DOI: 10.1002/cmdc.200((Please insert last 6 DOI digits))

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

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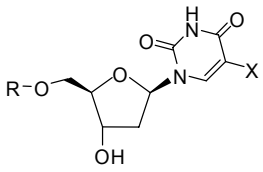
*In the search for novel inhibitors of the enzyme thymidine monophosphate kinase of Mycobacterium tuberculosis (TMPKmt), an attractive target for novel antituberculosis agents, we report here on the discovery of the first acyclic nucleoside analogues that potently and selectively inhibit TMPKmt. The most potent compounds in this series are (Z)-butenyl thymines carrying a naphtholactam or naphthosultam moiety at position 4 (compounds **34** and **40**), which display Ki values of 0.42 and 0.27 μM, respectively. Docking studies performed to rationalize the interaction of this new family of inhibitors with its target enzyme revealed a key interaction of the distal substituent with Arg95 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, make 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 8 million new cases and 2 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 of novel targets, *M. tuberculosis* thymidine monophosphate kinase (TMPKmt) is an attractive candidate to interfere with the replication of the pathogen.[7-10] TMPK catalyses 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]

Initially it was considered that the potential inhibitors of TMPKmt should resemble the natural substrate dTMP (**1**) (Chart 1) and therefore should incorporate a monophosphate moiety.



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

Chart 1. Structural formulae of the natural substrate dTMP (**1**) and the nucleosides thymidine (**2**) and 5-bromo-2′-deoxyuridine **(3**)

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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 non-phosphorylated nucleoside thymidine (**2**), with a free 5´-OH, has good affinity for TMPKmt.[9] Interestingly, the search of the *M. tuberculosis* genome[11] did not identify a gene coding for a thymidine kinase.[9] The observation that nucleosides like 5-bromo-2´deoxyuridine (**3**) (Chart 1), 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 on 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 derivatives were inhibitory to TMPKmt with Ki values around 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] Due to the relaxed substrate specificity of some viral enzymes, it has been found that acyclic nucleoside analogues may afford an increase of 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 (i.e 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** (Chart 2) showed a significant TMPKmt inhibition in the low micromolar range (*K*i = 42 μM).

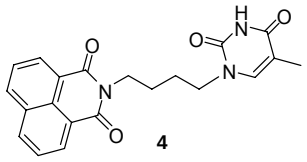

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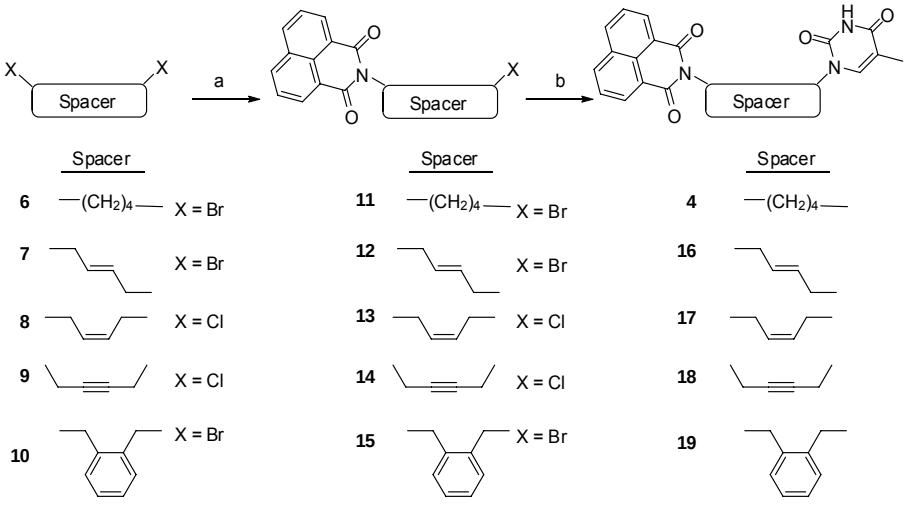
Chart 2. Acyclic thymine derivative identified as a hit in the initial screening against TMPKmt.

