pathogen enzymes than by their human counterparts. Moreover, acyclic nucleoside analogues lack a glycosidic linkage that makes them less prone to chemical or enzymatic degradation (that is, thymidine and structural analogues can be cleaved at the glycosidic bond by the human enzyme thymidine phosphorylase).[24]

We have screened against TMPKmt representative compounds from our own collection of acyclic nucleoside analogues containing a thymine base using a previously described coupled spectrophotometric enzymatic assay.[13] From such screening, compound **4** showed significant TMPKmt inhibition in the low micromolar range ($K_i = 42$ μM). Therefore compound



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4 was chosen as our lead compound to perform some structural variations. We started by keeping the thymine base intact and incorporating modifications in the spacer that connects the thymine base and the distal 1,8-naphthalimide. Keeping the thymine moiety was considered a wise starting point as it would maintain key interactions with a part of the enzyme active site that is also conserved in the apo form and is required to trigger the catalytic induced-fit mechanism.[25] In a second series of modifications, the thymine base was replaced by the closest structural analogues. Finally, modifications were performed at the distal substituent by reduction of one of the carbonyl groups of the naphthalimide moiety and further replacement of the naphthalimide by a naphtholactam or a naphthosultam moiety.

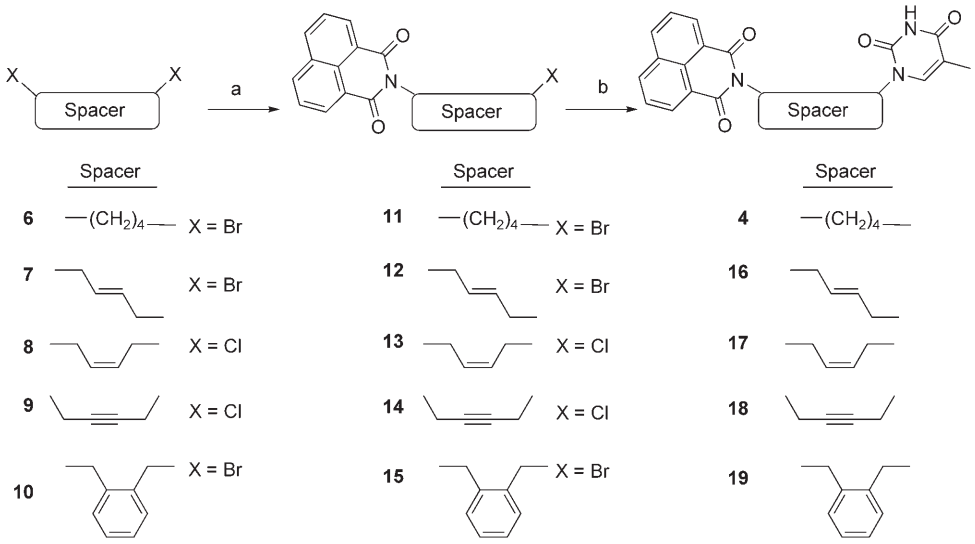
Results and Discussion

Chemistry

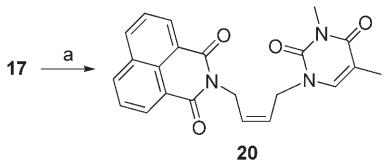
Our first series of compounds contain the thymine base to preserve the interaction at the nucleoside binding site of TMPKmt, connected to the distal 1,8-naphthalimide moiety through different spacers structurally related to the butyl chain present in our lead compound **4**. Such spacers included a (*E*) or (*Z*)-butenyl, butynyl, or a 2-methylbenzyl. The synthetic strategy consisted of a two-step procedure. Thus, treatment of 1,8-naphthalimide (**5**) with NaH in dry DMF followed by an in situ reaction with the corresponding dihalides (**6**–**10**) afforded the monohalo compounds **11**–**15** in good to excellent yields (65–95%) (Scheme 1). Bis-substitution is prevented by the employment of the dihalide in excess. Reaction of the halides **11**–**15** with silylated thymine in the presence of NaI in CH_3CN successfully afforded the target compounds **4**, **16**–**19**, with yields of approximately 80%, with the exception of compound **19** (45% yield). It should be mentioned that in all cases the only substitution product isolated is that of alkylation at N^1 of thymine.

When compounds **16**–**19** were evaluated on TMPKmt (see 'Enzymatic assays' below), it was found that the (*Z*)-butenyl derivative (**17**) improved the inhibitory potency against TMPKmt more than tenfold relative to the lead compound **4**. Thus, compound **17** was selected as a new lead to perform modifications both at the base and at the distal substituent.

The modifications performed at the base moiety of **17** involved methylation of the NH at the 3-position and replacement of the thymine base by the closest structural analogues, 5-bromo- and 5-iodouracil. Thus, reaction of **17** with MeI in the presence of DBU in DMAC afforded the N^3-methyl derivative (**20**) in 69% yield (Scheme 2).

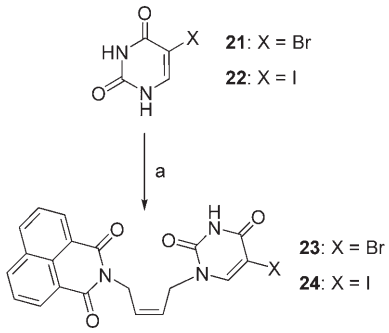


Scheme 1. Reagents and conditions: a) Benzo[*d*,*e*]isoquinoline-1,3(2*H*)-dione (**5**), NaH, DMF, 0 °C, 30 min, then addition of **6**–**10**, 80 °C, 5 h; b) thymine, *N*,*O*-bistrimethylsilylacetamide, CH_3CN, 80 °C till solubilization of thymine, then addition of **11**–**15**, NaI, CH_3CN, 80 °C, 8 h.



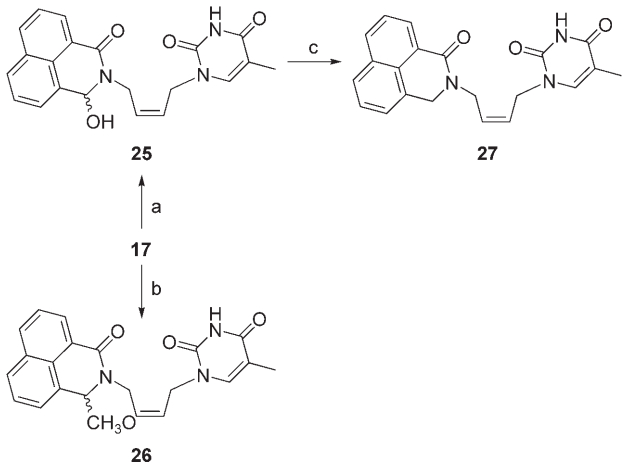
Scheme 2. Reagents and conditions: a) MeI, DBU, DMAC, RT.

On the other hand, reaction of the chloride **13** with silylated 5-bromouracil afforded the nucleoside analogue **23** in 85% yield (Scheme 3). Similarly, reaction of **13** with silylated 5-iodouracil led to the 5-iodouracil derivative **24** in 79% yield.



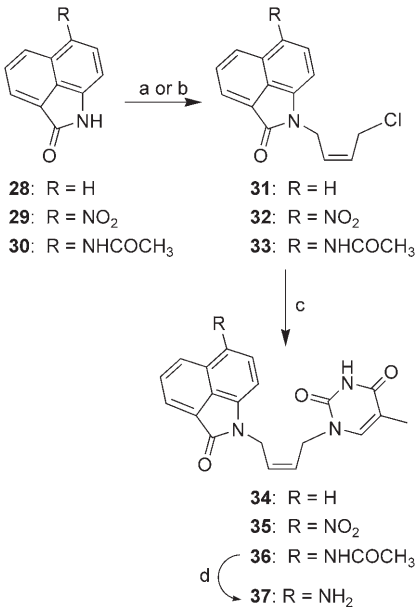
Scheme 3. Reagents and conditions: a) HMDS, $(NH_4)_2SO_4$ (cat), 150 °C, overnight, evaporation under vacuum, then addition of **13** (see Scheme 1), NaI, CH_3CN, 80 °C, 8 h.

Concerning the modifications at the distal substituent, it was considered of interest to explore how the reduction of one of the carbonyl groups of the naphthalimide moiety could affect the inhibitory properties of the lead compound **17**. Thus, reaction of **17** with $NaBH_4$ in EtOH[26] followed by treatment with NaOH afforded the hydroxy compound **25** in 66% yield (Scheme 4). Interestingly, if after performing the reduction of compound **17** with $NaBH_4$, the reaction was quenched by addition of MeOH, the corresponding methoxy derivative **26** was isolated. Further reduction of the hydroxy compound **25** with $NaBH_4$ in trifluoroacetic acid[26] led to **27** in 93% yield. As will be discussed later, compound **27** was found to be equipotent to the parent naphthalimide derivative **17**.



Scheme 4. Reagents and conditions: a) $NaBH_4$, EtOH, 55 °C, then HCl (3 N) and neutralization with NaOH (1 N); b) $NaBH_4$, EtOH, 55 °C, then HCl (3 N) and addition of MeOH; c) $NaBH_4$, TFA, RT.

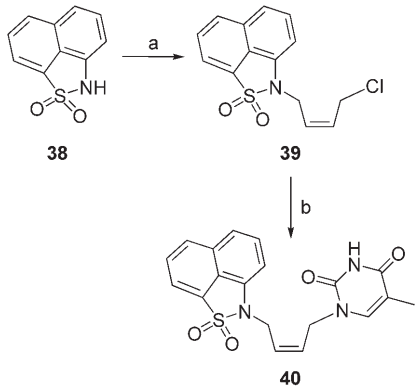
Therefore the next series of modifications involved replacement of the naphthalimide substituent by naphtholactams. Benzo[*c*,*d*]indol-2(1*H*)-one (naphtholactam) (**28**) (Scheme 5) was employed as the starting material for the next series of compounds. On one hand, the ease of alkylation of benzo[*c*,*d*]indol-2(1*H*)-one at the lactam nitrogen[27] under identical conditions to those already described in our first series of compounds is well described, allowing us to reach the targeted analogues in a two-step sequence. Moreover, the 6-position of benzo[*c*,*d*]indol-2(1*H*)-one can be easily nitrated permitting the study of how substituents at this position affect the interaction with the enzyme. Thus, the 6-nitrobenzo[*c*,*d*]indol-2(1*H*)-one and the 6-aminobenzo[*c*,*d*]indol-2(1*H*)-one were considered as interesting distal substituents to be incorporated in our lead structure. The 6-aminobenzo[*c*,*d*]indol-2(1*H*)-one was synthesized, as described,[28] through catalytic hydrogenation of the 6-nitrobenzo[*c*,*d*]indol-2(1*H*)-one (**29**). However, as this amino compound was quite unstable in our hands, the amino derivative obtained after hydrogenation was directly transformed into its acetamide derivative (**30**) and was used as such in the next steps. Thus, reaction of benzo[*c*,*d*]indol-2(1*H*)-one (**28**) or its 6-nitro analogue (**29**)[28] with (*Z*)-1,4-dichloro-2-butene in the presence of NaH, as described for other halides,[27,29] afford-



Scheme 5. Reagents and conditions: a) NaH, DMF, 0 °C, 30 min, then addition of (*Z*)-1,4-dichlorobut-2-ene, 80 °C, 1 h (conditions for **31** and **32**); b) NaOMe, DMF, RT, 15 min, then addition of (*Z*)-1,4-dichlorobut-2-ene, RT, 1 h (for **33**); c) thymine, *N*,*O*-bistrimethylsilylacetamide, CH_3CN, 80 °C till solubilization of thymine, then addition of **31**–**33**, NaI, CH_3CN, 80 °C, 8 h; d) HCl (1.6 M), dioxane, Δ, 3 h.

ed the monosubstituted compounds **31** and **32** in 84 and 54% yields, respectively. Alternatively, the 6-acetamide derivative **30** reacted with (*Z*)-1,4-dichloro-2-butene in the presence of NaOMe afforded **33** in 67% yield. Reaction of the chloro derivatives **31**–**33** with silylated thymine in the presence of NaI yielded the target compounds **34**–**36** in 77, 66, and 65% yields, respectively. Further treatment of **36** with HCl in dioxane at reflux afforded the 6-amino derivative **37** in 98% yield.

As a naphthosultam ring can be considered a good isosteric replacement for a naphtholactam,[27] the synthesis of the naphthosultam derivative **40** was undertaken following an analogous synthetic approach. Reaction of **38** with (*Z*)-1,4-dichloro-2-butene in the presence of NaH afforded the chloro intermediate **39** that reacted with silylated thymine (Scheme 6).



Scheme 6. Reagents and conditions: a) NaH, DMF, 0 °C, 30 min, then addition of (*Z*)-1,4-dichlorobut-2-ene, 80 °C, 2 h; b) thymine, *N*,*O*-bistrimethylsilylacetamide, CH_3CN, 80 °C till solubilization of thymine, then addition of **39**, NaI, CH_3CN, 80 °C.

Thus, the naphthosultam derivative **40** was obtained with a global yield of 48% for the two steps.

Enzymatic assays

The inhibitory potencies of the synthesized compounds **16**–**20**, **23**–**27**, **34**–**37**, and **40** were evaluated on TMPKmt using a spectrophotometric assay.[30] This assay is routinely used for TMPK activity determination and, in general, for other members of the nucleoside monophosphate kinase family, as it is fast and easily carried out. The results are shown in Table 1, where dTMP (**1**) and thymidine (dT, **2**) are also included for comparative purposes. From the testing of our initial series of compounds **16**–**20**, it became clear that the nature of the spacer has a great impact on the affinity of the compounds for the target enzyme. Thus, the (*Z*)-butenyl derivative (**17**) improved the inhibitory potency against TMPKmt more than tenfold relative to the lead compound **4**, whereas the (*E*)-butenyl (**16**) or the butynyl (**18**) derivatives were devoid of inhibitory activity at the tested concentration (100 μM). The benzylic derivative (**19**) could not be tested because of low solubility. From these data, it was concluded that the (*Z*)-butenyl derivative **17** was the most potent compound against TMPKmt in this series ($K_i = 1.9$ μM). Interestingly, the K_M of TMPKmt for dTMP is 4.5 μM, and the K_i for thymidine is 27 μM.

Table 1. Evaluation of acyclic nucleoside analogues on TMPKmt.

Compd	K_i [μM]	Compd	K_i [μM]
dTMP (**1**)	4.5[a]	**24**	NS[c]
dT (**2**)	27 ± 1.4	**25**	4.7 ± 0.9
4	42.0 ± 1.4	**26**	6.2 ± 0.8
16	NI[b]	**27**	1.4 ± 0.2
17	1.9 ± 0.6	**34**	0.42 ± 0.01
18	NI[b]	**35**	0.75 ± 0.05
19	NS[c]	**36**	6.0 ± 0.2
20	NI[b]	**37**	2.4 ± 0.4
23	1.1 ± 0.3	**40**	0.27 ± 0.01

[a] K_M value. [b] No inhibition at 100 μM. [c] Not soluble in the assay medium.

Methylation of the NH group at position 3 of the thymine moiety (compound **20**) renders a compound that shows no inhibition at 50 μM, pointing to the importance of the intact thymine base of the lead compound **17** for recognition by the target enzyme. This was further confirmed by molecular modeling studies. As mentioned in the introduction, 5-bromo-2′-deoxyuridine (**3**) is more potent than thymidine to inhibit the catalytic activity of TMPKmt.[9] Thus, it was reasonable to replace the thymine base in our new lead compound **17** by 5-bromouracil. The 5-bromo derivative **23** was as inhibitory to TMPKmt as the thymine analogue **17** (Table 1). Unfortunately, the K_i value of the 5-iodo derivative **24** could not be determined because of its low solubility in inorganic solvents.

Concerning the modifications at the distal substituent, the initial set of compounds (**25**–**27**) kept the inhibitory potency against the dTMP phosphorylation catalyzed by TMPKmt, as shown in Table 1. In particular, compound **27** ($K_i = 1.4$ μM) was equipotent to the parent naphthalimide derivative **17**, indicating that at least one of the carbonyl groups of the naphthalimide substituent was not required for interaction with the target enzyme. Thus, the next series of compounds incorporated a naphtholactam substituent instead of a naphthalimide. Interestingly, the naphtholactam derivative (**34**) exhibited a K_i value of 0.42 μM against dTMP phosphorylation, thus being fourfold more potent that the parent naphthalimide **17**. Introduction of a nitro group at position 6 of the naphtholactam (**35**) did not significantly affect the K_i value, whereas the 6-amino and 6-acetamide derivatives (**37** and **36**, respectively) afforded compounds that were still active although their K_i values were six- and 14-fold greater than that of the unsubstituted compound **34**. Finally, the naphthosultam derivative **40** gave the best K_i value of all the tested compounds ($K_i =$ 0.27 μM). This value makes this molecule, to the best of our knowledge, the most potent inhibitor described so far against the target enzyme TMPKmt.

Interestingly, two of the more potent acyclic nucleosides described herein (**17** and **27**) gave no inhibition of TMPKh at 0.02 mM, thus affording a selectivity index (SI = K_i (μM) TMPKh/ K_i (μM) TMPKmt) greater than 50. This great selectivity is corre-

lated to the absence of toxicity against VERO cells at concentrations up to 100 μg mL^{-1}.