Therefore compound **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 as a wise starting point since 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 carbonyls 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, in order 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 in a two-step procedure. Thus, treatment of 1,8-naphthalimide (**5**) with NaH in dry DMF followed by "in situ" reaction with the corresponding dihalides (**6**-**10**) afforded the monohalo compounds **11**-**15** in good to excellent yields (65-95%) (Scheme 1).

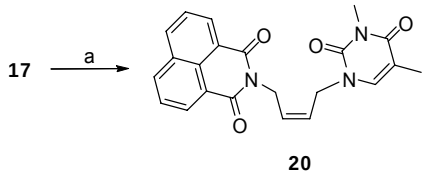


Scheme 1. (a) benzo[*de*]isoquinoline-1,3(2*H*)-dione (**5**); NaH; DMF, 0 ºC, 30 min; then addition of **6**-**10**; 80 °C; 5 h; (b) Thymine; *N*,*O*-bistrimethylsilylacetamide; CH_3CN; 80 °C till solubilization of thymine; then addition of **11**-**15**; NaI; CH_3CN; 80 °C; 8h

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 around 80%, with the only exception of compound **19** (45% yield). It should be mentioned that in all the cases the only substitution product isolated is that of alkylation at N^1 of thymine.

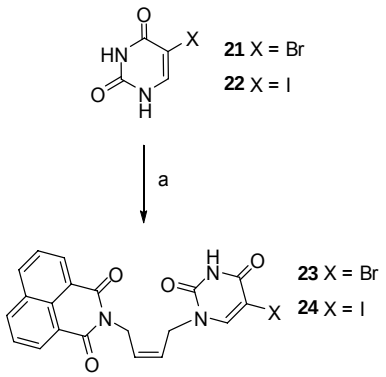
When compounds **16-19** were evaluated on TMPKmt (see Enzymatic Assays), it was found that the (*Z*)-butenyl derivative (**17**) improved more than 10-fold the inhibitory potency against TMPKmt compared to the lead compound **4**. Thus, compound **17** was selected as a new lead to perform modifications 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).



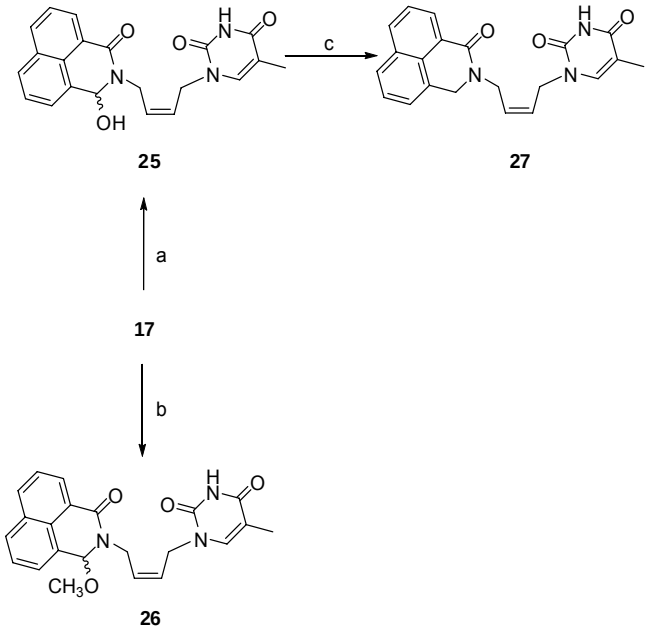
Scheme 2. (a) MeI; DBU; DMAC; rt.

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 lead to the 5-iodouracil derivative **24** in 79% yield.