Molecular modeling

Binding of representative compounds to the active binding site of TMPKmt was explored in silico using the automated docking program AutoDock, followed by molecular dynamics simulations. With respect to the crystal structure of the TMPKmt–dTMP complex,[8] only minor changes were observed in the complexes of the enzyme with the inhibitors, mostly affecting the rotamers of Tyr103 and Tyr165, together with a slight reorganization of the magnesium binding site although the cation maintained its co-ordination sphere with the oxygen atoms of Asp9, Asp163, Glu166, and a water molecule. The refined complexes indeed placed the inhibitors **17**, **34**, and **40** buried within the active site (Supporting Information, Figure S1) giving rise to a number of interactions. Thus, the thymine moiety binds inside the dTMP-binding cavity and stacks on the phenyl ring of Phe70, with NH3 establishing a good hydrogen bond with the side-chain oxygen group of Asn100 and O4 hydrogen bonding to the terminal guanidinium nitrogens of Arg74 (Figure 1). The spacer attached to the

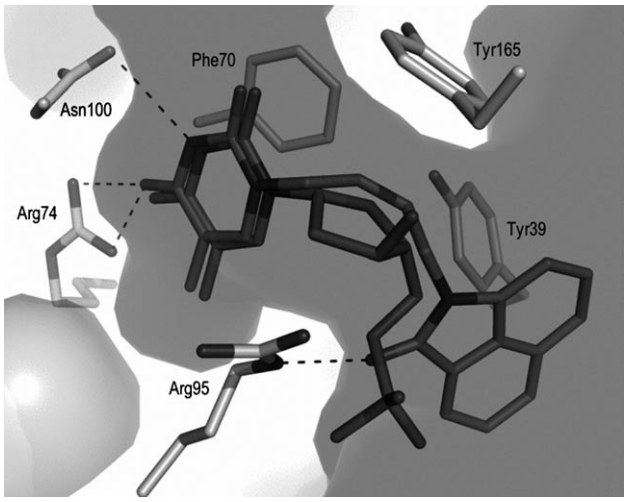


Figure 1. Detail of the substrate binding site in the complex of **34** with TMPKmt. Only the side chains of relevant residues (discussed in the text) are shown as sticks and are labeled, except for Pro37 and Tyr103, which have been removed for clarity. The black dashes represent the proposed hydrogen bonds involved in binding of the inhibitor. For comparison, the natural substrate dTMP, as found in PDB entry 1G3U following protein superimposition, is also displayed. Note the good alignment of anchoring points between substrate and inhibitor.

thymine interacts with the aromatic side chains of Tyr103 and Tyr165, which are oriented at a right angle with respect to each other whereas the naphthyl moiety establishes an edge-to-face stacking interaction with the aromatic ring of Tyr39.

The aromatic stacking interaction with the spacer's *cis* double bond seems to be crucial for compound stabilization because the inhibitory potency against TMPKmt decreases more than tenfold when the double bond is replaced by a single one (**4**) and is completely lost when the double bond is *trans* (**16**) or is replaced with a triple bond (**18**). It must be noted that for compound **4**, adoption of a docked geometry similar to that proposed for **17**, **34**, or **40** would involve an unfavorable *syn*-periplanar conformation about the central methylene bond. Besides, the latter three compounds containing either a naphthalimide (**17**), or a naphtholactam (**34**), or a naphthosultam (**40**) can engage an oxygen atom from these substituents in a good hydrogen bond with the guanidinium group of Arg95. This interaction thus mimics that observed between this same amino acid and the dTMP substrate. The observed differences in inhibitory activity among **17**, **34**, and **40** are likely due to differential interactions with residues Tyr39 and Pro37. All in all, a common pharmacophore for binding of substrate and inhibitors can be observed, as displayed in Figure 1 for representative compound **34**. The model also explains the experimental findings that the isosteric replacement of the thymine base in **17** by 5-bromouracil, as in **23**, does not affect the potency because either the methyl group or the bromine atom equally face the Cβ of residues Phe36 and Arg95.

Antimycobacterial evaluation

Selected compounds among those with the highest inhibitory activity in the enzymatic assays (that is, **17**, **34**, and **35**) were evaluated for their growth inhibitory activity against *M. bovis* BCG.[31] The concentrations tested ranged from 0.25 to 7.5 μg mL^{-1}. Unfortunately no significant inhibition of bacterial growth was observed.

Compounds **34** and **40** were also tested in vitro against *M. tuberculosis* following two well-established procedures: the proportion method on Middlebrook 7H11 agar medium[32] and the colorimetric REMA (resazurin microtiter plate assay) method.[31] Several mycobacterial strains were included: the H37Rv reference strain, an internationally used reference strain for genotyping of *M. tuberculosis*-complex (MT14323), and four clinical isolates (ITM 071951, ITM 071952, ITM 071960, ITM 021716). These isolates were previously found to show no (H37Rv, Mt14323, ITM 071952), limited (ITM 071951), or complete (ITM 021716, ITM 071960) resistance to the first-line anti-TB drugs. No anti-TB activity was detected at the concentrations tested (from 32 to 1 μg mL^{-1}) for both compounds.

Selected compounds (**17**, **34**, **36**, and **40**) were also tested by the TAACF service against *M. tuberculosis* H37Rv in BATEC medium using the microplate Alamar blue assay (MABA). In this assay, compound **36** showed weak activity with an IC_{50} value of 19 μg mL^{-1}.

Thus, the testing performed so far with the inhibitors described herein has not led to potent mycobacterial inhibition. At least two arguments can be raised to justify these experimental data: either the compounds are not able to efficiently cross the bacterial wall and reach the target enzyme and/or the concentrations attained are not enough to lead to a significant effect against mycobacterial growth. Still, we have now in hand compounds with K_i values against the target enzyme in the submicromolar range that deserve further exploration, in-

cluding, for example, further derivatization that should facilitate the crossing of the bacterial wall.

Conclusions

A series of acyclic nucleoside analogues containing a thymine base have been identified as novel and selective TMPKmt inhibitors. These compounds have been prepared following a designed synthetic strategy, in most cases in a two-step procedure using commercial products, so that the target compounds are readily obtained in good to excellent yields.

The compounds described herein are among the most potent TMPKmt inhibitors reported to date. In the naphthalimide series, compounds such as **17**, **23**, and **27** show K_i values in the low micromolar range (1.9, 1.1, and 1.4 μM). Moreover, replacement of the naphthalimide ring by a naphtholactam or naphthosultam (compounds **34**, **35**, and **40**) led to K_i values in the submicromolar range (0.42, 0.75, and 0.27 μM, respectively).

Experimental Section

Chemical procedures

Melting points were obtained on a Reichert-Jung Kofler apparatus and are uncorrected. Microanalyses were obtained with a Heraeus CHN-O-RAPID instrument. Electrospray mass spectra were measured on a quadrupole mass spectrometer equipped with an electrospray source (Hewlett–Packard, LC/MS HP 1100). ^{1}H and ^{13}C NMR spectra were recorded on a Varian Gemini operating at 200 MHz (^{1}H) and 50 MHz (^{13}C), and on a Varian INNOVA 300 operating at 299 MHz (^{1}H) and 75 MHz (^{13}C).

Analytical TLC was performed on silica gel 60 F_{254} (Merck) precoated plates (0.2 mm). Spots were detected under UV light (254 nm) and/or by charring with phosphomolybdic acid. Separations on silica gel were performed by preparative centrifugal circular thin layer chromatography (CCTLC) on a Chromatotron^R (Kieselgel 60 PF_{254} gipshaltig (Merck)); layer thickness: 1 or 2 mm; flow rate: 4 or 8 mL min^{-1}, respectively. Liquid chromatography was performed using a force flow (flash chromatography) Horizon HPFG system (Biotage) with Flash 25 or 40 silica gel cartridges. All experiments involving water-sensitive compounds were conducted under scrupulously dry conditions. Triethylamine and acetonitrile were dried by heating at reflux over calcium hydride. Tetrahydrofuran was dried by heating at reflux over sodium/benzophenone. Anhydrous *N*,*N*-dimethylformamide (DMF) was purchased from Aldrich. Anhydrous *N*,*N*-dimethylacetamide (DMAC) was purchased from Aldrich.

General procedure for the alkylation of benzo[*d*,*e*]isoquinoline-1,3(2*H*)-dione. NaH (60% oil suspension) (1.5 mmol) was added to a solution of benzo[*d*,*e*]isoquinoline-1,3(2*H*)-dione (**5**) (1.0 mmol) in dry DMF (1.0 mL) at 0 °C. The mixture was stirred at 0 °C for 30 min. Then, the corresponding alkyl or benzyl halide (1.7 mmol) was added and the mixture was heated at 80 °C for 5 h or till disappearance of starting material. After reaching room temperature, the reaction was quenched by addition of a NH_4Cl solution and evaporated to dryness. The resulting residue was purified by column chromatography.

2-(4-Bromobutyl)-1*H*-benzo[*d*,*e*]isoquinoline-1,3(2*H*)-dione (11).[33] Following the general procedure, benzo[*d*,*e*]isoquinoline-1,3(2*H*)-dione (197 mg, 1.0 mmol) reacted with 1,4-dibromobutane (**6**) (363 mg, 1.7 mmol) for 7 h. The residue was purified by flash column chromatography (hexane/EtOAc, 4:1) to yield 242 mg (73%) of **11** as a white solid. MS (ES, positive mode): *m/z* 332 $[M+1]^+$, with a Br isotopic pattern; ^{1}H NMR ([D$_6$]DMSO): $\delta = 1.63$–1.70 (m, 4H, CH_2CH_2), 3.56 (d, $J = 6.3$ Hz, 2H, CH_2), 4.06 (d, $J = 6.8$ Hz, 2H, CH_2), 7.85 (t, $J = 7.9$ Hz, 2H, Ar), 8.43–8.49 ppm (m, 4H, Ar); ^{13}C NMR ([D$_6$]DMSO): $\delta = 26.3$, 29.9, 34.7, 38.9 (CH_2), 122.0, 127.2, 127.4, 130.7, 131.3, 134.3 (Ar), 163.5 ppm (CO).