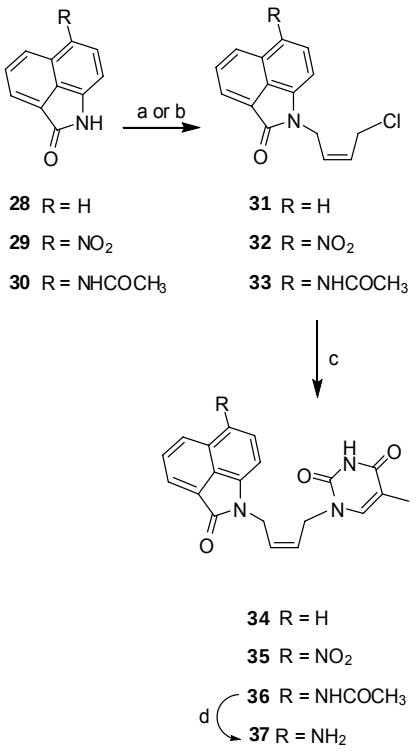
Scheme 3. (a) HMDS, $(NH_4)_2SO_4$ (cat), 150 ºC, overnight; evaporation under vacuum; then addition of **13** (see Scheme 1); NaI; CH_3CN; 80 ºC; 8h.

Concerning the modifications at the distal substituent, it was considered of interest to explore how the reduction of one of the carbonyls 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 hydroxyl 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 hydroxyl 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. (a) $NaBH_4$; EtOH, 55 ºC; then HCl 3N and neutralization with NaOH 1N; (b) $NaBH_4$; EtOH, 55 ºC; then HCl 3N and addition of MeOH; (c) $NaBH_4$; TFA; rt.

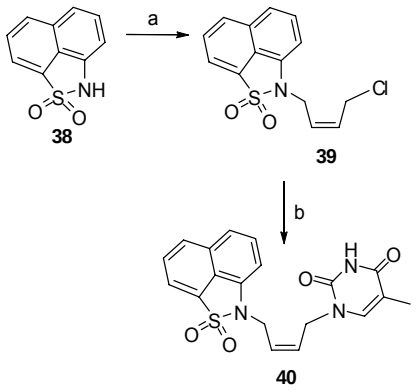
Therefore the next series of modifications involved replacement of the naphthalimide substituent by naphtholactams. Benzo[*cd*]indol-2(1*H*)-one (naphtholactam) (**28**) (Scheme 5) was employed as the starting material for the next series of compounds. On one hand, the ease of alkylation of benzo[*cd*]indol-2(1*H*)-one 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[*cd*]indol-2(1*H*)-one can be easily nitrated giving the opportunity to study how substituents at this position affect the interaction with the enzyme. Thus, the 6-nitrobenzo[*cd*]indol-2(1*H*)-one and the 6-aminobenzo[*cd*]indol-2(1*H*)-one were



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

considered as interesting distal substituents to be incorporated in our lead structure. The 6-aminobenzo[*cd*]indol-2(1*H*)-one was synthesized, as described,[28] through catalytic hydrogenation of the 6-nitrobenzo[*cd*]indol-2(1*H*)-one (**29**). However, since this amino compound was quite unstable in our hands, the amino derivative obtained after hydrogenation was directly transformed into its acetamide derivative (**30**) and as such was used in the next steps. Thus, reaction of benzo[*cd*]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] afforded 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 to afford **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.

Since a naphthosultam ring can be considered as 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). Thus, the naphthosultam derivative **40** was obtained with a global yield of 48% for the two steps.



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

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, since 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 more than 10-fold the inhibitory potency against TMPKmt compared to the lead compound **4**, while 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 due to low solubility. From these data, it was concluded that the (*Z*)-butenyl derivative **17** was the most potent compound against TMPKmt in this series (Ki = 1.9 μM). Interestingly, the *K*m of TMPKmt for dTMP is 4.5 μM, and the *K*i for dT is 27 μM.

Methylation of the NH 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 the 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 due to its low solubility in non organic 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** (Ki = 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 4-fold 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, while the 6-amino and 6-acetamide derivatives (**37** and **36**, respectively) afforded compounds that were still active although their *K*i values were 6 and 14-fold higher 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.

Table 1. Evaluation of acyclic nucleoside analogues on TMPKmt

Compound	Ki (μM)	Compound	Ki (μM)
dTMP (**1**)	4.5[a]	**24**	NS[c]
dT (**2**)	27	**25**	4.7
4	42	**26**	6.2
16	NI[b]	**27**	1.4
17	1.9	**34**	0.42
18	NI[b]	**35**	0.75
19	NS[c]	**36**	6.0
20	NI[b]	**37**	2.4
23	1.1	**40**	0.27

[a] *K*m value; [b] No inhibition at 100 μM; [c] Not soluble in the assay medium.

Interestingly, two of the more potent acyclic nucleosides described here (**17** and **27**) gave no inhibition of TMPKh at 0.02 mM, thus affording a selectivity index (SI= Ki (μM) TMPKh/Ki (μM) TMPKmt) higher than 50. This great selectivity is correlated to the absence of toxicity against VERO cells at concentrations up to 100 μg/mL.

Molecular modelling

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 coordination sphere with the oxygens 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 NH-3 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 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.

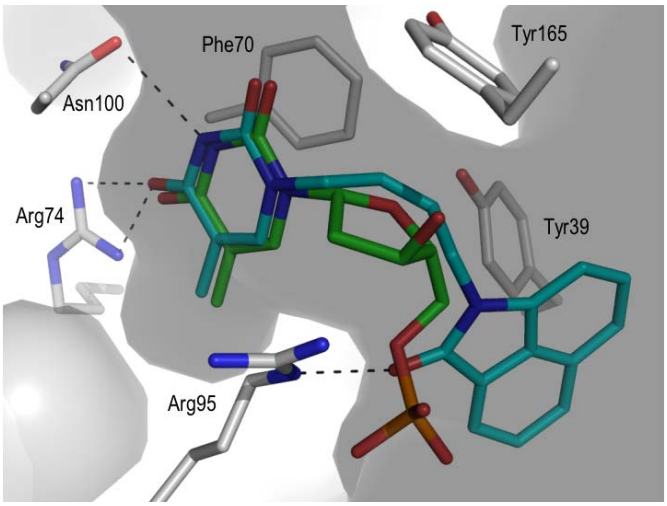


Figure 1. Detail of the substrate binding site in the complex of **34** (C atoms in cyan) with TMPKmt (C atoms in grey). Only the side chains of relevant residues (discussed in the text) are shown as sticks and have been labeled, except for Tyr103 which has been removed for clarity. The black dashes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

The aromatic stacking interaction with the spacer's double bond seems to be crucial for compound stabilization, because when the double bond is replaced by a single one (**4**), the inhibitory potency against TMPKmt decreases more than 10–fold. This interaction is not feasible for **16** and **18**, which provides a rationale for the lack of activity of these compounds. On the other hand, the greater inhibitory activity brought about by the replacement of the naphthalimide substituent (**17**) with either a naphtholactam (**34**) or a naphthosultam (**40**) can be accounted for by an improved hydrogen bonding interaction between the oxygen atom and the side chain of Arg95, which thus mimics the interaction observed between this same amino acid and the dTMP substrate. 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 accounts for 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 (i.e. **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. 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 4 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) 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} of 19 μg/mL.

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

Conclusion

In conclusion, 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**) lead 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), respectively, on a Varian INNOVA 300 operating at 299 MHz (^{1}H) and 75 MHz (^{13}C), respectively.

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 (Kiesegel 60 PF_{254} gipshaltig (Merck)), layer thickness (1 or 2 mm), flow rate (4 or 8 mL/min, 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 refluxing over calcium hydride. Tetrahydrofuran was dried by refluxing 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[*de*]isoquinoline-1,3(2*H*)-dione. To a solution of benzo[*de*]isoquinoline-1,3(2*H*)-dione (**5**) (1.0 mmol) in dry DMF (1.0 mL) at 0 °C, NaH (60% oil suspension) (1.5 mmol) was added. 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 5h or till disappearance of starting material. After reaching rt, 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[*de*]isoquinoline-1,3(2*H*)-dione (11).[33] Following the general procedure, benzo[*de*]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 (DMSO-d_6) δ: 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 (m, 4H, Ar); ^{13}C NMR (DMSO-d_6) δ: 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 (CO).