(*E*)-2-(4-Bromobut-2-enyl)-1*H*-benzo[*d*,*e*]isoquinoline-1,3(2*H*)-dione (12). Following the general procedure, benzo[*d*,*e*]isoquinoline-1,3(2*H*)-dione (197 mg, 1.0 mmol) reacted with (*E*)-1,4-dibromo-2-butene (**7**) (363 mg, 1.7 mmol) for 4 h. The residue was purified by flash column chromatography (CH_2Cl_2/MeOH, 20:1) to yield 287 mg (87%) of **12** as a white solid. MS (ES, positive mode): *m/z* 330 $[M+1]^+$, with a Br isotopic pattern; ^{1}H NMR ($CDCl_3$): $\delta = 3.91$ (d, $J = 5.6$ Hz, 2H, CH_2), 4.82 (d, $J = 3.4$ Hz, 2H, CH_2), 6.00 (m, 2H, CH=CH), 7.76 (t, $J = 8.0$ Hz, 2H, Ar), 8.23 (d, $J = 8.4$ Hz, 2H, Ar), 8.60 ppm (d, $J = 8.0$ Hz, 2H, Ar); ^{13}C NMR ($CDCl_3$): $\delta = 31.6$ 40.9 (CH_2), 127.0, 129.2 (CH=CH), 122.5, 130.0, 131.4, 131.6, 134.1 (Ar), 163.9 ppm (CO).

(*Z*)-2-(4-Chlorobut-2-enyl)-1*H*-benzo[*d*,*e*]isoquinoline-1,3(2*H*)-dione (13). Following the general procedure, benzo[*d*,*e*]isoquinoline-1,3(2*H*)-dione (197 mg, 1.0 mmol) reacted with (*Z*)-1,4-dichloro-2-butene (**8**) (0.18 mL, 1.7 mmol) for 5 h. The residue was purified by flash column chromatography (hexane/EtOAc, 1:1) to yield 270 mg (95%) of **13** as a white solid; MS (APCI): *m/z* 285 $[M]^+$, with a Cl isotopic pattern; ^{1}H NMR ($CDCl_3$): $\delta = 4.44$ (d, $J = 6.8$ Hz, 2H, CH_2), 4.87 (d, $J = 6.2$ Hz, 2H, CH_2), 5.83 (m, 2H, CH=CH), 7.75 (t, $J = 7.5$ Hz, 2H, Ar), 8.22 (dd, $J = 7.3$, 0.9 Hz, 2H, Ar), 8.60 ppm (dd, $J = 6.2$, 1.1 Hz, 2H, Ar); ^{13}C NMR ($CDCl_3$): $\delta = 36.8$, 39.2 (CH_2), 127.0, 128.0 (CH=CH), 122.5, 129.6, 131.4, 134.1 (Ar), 163.9 ppm (CO).

2-(4-Chlorobut-2-ynyl)-1*H*-benzo[*d*,*e*]isoquinoline-1,3(2*H*)-dione (14). Following the general procedure, benzo[*d*,*e*]isoquinoline-1,3(2*H*)-dione (197 mg, 1.0 mmol) reacted with 1,4-dichloro-2-butyne (**9**) (0.17 mL, 1.7 mmol) for 7 h. The residue was purified by flash column chromatography (CH_2Cl_2/MeOH, 30:1) to yield 152 mg (64%) of **14** as a white solid. MS (ES, positive mode): *m/z* 306 $[M+Na]^+$, with a Cl isotopic pattern; ^{1}H NMR ($CDCl_3$): $\delta = 4.12$ (t, $J = 2.0$ Hz, 2H, CH_2), 5.00 (t, $J = 2.0$ Hz, 2H, CH_2), 7.77 (dd, $J = 7.3$, 1.1 Hz, 2H, Ar), 8.24 (dd, $J = 7.4$, 1.1 Hz, 2H, Ar), 8.64 ppm (d, $J = 6.3$, 1.1 Hz, 2H, Ar); ^{13}C NMR ($CDCl_3$): $\delta = 29.6$, 30.4 (CH_2), 76.6, 81.4 (C≡C), 122.3, 127.0, 128.2, 131.6, 131.7, 134.4 (Ar), 163.4 ppm (CO).

2-[(2-Bromomethyl)benzyl]-1*H*-benzo[*d*,*e*]isoquinoline-1,3(2*H*)-dione (15). Following the general procedure, benzo[*d*,*e*]isoquinoline-1,3(2*H*)-dione (197 mg, 1.0 mmol) reacted with 1,2-bis(bromomethyl)benzene (**10**) (480 mg, 1.7 mmol) for 18 h. The residue was purified by column chromatography (hexane/EtOAc, 3:1) to yield 246 mg (64%) of **15** as a white solid. MS (ES, positive mode): *m/z* 380 $[M+1]^+$, with a Br isotopic pattern; ^{1}H NMR ($CDCl_3$): $\delta = 4.91$ (s, 2H, CH_2), 5.52 (s, 2H, CH_2), 7.19–7.21 (m, 2H, Ar), 7.35 (m, 2H, Ar), 7.77 (t, $J = 9.0$ Hz, 2H, Ar), 8.24 (d, $J = 8.4$ Hz, 2H, Ar), 8.62 ppm (d, $J = 7.2$ Hz, 2H, Ar); ^{13}C NMR ($CDCl_3$): $\delta = 31.8$, 40.3 (CH_2), 122.5, 127.0, 127.7, 128.2, 128.6, 129.1, 130.4, 131.6, 134.2, 135.9, 136.1 (Ar), 164.4 ppm (CO).

General Procedure for the reaction of compounds 11–15 with thymine. *N*,*O*-bis(trimethylsilyl)acetamide (0.6 mL) was added to a suspension of thymine (214 mg, 1.70 mmol) in dry CH_3CN (6 mL) and the mixture was heated at 80 °C until total solubilization of thymine. Then, NaI (73 mg, 0.5 mmol) and a solution of the corresponding halide **11**–**15** (1.0 mmol) in CH_3CN (4 mL) were added.

The reaction mixture was kept at 80 °C for 8 h. The mixture was filtered and the isolated solid contained the target compound. The filtrate was treated with a solution of sodium bisulfite (10 mL) and extracted with EtOAc (25 mL). The organic phase was dried on $MgSO_4$, filtered, and evaporated. The residue obtained was purified by column chromatography. The yields given include the amount obtained from the isolated solid together with that obtained after column chromatography.

2-(4-(Thymin-1-yl)butyl)-1*H*-benzo[*d*,*e*]isoquinoline-1,3(2*H*)-dione (4). According to the general procedure, thymine (128 mg, 1.02 mmol) reacted with the bromide **11** (200 mg, 0.60 mmol). After purification by column chromatography (CH_2Cl_2/MeOH, 20:1), 192 mg (85%) of **11** was obtained; mp: 219–220 °C; MS (ES, positive mode): *m/z* 378 $[M+1]^+$; ^{1}H NMR ([D$_6$]DMSO): $\delta = 1.62$ (s, 3H, 5-CH_3), 1.60–1.70 (m, 4H, CH_2), 3.51 (m, 2H, CH_2), 4.10 (m, 2H, CH_2), 7.52 (s, 1H, H-6), 7.85 (t, $J = 7.5$ Hz, 2H, Ar), 8.42–8.50 (m, 4H, Ar), 11.17 ppm (br s, 1H, NH); ^{13}C NMR ([D$_6$]DMSO): $\delta = 12.3$ (5-CH_3), 24.9, 26.5, 39.1, 47.3 (CH_2), 108.8 (C-5), 122.4, 127.6, 131.1, 131.6, 134.7 (Ar), 141.8 (C6), 151.2 (C2), 163.8 (CO), 164.6 ppm (C4); Anal. ($C_{21}H_{19}N_3O_4$): C, H, N.

(*E*)-2-(4-(Thymin-1-yl)but-2-enyl)-1*H*-benzo[*d*,*e*]isoquinoline-1,3(2*H*)-dione (16). According to the general procedure, thymine (130 mg, 1.03 mmol) reacted with **12** (200 mg, 0.61 mmol). After purification by column chromatography (CH_2Cl_2/MeOH, 20:1), 181 mg (79%) of **16** was obtained; mp: 265–266 °C; MS (ES, positive mode): *m/z* 376 $[M+1]^+$; ^{1}H NMR ([D$_6$]DMSO): $\delta = 1.70$ (s, 3H, 5-CH_3), 4.18 (d, $J = 4.6$ Hz, 2H, CH_2), 4.64 (d, $J = 4.4$ Hz, 2H, CH_2), 5.71 (m, 2H, CH=CH), 7.41 (s, 1H, H-6), 7.86 (t, $J = 9.4$ Hz, 2H, Ar), 8.44–8.50 (m, 4H, Ar), 11.20 ppm (br s, 1H, NH); ^{13}C NMR ([D$_6$]DMSO): $\delta = 11.8$ (5-CH_3), 40.6, 47.8 (CH_2), 108.6 (C5), 126.8, 127.9 (CH=CH), 122.0, 127.2, 127.4, 130.8, 131.3, 134.4 (Ar), 140.9 (C6), 150.6 (C2), 163.1 (CO), 164.2 ppm (C4); Anal. ($C_{21}H_{17}N_3O_4 \cdot 0.5\,H_2O$): C, H, N.