(*E*)-2-(4-Bromobut-2-enyl)-1*H*-benzo[*de*]isoquinoline-1,3(2*H*)-dione (12). Following the general procedure, benzo[*de*]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 ($CDCl_3$) δ: 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 (d, *J* = 8.0 Hz, 2H, Ar); ^{13}C NMR ($CDCl_3$) δ: 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 (CO).

(*Z*)-2-(4-Chlorobut-2-enyl)-1*H*-benzo[*de*]isoquinoline-1,3(2*H*)-dione (13). Following the general procedure, benzo[*de*]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:ethyl acetate, 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 ($CDCl_3$) δ: 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 (dd, *J* = 6.2, 1.1 Hz, 2H, Ar); ^{13}C NMR ($CDCl_3$) δ: 36.8, 39.2 (CH_2), 127.0, 128.0 (CH=CH), 122.5, 129.6, 131.4, 134.1 (Ar), 163.9 (CO).

2-(4-Chlorobut-2-ynyl)-1*H*-benzo[*de*]isoquinoline-1,3(2*H*)-dione (14). Following the general procedure, benzo[*de*]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 ($CDCl_3$) δ: 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 (d, *J* = 6.3, 1.1 Hz, 2H, Ar). ^{13}C NMR ($CDCl_3$) δ: 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 (CO).

2-[(2-Bromomethyl)benzyl]-1*H*-benzo[*de*]isoquinoline-1,3(2*H*)-dione (15). Following the general procedure, benzo[*de*]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 ($CDCl_3$) δ: 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 (d, *J* = 7.2 Hz, 2H, Ar); ^{13}C NMR ($CDCl_3$) δ: 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 (CO).

General Procedure for the reaction of compounds 11-15 with thymine. To a suspension of thymine (214 mg, 1.70 mmol) in dry CH_3CN (6 mL), *N,O*-bis(trimethylsilyl)acetamide (0.6 mL) was added and the mixture was heated at 80 ºC 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 ºC for 8 h. The mixture was filtered and the isolated solid contained the target compound. The filtrate was treated with a solution of sodium bisulphite (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[*de*]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** were obtained. Mp 219-220 ºC; MS (ES, positive mode): m/z 378 $(M+1)^+$; ^{1}H NMR (DMSO-d_6) δ: 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 (br s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ: 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 (C-6), 151.2 (C-2), 163.8 (CO), 164.6 (C-4). Anal ($C_{21}H_{19}N_3O_4$): C, H, N.

(*E*)-2-(4-(Thymin-1-yl)but-2-enyl)-1*H*-benzo[*de*]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** were obtained. Mp 265-266 ºC; MS (ES, positive mode): m/z 376 $(M+1)^+$; ^{1}H NMR (DMSO-d_6) δ: 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 (br s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ: 11.8 (5-CH_3), 40.6, 47.8 (CH_2), 108.6 (C-5), 126.8, 127.9 (CH=CH), 122.0, 127.2, 127.4, 130.8, 131.3, 134.4 (Ar), 140.9 (C-6), 150.6 (C-2), 163.1 (CO), 164.2 (C-4). Anal (**$C_{21}H_{17}N_3O_4 . 0.5 H_2O$**): C, H, N

(*Z*)-2-(4-(Thymin-1-yl)but-2-enyl)-1*H*-benzo[*de*]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** were obtained. Mp 235-236 °C, MS (ES, positive mode): m/z 376 $(M+1)^+$; ^{1}H NMR (DMSO-d_6) δ: 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 (br s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ: 12.4 (5-CH_3), 37.4, 44.1 (CH_2), 109.1 (C-5), 127.6, 128.7 (CH=CH), 122.4, 131.1, 131.7 , 134.9 (Ar) 141.3 (C-6), 151.2 (C-2), 163.7 (CO), 164.6 (C-4). Anal ($C_{21}H_{17}N_3O_4$): C, H, N.