(*Z*)-2-(4-(Thymin-1-yl)but-2-enyl)-1*H*-benzo[*d*,*e*]isoquinoline-1,3(2*H*)-dione (17). According to the general procedure, thymine (150 mg, 1.19 mmol) reacted with **13** (200 mg, 0.70 mmol). After purification by flash column chromatography (CH_2Cl_2/MeOH, 20:1), 207 mg (79%) of **17** was obtained; mp: 235–236 °C, MS (ES, positive mode): *m/z* 376 $[M+1]^+$; ^{1}H NMR ([D$_6$]DMSO): $\delta = 1.78$ (s, 3H, 5-CH_3), 4.55 (d, $J = 5.9$ Hz, 2H, CH_2), 4.82 (d, $J = 5.8$ Hz, 2H, CH_2), 5.65 (m, 2H, CH=CH), 7.57 (s, 1H, H-6), 7.88 (t, $J = 8.0$ Hz, 2H, Ar), 8.46–8.53 (m, 4H, Ar), 11.30 ppm (br s, 1H, NH); ^{13}C NMR ([D$_6$]DMSO): $\delta = 12.4$ (5-CH_3), 37.4, 44.1 (CH_2), 109.1 (C5), 127.6, 128.7 (CH=CH), 122.4, 131.1, 131.7, 134.9 (Ar) 141.3 (C6), 151.2 (C2), 163.7 (CO), 164.6 ppm (C4); Anal. ($C_{21}H_{17}N_3O_4$): C, H, N.

2-(4-(Thymin-1-yl)butynyl)-1*H*-benzo[*d*,*e*]isoquinoline-1,3(2*H*)-dione (18). According to the general procedure, thymine (90 mg, 0.72 mmol) reacted with **14** (120 mg, 0.42 mmol). The solid isolated by filtration afforded 132 mg (84%) of **18**; mp: 245–246 °C; MS (ES, positive mode): *m/z* 374 $[M+1]^+$; ^{1}H NMR ([D$_6$]DMSO): $\delta = 1.72$ (s, 3H, 5-CH_3), 4.45 (s, 2H, CH_2), 4.84 (s, 2H, CH_2), 7.53 (s, 1H, H-6), 7.89 (t, $J = 7.6$ Hz, 2H, Ar), 8.48–8.52 (m, 4H, Ar), 11.30 ppm (br s, 1H, NH); ^{13}C NMR ([D$_6$]DMSO): $\delta = 12.0$ (5-CH_3), 29.7, 36.8 (CH_2), 76.7, 81.2 (C≡C), 109.7 (C5), 122.3, 127.5, 128.4, 130.8, 131.9, 135.0 (Ar), 139.8 (C6), 150.6 (C2), 163.0 (CO), 163.8 ppm (C4); Anal. ($C_{21}H_{15}N_3O_4$): C, H, N.

2-[2-(Thymin-1-yl)methyl)benzyl]-1*H*-benzo[*d*,*e*]isoquinoline-1,3(2*H*)-dione (19). According to the general procedure, thymine (86 mg, 0.68 mmol) reacted with **15** (150 mg, 0.40 mmol). After purification by column chromatography (CH_2Cl_2/MeOH, 30:1), 76 mg (45%) of **19** was obtained; mp: 290–291 °C; MS (ES, positive mode): *m/z* 448 $[M+Na]^+$; ^{1}H NMR ([D$_6$]DMSO): $\delta = 1.76$ (s, 3H, 5-CH_3), 5.11 (s, 2H, CH_2), 5.31 (s, 2H, CH_2), 7.06–7.25 (m, 4H, Ar), 7.57 (s, 1H, H-6), 7.75 (t, $J = 7.4$ Hz, 2H, Ar), 8.51 (m, 4H, Ar), 11.41 ppm (br s, 1H, NH); ^{13}C NMR ([D$_6$]DMSO): $\delta = 12.0$ (5-CH_3), 40.3, 47.2 (CH_2), 109.3, 121.9, 126.0, 127.1, 127.2, 127.4, 127.6, 127.8, 131.1, 131.4, 134.0, 134.7, 134.9 (Ar), 141.0 (C6), 151.1 (C2), 163.6 (CO), 164.4 ppm (C4); Anal. ($C_{25}H_{19}N_3O_4 \cdot 2\,H_2O$): C, H, N.

(*Z*)-2-(4-(3-Methylthymin-1-yl)but-2-enyl)-1*H*-benzo[*d*,*e*] isoquinoline-1,3(2*H*)-dione (20). DBU (0.09 mL, 0.6 mmol) and MeI (0.09 mL, 1.5 mmol) were added to a suspension containing **17** (192 mg, 0.5 mmol) in *N*,*N*-dimethylacetamide (5 mL). The reaction was stirred at room temperature overnight. Volatiles were removed and a mixture of hexane/ethyl ether (1:1) (20 mL) was added. The mixture was kept at −20 °C overnight. Solvents were decanted and the residue was purified by CCTLC in the Chromatotron (CH_2Cl_2/MeOH, 20:1) to yield 133 mg (69%) of **20** as a white solid; mp: 219–220 °C; MS (ES, positive mode): *m/z* 390 $[M+1]^+$; ^{1}H NMR ([D$_6$]DMSO): $\delta = 1.84$ (s, 3H, 5-CH_3), 3.18 (s, 3H, NCH_3), 4.62 (d, $J = 5.7$ Hz, 2H, CH_2), 4.83 (d, $J = 5.9$ Hz, 2H, CH_2), 5.58–5.79 (m, 2H, CH=CH), 7.69 (s, 1H, H-6), 7.87 (t, $J = 7.3$ Hz, 2H, Ar), 8.45–8.53 ppm (m, 4H, Ar); ^{13}C NMR ([D$_6$]DMSO): $\delta = 12.6$ (5-CH_3), 30.6 (NCH_3), 37.0, 45.0 (CH_2), 107.8 (C5), 127.1, 128.7 (CH=CH), 122.0, 130.8, 131.3, 134.5 (Ar), 139.4 (C6), 150.1 (C2), 163.3 (CO), 165.1 ppm (C4); Anal. ($C_{22}H_{19}N_3O_4$): C, H, N.

(*Z*)-2-(4-(5-Bromouracil-1-yl)but-2-enyl)-1*H*-benzo[*d*,*e*] isoquinoline-1,3(2*H*)-dione (23). A suspension of 5-bromouracil (**21**) (304 mg, 1.6 mmol) in hexamethyldisilazane (HMDS) (2.5 mL) was heated at 150 °C in the presence of ammonium sulfate (5 mg) overnight till it became a clear solution. The excess of HMDS was removed under reduced pressure. Then, the halide **13** (228 mg, 0.8 mmol), NaI (58 mg, 0.4 mmol) in dry CH_3CN (12 mL) were added to the silylated base. The mixture was heated at 80 °C overnight. The reaction was allowed to reach room temperature, diluted with EtOAc (10 mL) and washed with a cooled $NaHCO_3$ solution (10 mL). The organic phase was decanted, dried on $MgSO_4$, filtered, and evaporated. The residue was purified by flash column chromatography (CH_2Cl_2/MeOH, 30:1) to yield 298 mg (85%) of **23**; mp: 258–259 °C; MS (ES, positive mode): *m/z* 440 $[M+1]^+$ with a Br isotopic pattern; ^{1}H NMR ([D$_6$]DMSO): $\delta = 4.72$ (d, $J = 6.1$ Hz, 2H, CH_2), 4.94 (d, $J = 6.2$ Hz, 2H, CH_2), 5.81 (m, 2H, CH=CH), 8.01 (t, $J = 9.0$ Hz, 2H, Ar), 8.40 (s, 1H, H-6), 8.58–8.61 (m, 4H, Ar), 12.2 ppm (br s, 1H, NH); ^{13}C NMR ([D$_6$]DMSO): $\delta = 37.0$, 44.4 (CH_2), 94.8 (C5), 127.0, 128.7 (CH=CH), 122.0, 127.3, 127.4, 130.8, 131.4, 134.5 (Ar), 145.0 (C6), 150.3 (C2), 159.6, 163.5 ppm (CO, C4); Anal. ($C_{20}H_{14}BrN_3O_4$): C, H, N.

(*Z*)-2-(4-(5-Iodouracil-1-yl)but-2-enyl)-1*H*-benzo[*d*,*e*]isoquinoline-1,3(2*H*)-dione (24). Following a procedure analogous to that described for the synthesis of **23**, 5-Iodouracil (**22**) (237 mg, 1.0 mmol) reacted with **13** (142 mg, 0.5 mmol). The final residue was purified by flash column chromatography (CH_2Cl_2/MeOH, 30:1) to yield 192 mg (79%) of **24**; mp: 259–260 °C; MS (ES, positive mode): *m/z* 488 $[M+1]^+$; ^{1}H NMR ([D$_6$]DMSO): $\delta = 4.59$ (d, $J = 6.2$ Hz, 2H, CH_2), 4.81 (d, $J = 6.3$ Hz, 2H, CH_2), 5.63 (m, 2H, CH=CH), 7.88 (t, $J = 8.1$ Hz, 2H, Ar), 8.29 (s, 1H, H-6), 8.45–8.53 (m, 4H, Ar), 11.68 ppm (br s, 1H, NH); ^{13}C NMR ([D$_6$]DMSO): $\delta = 37.0$, 44.4 (CH_2), 68.4 (C-5), 127.0, 128.7 (CH=CH), 122.1, 127.3, 127.4, 130.8, 131.4, 134.5 (Ar), 149.7 (C6), 150.7 (C2), 161.1, 163.7 ppm (CO, C4); Anal. ($C_{20}H_{14}IN_3O_4$): C, H, N.