2-(4-(Thymin-1-yl)butynyl)-1*H*-benzo[*de*]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 ºC; MS (ES, positive mode): m/z 374 $(M+1)^+$; ^{1}H NMR (DMSO-d_6) δ: 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 (br s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ: 12.0 (5-CH_3), 29.7, 36.8 (CH_2), 76.7, 81.2 (C≡C), 109.7 (C-5), 122.3, 127.5, 128.4, 130.8, 131.9, 135.0 (Ar), 139.8 (C-6), 150.6 (C-2), 163.0 (CO), 163.8 (C-4). Anal ($C_{21}H_{15}N_3O_4$): C, H, N.

2-[2-(Thymin-1-yl)methyl)benzyl]-1*H*-benzo[*de*]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** were obtained. Mp 290-291 ºC; MS (ES, positive mode): m/z 448 $(M+Na)^+$; ^{1}H NMR (DMSO-d_6) δ: 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 (br s, 1H, NH); ^{13}C NMR (DMSO-d_6): 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 (C-6), 151.1 (C-2), 163.6 (CO), 164.4 (C-4). Anal ($C_{25}H_{19}N_3O_4 . 2H_2O$)**:** C, H, N.

(*Z*)-2-(4-(3-Methylthymin-1-yl)but-2-enyl)-1*H*-benzo[*de*] isoquinoline-1,3(2*H*)-dione (20). To a suspension containing **17** (192 mg, 0.5 mmol) in *N*,*N*-dimethylacetamide (5 mL), DBU (0.09 mL, 0.6 mmol) and MeI (0.09 mL, 1.5 mmol) were added. The reaction was stirred at rt 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 ºC; MS (ES, positive mode): m/z 390 $(M+1)^+$; ^{1}H NMR (DMSO-d_6) δ: 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 (m, 4H, Ar); ^{13}C NMR (DMSO-d_6) δ: 12.6 (5-CH_3), 30.6 (NCH_3), 37.0, 45.0 (CH_2), 107.8 (C-5), 127.1, 128.7 (CH=CH), 122.0, 130.8, 131.3, 134.5 (Ar), 139.4 (C-6), 150.1 (C-2), 163.3 (CO), 165.1 (C-4). Anal ($C_{22}H_{19}N_3O_4$): C, H, N.

(*Z*)-2-(4-(5-Bromouracil-1-yl)but-2-enyl)-1*H*-benzo[*de*] 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 ºC 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 ºC overnight. The reaction was allowed to reach rt, 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 ºC; MS (ES, positive mode): m/z 440 $(M+1)^+$ with a Br isotopic pattern; ^{1}H NMR (DMSO-d_6) δ: 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 (br s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ: 37.0, 44.4 (CH_2), 94.8 (C-5), 127.0, 128.7 (CH=CH), 122.0, 127.3, 127.4, 130.8, 131.4, 134.5 (Ar), 145.0 (C-6), 150.3 (C-2), 159.6, 163.5 (CO, C-4). Anal ($C_{20}H_{14}BrN_3O_4$): C, H, N.

(*Z*)-2-(4-(5-Iodouracil-1-yl)but-2-enyl)-1*H*-benzo[*de*]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 ºC; MS (ES, positive mode): m/z 488 $(M+1)^+$; ^{1}H NMR (DMSO-d_6) δ: 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 (br s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ: 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 (C-6), 150.7 (C-2), 161.1, 163.7 (CO, C-4). 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[*de*]isoquinolin-1-one (25). To a suspension containing **17** (375 mg, 1.0 mmol) in EtOH (40 mL), $NaBH_4$ (378 mg, 10.0 mmol) was added in small portions for 10 min. The mixture was heated at 55 °C for 2 h, while it became into a yellow solution. Then, HCl 3N (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: diethyl ether, 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 (DMSO-d_6) δ: 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 (br s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ: 12.0 (5-CH_3), 41.2, 43.7 (CH_2), 79.8 (CHOH), 108.8 (C-5), 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 (C-6), 151.0 (C-2), 161.8 (CO), 164.3 (C-4). Anal **(**$C_{21}H_{19}N_3O_4.\ 0.5H_2O$**):** C, H, N.