(*Z*)-2-(4-Thymin-1-yl)but-2-enyl)-3-hydroxy-2,3-dihydro-1*H*-benzo[*d*,*e*]isoquinolin-1-one (25). $NaBH_4$ (378 mg, 10.0 mmol) was added in small portions for 10 min to a suspension containing **17**

(375 mg, 1.0 mmol) in EtOH (40 mL). The mixture was heated at 55 °C for 2 h, while it became into a yellow solution. Then, HCl·3 N (4 mL) was added and a white precipitate was formed. The reaction was finally neutralized by addition of NaOH 50%. Volatiles were removed and the residue was purified by flash column chromatography (EtOAc/Et_2O, 3:1) to yield 249 mg (66%) of **25** as a white solid; mp: 150–151 °C; MS (ES, positive mode): *m/z* 360 $[M+1-H_2O]^+$; ^{1}H NMR ([D$_6$]DMSO): $\delta = 1.76$ (s, 3H, 5-CH_3), 4.39–4.63 (m, 4H, CH_2), 5.57 (m, 1H, CH=CH), 5.77 (m, 1H, CH=CH), 6.20 (d, $J = 9.2$ Hz, 1H, CH), 6.82 (d, $J = 9.2$ Hz, 1H, OH), 7.62–7.72 (m, 4H, Ar, H-6), 8.04 (m, 1H, Ar), 8.03–8.09 (m, 2H, Ar), 11.30 ppm (br s, 1H, NH); ^{13}C NMR ([D$_6$]DMSO): $\delta = 12.0$ (5-CH_3), 41.2, 43.7 (CH_2), 79.8 (CHOH), 108.8 (C5), 126.8, 127.1 (CH=CH), 123.7, 126.0, 126.4, 126.6, 127.3, 127.7, 130.5 131.6, 131.8, 131.9 (Ph), 141.2 (C6), 151.0 (C2), 161.8 (CO), 164.3 ppm (C4); Anal. ($C_{21}H_{19}N_3O_4 \cdot 0.5\,H_2O$): C, H, N.

(*Z*)-2-(4-Thymin-1-yl)but-2-enyl)-3-methoxy-2,3-dihydro-1*H*-benzo[*d*,*e*]isoquinolin-1-one (26). Compound **17** (375 mg, 1.0 mmol) reacted with $NaBH_4$ (378 mg, 10.0 mmol) as described for the synthesis of **25**. After addition of 3 N·HCl, MeOH (3 mL) was added. Volatiles were removed and the residue was purified by flash column chromatography (EtOAc/Et_2O, 3:1) to yield 133 mg (34%) of **26** as a white solid; mp: 154–155 °C; MS (ES, positive mode): *m/z* 414 $[M+1]^+$; ^{1}H NMR ([D$_6$]DMSO): $\delta = 1.76$ (s, 3H, 5-CH_3), 2.76 (s, 3H, OCH_3), 4.26–4.63 (m, 4H, CH_2), 5.60 (m, 1H, CH=CH), 5.79 (m, 1H, CH=CH), 6.36 (s, 1H, CH), 7.64 (s, 1H, H-6), 7.76–7.71 (m, 2H, Ar), 8.10 (dd, $J = 6.1$, 3.5 Hz, 1H, Ar), 8.27 (d, $J = 6.8$ Hz, 3H, Ar), 11.30 ppm (br s, 1H, NH); ^{13}C NMR ([D$_6$]DMSO): $\delta = 12.2$ (5-CH_3), 40.0, 44.0 (CH_2), 49.4 (OCH_3), 86.0 ($CHOCH_3$), 109.1 (C5), 126.7, 128.9 (CH=CH), 123.4, 127.0, 127.1, 127.2, 127.5, 129.4, 131.6, 132.7 (Ar), 141.4 (C6), 151.1 (C2), 163.0 (CO), 164.4 ppm (C4); Anal. ($C_{22}H_{21}N_3O_4 \cdot H_2O$): C, H, N.

(*Z*)-2-(4-Thymin-1-yl)but-2-enyl)-2,3-dihydro-1*H*-benzo-[*d*,*e*]isoquinolin-1-one (27). $NaBH_4$ (378 mg, 10.0 mmol) was added in portions to a solution of **25** (377 mg, 1.0 mmol) in trifluoroacetic acid (TFA) (2 mL) at 0 °C. The reaction was allowed to reach room temperature and was further stirred for 8 h. The reaction was quenched by addition of cold MeOH and volatiles were removed. The residue was washed with H_2O, filtered, and the white solid was dried to yield 337 mg (93%) of **27**; mp: 202–203 °C; MS (ES, positive mode): *m/z* 362 $[M+1]^+$; ^{1}H NMR ([D$_6$]DMSO): $\delta = 1.75$ (s, 3H, 5-CH_3), 4.39 (d, $J = 6.4$ Hz, 2H, CH_2), 4.48 (d, $J = 6.4$ Hz, 2H, CH_2), 5.08 (s, 2H, CH_2), 5.70 (m, 2H, CH=CH), 7.47 (d, $J = 6.2$ Hz, 1H, Ar), 7.56–7.66 (m, 2H, Ar), 7.65 (s, 1H, H-6), 7.89 (d, $J = 8.1$ Hz, 1H, Ar), 8.08–8.15 (m, 2H, Ar), 11.30 ppm (br s, 1H, NH); ^{13}C NMR ([D$_6$]DMSO): $\delta = 12.6$ (5-CH_3), 44.1, 45.0, 50.6 (CH_2), 110.0 (C5), 126.3, 127.1 (CH=CH), 124.0, 125.0, 126.6, 127.4, 128.4, 128.7, 128.9, 129.1, 132.5, 132.7 (Ar), 142.2 (C6), 151.1 (C-2), 162.7 (CO), 164.4 ppm (C4); Anal. ($C_{21}H_{19}N_3O_3$): C, H, N.

6-Acetamidobenzo[*c*,*d*]indol-2(1*H*)-one (30). A solution of 6-nitrobenzo[*c*,*d*]indol-2(1*H*)-one (**29**)[28] (1.0 g, 4.67 mmol) in THF (75 mL) was placed in a Parr hydrogenation bottle and 110 mg of 5% Pd/C were added. The mixture was hydrogenated under 40 psi of H_2 for 9 h. The reaction mixture was filtered through Celite and the filtrate evaporated. The residue was purified by column chromatography (CH_2Cl_2/MeOH, 50:1) to yield 740 mg (86%) of 6-aminobenzo[*c*,*d*]indol-2(1*H*)-one; mp: 269–270 °C; mp (lit.): 266–268 °C.[28] In our hands, this compound tends to decompose and therefore was transformed into its corresponding acetamido derivative as follows. A solution containing 6-aminobenzo[*c*,*d*]indol-2(1*H*)-one (700 mg, 3.80 mmol) in glacial acetic acid (12 mL) and acetic anhydride (4.2 mL) was stirred at reflux for 1 h. Volatiles were removed and the residue was purified by flash column chromatography (CH_2Cl_2/MeOH, 20:1) to yield 584 mg (68%) of **30** as a yellow solid; mp: 260–262 °C; MS (ES): *m/z* 227 $[M+1]^+$; ^{1}H NMR ([D$_6$]DMSO): $\delta = 2.15$ (s, 3H, CH_3), 6.92 (d, 1H, $J = 7.6$ Hz, Ar), 7.70 (d, 1H, $J = 7.6$ Hz, Ar), 7.80 (dd, 1H, $J = 8.2$, 7.0 Hz, Ar), 8.00 (d, 1H, $J = 7.0$ Hz, Ar), 8.29 (d, 1H, $J = 8.2$ Hz, Ar), 9.97 (br s, 1H, NH), 10.7 ppm (br s, 1H, NH); ^{13}C NMR ([D$_6$]DMSO): $\delta = 24.0$ (CH_3), 106.7, 122.5, 124.3, 124.8, 126.1, 127.5, 128.1, 128.8, 129.0, 135.3 (Ar), 169.3, 169.4 ppm (CO).

1-((*Z*)-4-Chlorobut-2-enyl)benzo[*c*,*d*]indol-2(1*H*)-one (31). Following a procedure similar to that described in the general procedure for the alkylation of benzo[*d*,*e*]isoquinoline-1,3(2*H*)-dione, benzo-[*c*,*d*]indol-2-(1*H*)-one (**28**) (169 mg, 1.0 mmol) reacted with (*Z*)-1,4-dichloro-2-butene (180 μL, 1.7 mmol) in DMF (1 mL) for 1 h. The final residue was purified by flash column chromatography (hexane/EtOAc, 3:1) to yield 216 mg (84%) of **31** as a pale yellow solid. MS (ES): *m/z* 258 $[M+1]^+$, with a Cl isotopic pattern; ^{1}H NMR ([D$_6$]DMSO): $\delta = 4.34$ (d, 2H, $J = 7.9$ Hz, CH_2), 4.66 (d, 2H, $J = 6.6$ Hz, CH_2), 5.76 (m, 1H, CH=CH), 5.89 (m, 1H, CH=CH), 6.96 (d, 1H, $J = 7.0$ Hz, Ar), 7.47 (m, 1H, Ar), 7.55 (d, 1H, $J = 8.4$ Hz, Ar), 7.72 (m, 1H, Ar), 8.01 (d, 1H, $J = 8.1$ Hz, Ar), 8.08 ppm (d, 1H, $J = 7.0$ Hz, Ar); ^{13}C NMR ([D$_6$]DMSO): $\delta = 37.0$, 38.9 (CH_2), 127.2, 128.7 (CH=CH), 105.6, 120.8, 124.7, 125.5, 126.5, 128.9, 129.4, 129.5, 131.2, 139.0 (Ar), 167.8 ppm (CO).

1-((*Z*)-4-Chlorobut-2-enyl)-6-nitrobenzo[*c*,*d*]indol-2(1*H*)-one (32). Following a procedure similar to that described in the general procedure for the alkylation of benzo[*d*,*e*]isoquinoline-1,3(2*H*)-dione, 6-nitrobenzo[*c*,*d*]indol-2-(1*H*)-one (**29**)[28] (216 mg, 1.0 mmol) reacted with (*Z*)-1,4-dichloro-2-butene (180 μL, 1.7 mmol) for 1 h. The final residue was purified by flash chromatography (hexane/EtOAc, 3:1) to yield 164 mg (54%) of **32** as a yellow solid. MS (ES): *m/z* 302 $[M+1]^+$, with a Cl isotopic pattern; ^{1}H NMR ([D$_6$]DMSO): $\delta = 4.51$ (d, 2H, $J = 7.9$ Hz, CH_2), 4.68 (d, 2H, $J = 5.6$ Hz, CH_2), 5.74 (m, 1H, CH=CH), 5.85 (m, 1H, CH=CH), 7.27 (d, 1H, $J = 8.1$ Hz, Ar), 8.02 (d, 1H, $J = 7.0$ Hz, Ar), 8.19 (d, 1H, $J = 6.8$ Hz, Ar), 8.64 (d, 1H, $J = 8.1$ Hz, Ar), 8.82 ppm (d, 1H, $J = 8.3$ Hz, Ar); ^{13}C NMR ([D$_6$]DMSO): $\delta = 38.6$, 40.3 (CH_2), 128.5, 129.3 (CH=CH), 104.7, 122.7, 124.6, 125.4, 126.0, 129.5, 130.6, 132.6, 138.5, 145.1 (Ar), 167.0 ppm (CO).

***N*-1-((*Z*)-4-Chlorobut-2-enyl)-6-acetamidobenzo[*c*,*d*]indol-2(1*H*)-one (33).** NaOMe (54 mg, 1.0 mmol) was added to a solution of **30** (226 mg, 1.0 mmol) in dry DMF (6.3 mL) and the mixture was stirred at room temperature for 15 min. Then, the (*Z*)-1,4-dichloro-2-butene (370 μL, 3.5 mmol) was added and the mixture was stirred for 1 h. The reaction was quenched by addition of an NH_4Cl solution and evaporated to dryness. The resulting residue was purified by flash chromatography (CH_2Cl_2/MeOH, 50:1) to yield 210 mg (67%) of **33** as a yellow solid. MS (ES): *m/z* 315 $[M+1]^+$ with a Cl isotopic pattern; ^{1}H NMR ([D$_6$]DMSO): $\delta = 2.16$ (CH_3), 4.51 (d, 2H, $J = 7.6$ Hz, CH_2), 4.63 (d, 2H, $J = 6.6$ Hz, CH_2), 5.73 (m, 1H, CH=CH), 5.82 (m, 1H, CH=CH), 7.09 (d, 1H, $J = 7.6$ Hz, Ar), 7.77 (dd, 1H, $J = 7.8$, 6.6 Hz, Ar), 7.83 (d, 1H, $J = 7.0$ Hz, Ar), 8.08 (d, 1H, $J = 7.0$ Hz, Ar), 8.33 (d, 1H, $J = 8.2$ Hz, Ar), 10.01 ppm (br s, 1H, NHAc); ^{13}C NMR ([D$_6$]DMSO): $\delta = 23.5$ (CH_3), 36.3, 40.3 (CH_2), 128.0, 128.5 (CH=CH), 106.2, 121.6, 124.0, 124.4, 125.8, 128.9, 129.3, 129.4, 135.0 (Ar), 166.5, 169.0 ppm (CO).

(*Z*)-1-(4-(Thymin-1-yl)but-2-enyl)-1*H*-benzo[*c*,*d*]indol-2(1*H*)one (34). The halide **31** (258 mg, 1.0 mmol) was made to react with thymine (252 mg, 2.0 mmol) as already described for compounds **11**–**15**. The solid isolated by filtration afforded 267 mg (77%) of **31** as a white solid; mp: 208–209 °C; MS (ES): *m/z* 348 $[M+1]^+$; ^{1}H NMR ([D$_6$]DMSO): $\delta = 1.75$ (s, 3H, 5-CH_3) 4.53 (d, 2H, $J = 6.0$ Hz,

CH_2), 4.71 (d, 2H, $J=6.3$ Hz, CH_2), 5.66 (m, 2H, CH=CH), 7.22 (d, 1H, $J=7.0$ Hz, Ar), 7.53 (d, 1H, $J=8.4$ Hz, Ar), 7.60 (s, 1H, H-6), 7.65 (d, 1H, $J=8.4$ Hz, Ar), 7.80 (t, 1H, $J=7.1$ Hz, Ar), 8.07 (d, 1H, $J=6.9$ Hz, Ar), 8.19 (d, 1H, $J=8.1$ Hz, Ar), 8.86 (d, 1H, Ar), 11.20 ppm (brs, 1H, NH); ^{13}C NMR ([D$_6$]DMSO): $\delta=12.0$ (5-CH_3), 36.9, 44.1 (CH_2), 108.8 (C5), 127.6, 128.2 (CH=CH), 106.0, 120.3, 124.2, 124.3, 125.7, 129.0, 132.2, 138.4 (Ar), 141.9 (C6), 150.9 (C2), 164.2 (C4), 166.7 ppm (CO); Anal. ($C_{20}H_{17}N_3O_3\cdot H_2O$): C, H, N.

(*Z*)-2-(4-(Thymin-1-yl)but-2-enyl)-6-nitrobenzo[*c*,*d*]indol-2(1*H*)one (35). The chloride **32** (304 mg, 1.0 mmol) reacted with thymine (252 mg, 2.0 mmol) as already described for compounds **11**–**15**. The final residue was purified by flash column chromatography (CH_2Cl_2/MeOH, 40:1) to yield 259 mg (66%) of **35** as a pale yellow solid; mp: 260–261 °C; MS (ES): *m/z* 415 $[M+Na]^+$; ^{1}H NMR ([D$_6$]DMSO): $\delta=1.76$ (s, 3H, 5-CH_3), 4.53 (d, 2H, $J=5.8$ Hz, CH_2), 4.76 (d, 2H, $J=5.5$ Hz, CH_2), 5.68 (m, 2H, CH=CH), 7.40 (d, 1H, $J=8.1$ Hz, Ar), 7.59 (1 s, 1H, H-6), 8.03–8.09 (m, 2H, Ar), 8.24 (d, 1H, $J=7.0$ Hz, Ar), 8.65 (d, 1H, $J=8.5$ Hz, Ar), 11.30 ppm (brs, 1H, NH); ^{13}C NMR ([D$_6$]DMSO): $\delta=12.2$ (5-CH_3), 37.5, 44.5 (CH_2), 109.4 (C5), 128.2, 128.4 (CH=CH), 105.2, 122.1, 124.0, 125.7, 126.3, 129.8, 130.9, 132.9, 138.8 (Ar), 141.4 (C6), 151.2 (C2), 164.0 (C4), 167.1 ppm (CO); Anal. ($C_{20}H_{16}N_4O_5$): C, H, N.

(*Z*)-1-(4-(Thymin-1-yl)but-2-enyl)-6-acetamidobenzo[*c*,*d*]indol-2(1*H*)one (36). The chloride **33** (315 mg, 1.0 mmol) reacted with thymine (189 mg, 1.5 mmol) as already described for compounds **11**--**15.** The final residue was purified by flash chromatography (CH_2Cl_2/MeOH, 40:1) to yield 263 mg (65%) of **36** as a yellow solid; mp: 265–266 °C; MS (ES): *m/z* 405 $[M+1]^+$; ^{1}H NMR ([D$_6$]DMSO): $\delta=$ 1.75 (s, 3H, 5-CH_3), 2.16 (s, 3H, CH_3), 4.53 (d, 2H, $J=6.0$ Hz, CH_2), 4.68 (d, 2H, $J=6.0$ Hz, CH_2), 5.65 (m, 2H, CH=CH), 7.19 (d, 1H, $J=$ 7.7 Hz, Ar), 7.59 (s, 1H, H-6), 7.77 (d, 1H, $J=7.6$ Hz, Ar), 7.83 (d, 1H, $J=8.1$ Hz, Ar), 8.08 (d, 1H, $J=6.9$ Hz, Ar), 8.35 (d, 1H, $J=8.3$ Hz, Ar), 10.01 (brs, 1H, NHAc), 11.30 ppm (brs, 1H, NH); ^{13}C NMR ([D$_6$]DMSO): $\delta=12.3$ (5-CH_3), 23.9 ($COCH_3$), 37.2, 44.5 (CH_2), 109.5 (C5), 128.0, 128.3 (CH=CH), 106.7, 121.9, 124.3, 124.7, 126.3, 128.8, 129.1, 129.7 (Ar), 141.4 (C6), 151.3 (C2), 164.6 (C4), 167.0, 169.3 ppm (CO); Anal. ($C_{22}H_{20}N_4O_4$): C, H, N.