(*Z*)-2-(4-Thymin-1-yl)but-2-enyl)-3-methoxy-2,3-dihydro-1*H*-benzo[*de*]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 3N HCl, MeOH (3 mL) was added. Volatiles were removed and the residue was purified by flash column chromatography (EtOAc:diethyl ether, 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 (DMSO-d_6) δ: 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 (br s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ: 12.2 (5-CH_3), 40.0, 44.0 (CH_2), 49.4 (OCH_3), 86.0 ($CHOCH_3$), 109.1 (C-5), 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 (C-6), 151.1 (C-2), 163.0 (CO), 164.4 (C-4). Anal ($C_{22}H_{21}N_3O_4.H_2O$): C, H, N.

(*Z*)-2-(4-Thymin-1-yl)but-2-enyl)-2,3-dihydro-1*H*-benzo[*de*]isoquinolin-1-one (27). To a solution of **25** (377 mg, 1.0 mmol) in trifluoroacetic acid (TFA) (2 mL) at 0 °C, $NaBH_4$ (378 mg, 10.0 mmol) was added in portions. The reaction was allowed to reach rt 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 (DMSO-d_6) δ: 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 (br s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ: 12.6 (5-CH_3), 44.1, 45.0, 50.6 (CH_2), 110.0 (C-5), 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 (C-6), 151.1 (C-2), 162.7 (CO), 164.4 (C-4). Anal ($C_{21}H_{19}N_3O_3$): C, H, N.

6-Acetamidobenzo[*cd*]indol-2(1*H*)-one (30). A solution of 6-nitrobenzo[*cd*]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[*cd*]indol-2(1H)-one; mp: 269-270 °C; mp^{lit} 266-268 °C.[28] In our hands, this compound tends to decompose and therefore was transformed to its corresponding acetamido derivative as follows. A solution containing 6-aminobenzo[*cd*]indol-2(1H)-one (700 mg, 3.80 mmol) in glacial acetic acid (12 mL) and acetic anhydride (4.2 mL) was stirred at refluxed for 1h. 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 ºC; MS (ES): m/z 227 $(M + 1)^+$; ^{1}H NMR (DMSO-d_6) δ: 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 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ: 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 (CO).

1-((*Z*)-4-Chlorobut-2-enyl)benzo[*cd*]indol-2(1*H*)-one (31). Following a procedure similar to that described in the general procedure for the alkylation of benzo[*de*]isoquinoline-1,3(2*H*)-dione, benzo[*cd*]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 (DMSO-d_6) δ: 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 (d, 1H, *J* = 7.0 Hz, Ar); ^{13}C NMR (DMSO-d_6) δ: 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 (CO).

1-((*Z*)-4-Chlorobut-2-enyl)-6-nitrobenzo[*cd*]indol-2(1*H*)-one (32). Following a procedure similar to that described in the general procedure for the alkylation of benzo[*de*]isoquinoline-1,3(2*H*)-dione, 6-nitrobenzo[*cd*]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 1h. 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 (DMSO-d_6) δ: 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 (d, 1H, *J* = 8.3 Hz, Ar); ^{13}C-NMR (DMSO-d_6): 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 (CO).

***N*-1-((*Z*)-4-Chlorobut-2-enyl)-6-acetamidobenzo[*cd*]indol-2(1*H*)-one (33).** To a solution of **30** (226 mg, 1.0 mmol) in dry DMF (6.3 mL), NaOMe (54 mg, 1.0 mmol) was added and the mixture was stirred at rt for 15 min. Then, the (*Z*)-1,4-dichloro-2-butene (370 µL, 3.5 mmol) was added and the mixture was stirred for 1h. The reaction was quenched by addition of a 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 (DMSO-d_6) δ: 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 (br s, 1H, NHAc). ^{13}C NMR (DMSO-d_6) δ: 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 (CO).