(*Z*)-1-(4-(Thymin-1-yl)but-2-enyl)-6-aminobenzo[*c*,*d*]indol-2(1*H*)one (37). A solution of **36** (23 mg, 0.056 mmol) in a mixture of dioxane (4 mL) and 1.6 M of HCl (1 mL) was held at reflux for 5 h. After cooling, the mixture was filtered and the filtrate was treated with a solution 30% of NH_3 (40 mL) and extracted with CH_2Cl_2 (100 mL). The organic phase was dried over $MgSO_4$, filtered, and evaporated to yield 20 mg (98%) of **37** as a red solid; mp: 204–205 °C; MS (ES): *m/z* 363 $[M+1]^+$; ^{1}H NMR ([D$_6$]DMSO): $\delta=1.74$ (s, 3H, 5-CH_3), 4.51 (d, 2H, $J=6.3$ Hz, CH_2), 4.62 (d, 2H, $J=6.2$ Hz, CH_2), 5.64 (m, 2H, CH=CH), 5.85 (brs, 2H, NH_2), 6.50 (d, 1H, $J=$ 6.1 Hz, Ar), 6.95 (d, 1H, $J=7.6$ Hz, Ar), 7.57 (s, 1H, H-6), 7.67 (d, 1H, $J=7.9$ Hz, Ar), 8.00 (d, 1H, $J=7.0$ Hz, Ar), 8.37 (d, 1H, $J=8.2$ Hz, Ar), 11.30 ppm (brs, 1H, NH); ^{13}C NMR ([D$_6$]DMSO): $\delta=12.1$ (5-CH_3), 37.1, 44.5 (CH_2), 109.0 (C5), 127.2, 127.4 (CH=CH), 107.6, 121.0, 124.7, 126.1, 127.6, 127.7, 129.5, 141.7 (Ar), 141.5 (C6), 141.8 (C-NH_2), 151.2 (C2), 164.9 (C4), 166.7 ppm (CO); Anal. ($C_{20}H_{18}N_4O_3\cdot 3H_2O$): C, H, N.

2-((*Z*)-4-Chlorobut-2-enyl)-2*H*-naphtho[1,8 *cd*]isothiazole-1,1-dioxide (39). Following a procedure similar to that described in the general procedure for the alkylation of benzo[*d*,*e*]isoquinoline-1,3(2*H*)-dione, 2*H*-naphtho[1,8*cd*]isothiazole-1,1-dioxide (**38**) (205 mg, 1.0 mmol) reacted with (*Z*)-1,4-dichloro-2-butene (180 μL, 1.7 mmol) for 2 h. The residue was purified by flash chromatography (CH_2Cl_2/MeOH, 30:1) to yield 182 mg (62%) of **39** as a white solid. MS (ES): *m/z* 294 $[M+1]^+$,with a Cl isotopic pattern; ^{1}H NMR ([D$_6$]DMSO): $\delta=4.51$ (d, 2H, $J=7.5$ Hz, CH_2), 4.65 (d, 2H, $J=5.7$ Hz, CH_2), 5.72 (m, 1H, CH=CH), 5.86 (m, 1H, CH=CH), 6.99 (m, 1H, Ar), 7.63 (m, 2H, Ar), 7.89 (pt, 1H, $J=7.3$ Hz, Ar), 8.27 ppm (pt, 2H, $J=$ 8.4 Hz, Ar); ^{13}C NMR ([D$_6$]DMSO): $\delta=38.4$, 40.7 (CH_2), 128.5, 129.1 (CH=CH), 104.9, 118.5, 119.0, 120.8, 130.0, 130.2, 130.4, 132.0, 135.5 ppm (Ar).

(*Z*)-2-(4-Thymin-1-yl)but-2-enyl)-2*H*-naphtho[1,8 *cd*]isothiazole-1,1-dioxide (40). According to the general procedure, thymine (252 mg, 2.0 mmol) reacted with the chloride **39** (294 mg, 1.0 mmol). After purification by flash column chromatography (CH_2Cl_2/MeOH, 40:1), 285 mg (77%) of **40** was obtained as a white solid; mp: 219–220 °C; MS (ES): *m/z* 406 $[M+Na]^+$; ^{1}H NMR ([D$_6$]DMSO): $\delta=1.76$ (s, 3H, 5-CH_3), 4.51 (d, 2H, $J=5.7$ Hz, CH_2), 4.71 (d, 2H, $J=4.6$ Hz, CH_2), 5.71 (m, 2H, CH=CH), 7.09 (m, 1H, Ar) 7.60 (m, 2H, Ar), 7.65 (s, 1H, H-6), 7.89 (pt, 1H, $J=7.3$ Hz, Ar), 8.28 (pt, 2H, $J=8.0$ Hz, Ar), 11.30 ppm (brs, 1H, NH); ^{13}C NMR ([D$_6$]DMSO): $\delta=12.3$ (5-CH_3), 40.7, 44.4 (CH_2), 109.3 (C5), 129.1, 130.0 (CH=CH), 105.0, 118.6, 119.0, 120.8, 128.2, 129.0, 130.4, 132.0, 135.6 (Ar), 141.3 (C6), 151.3 (C2), 164.6 ppm (C4); Anal. ($C_{19}H_{17}N_3O_4S$): C, H, N.

Enzymatic assays

In vitro tests were performed on recombinant proteins overexpressed in *E. coli* as native (TMPKmt) or fusion (TMPKh) enzymes. TMPKmt and TMPKh production and purification have been described elsewhere.[7,14] TMPK activity was determined using the coupled spectrophotometric assay according to Blondin et al.[30] at $\lambda=334$ nm in an Eppendorf ECOM 6122 spectrophotometer. 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, and two 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 0.005 and 0.2 mM. The K_i values were calculated using the classical competitive inhibition model (for more details see reference [14]).

Biological assays on *M. bovis* (BCG). The different compounds were assayed for their inhibitory potency toward *M. bovis* (variant BCG) growth in vitro using a micromethod as previously described.[17]

Biological assays on *M. tuberculosis*. These assays were done following the proportion method[32] on Middlebrook 7H11 agar medium and the REMA method as described above. For the proportion method, a bacterial suspension of 1 mg mL^{-1} bacilli was prepared in sterile distilled water. Drug-containing tubes and a growth-control tube were inoculated with 0.1 mL of a 10^{-1} dilution whereas a second control tube was inoculated with 0.1 mL of a 10^{-3} dilution. After overnight inclination at room temperature, tubes were incubated for 28 days at 37 °C with 5% CO_2. Tubes were inspected visually for growth; the lowest compound concentration that showed growth equal or minor to the growth observed on the most diluted control tube determined the MIC value for the given compound assayed. Other experiments have been performed against *M. tuberculosis* H37Rv in BATEC medium using the microplate Alamar blue assay (MABA) by the TAACF service. For more details visit the website: http://www.taacf.org.

Docking of representative inhibitors

Compounds **17**, **34**, and **40** were used as ligands for the automated docking experiments. The crystal structure of TMPKmt cocrystallized with its natural substrate dTMP and Mg^{2+} was used and it was retrieved from the Protein Data Bank[34] (PDB code: 1G3U). dTMP was removed from the structure but the magnesium ion was retained and assigned a charge of +0.8, following the suggestion of previous work.[35] AMBER compatible RESP point charges were used for the inhibitors, as reported previously.[36] The Lamarckian genetic algorithm implemented in AutoDock 3.0.5[37] was used to generate docked conformations of each inhibitor within the putative binding cavity by randomly changing the overall orientation of the molecule as well as the torsion angles of all rotatable bonds. Default settings were used except for the number of runs, population size, and maximum number of energy evaluations, which were fixed at 250, 100, and 250 000, respectively. Rapid intra- and intermolecular energy evaluation of each configuration was achieved by having the receptor's atomic affinity potentials for aliphatic and aromatic carbon, oxygen, nitrogen, and hydrogen atoms precalculated in a three-dimensional grid with a spacing of 0.375 Å. A distance-dependent dielectric function was used in the computation of electrostatic interactions.

Molecular dynamics (MD) simulations

The MD simulations for the complexes of TMPKmt and **17**, **34**, and **40** were carried out using the AMBER 8.0 suite of programs.[38] The magnesium ion was assigned a charge of +2 and the bonded and nonbonded parameters for the ligands were assigned, by analogy or through interpolation, from those already present in the AMBER database in a way consistent with the second generation AMBER force field[39] (parm99). Each molecular system was neutralized by the addition of four chloride ions,[40] and immersed in a truncated octahedron of ~8400 TIP3P water molecules.[41] Periodic boundary conditions were applied and electrostatic interactions were treated using the smooth particle mesh Ewald method[42] with a grid spacing of 1 Å. The cutoff distance for the nonbonded interactions was 9 Å. The SHAKE algorithm[43] was applied to all bonds and an integration step of 2.0 fs was used throughout. Solvent molecules and counterions were relaxed by energy minimization and allowed to redistribute around the positionally restrained solute (25 kcal mol^{-1} $Å^{-2}$) during 50 ps of MD at constant temperature (300 K) and pressure (1 atm). These initial harmonic restraints were gradually decreased in a series of progressive energy minimizations until they were completely removed. The resulting systems were heated again from 100 to 300 K during 20 ps and allowed to equilibrate in the absence of any restraints for 10.0 ns during which system coordinates were collected every 2 ps for further analysis. Representative snapshots were energy minimized and visually inspected using the computer graphics program PyMOL.[44]

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