(*Z*)-1-(4-(Thymin-1-yl)but-2-enyl)-1*H*-benzo[*cd*]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 ºC; MS (ES): m/z 348 $(M +1)^+$; ^{1}H NMR (DMSO-d_6) δ: 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 (br s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ: 12.0 (5-CH_3), 36.9, 44.1 (CH_2), 108.8 (C-5), 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 (C-6), 150.9 (C-2), 164.2 (C-4), 166.7 (CO). Anal ($C_{20}H_{17}N_3O_3.H_20$): C, H, N.

(*Z*)-2-(4-(Thymin-1-yl)but-2-enyl)-6-nitrobenzo[*cd*]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

ºC; MS (ES): m/z 415 $(M + Na)^+$; ^{1}H NMR (DMSO-d_6) δ: 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 (1s, 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 (br s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ: 12.2 (5-CH_3), 37.5, 44.5 (CH_2), 109.4 (C-5), 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 (C-6), 151.2 (C-2), 164.0 (C-4), 167.1 (CO). Anal ($C_{20}H_{16}N_4O_5$): C, H, N.

(*Z*)-1-(4-(Thymin-1-yl)but-2-enyl)-6-acetamidobenzo[*cd*]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 ºC; MS (ES): m/z 405 $(M + 1)^+$;. ^{1}H NMR (DMSO-d_6) δ: 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 (br s, 1H, NHAc), 11.30 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ: 12.3 (5-CH_3), 23.9 ($COCH_3$), 37.2, 44.5 (CH_2), 109.5 (C-5), 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 (C-6), 151.3 (C-2), 164.6 (C-4), 167.0, 169.3 (CO). Anal ($C_{22}H_{20}N_4O_4$): C, H, N.

(*Z*)-1-(4-(Thymin-1-yl)but-2-enyl)-6-aminobenzo[*cd*]indol-2(1*H*)one (37). A solution of **36** (23 mg, 0.056 mmol) in a mixture of dioxane (4 mL) and 1.6M of HCl (1 mL) was refluxed 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 ºC; MS (ES): m/z 363 $(M + 1)^+$; ^{1}H NMR (DMSO-d_6) δ: 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 (br s, 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 (br s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ: 12.1 (5-CH_3), 37.1, 44.5 (CH_2), 109.0 (C-5), 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 (C-6), 141.8 (C-NH_2), 151.2 (C-2), 164.9 (C-4), 166.7 (CO). Anal ($C_{20}H_{18}N_4O_3.3H_2O$): 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[*de*]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 2h. 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 (DMSO-d_6) δ: 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 (pt, 2H, *J* = 8.4 Hz, Ar); ^{13}C NMR (DMSO-d_6) δ: 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 (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** were obtained as a white solid. Mp: 219-220 ºC; MS (ES): m/z 406 $(M + Na)^+$; ^{1}H NMR (DMSO-d_6) δ: 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 (br s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ: 12.3 (5-CH_3), 40.7, 44.4 (CH_2), 109.3 (C-5), 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 (C-6), 151.3 (C-2), 164.6 (C-4). 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 λ=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 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 0.005 and 0.2 mM. The Ki values were calculated using the classical competitive inhibition model (for more details see ref[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 micro-method 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 bacilli was prepared in sterile distilled water. Drug-containing tubes as well 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 has 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: 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 rotable bonds. Default settings were used except for 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 0.375Å. 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 4 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

non–bonded 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 (25kcal·mol^{-1}·Å^{-2}) during 50 ps of MD at constant temperature (300 K) and pressure (1 atm). These initial harmonic restraints were gradually reduced 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]

Acknowledgements

O.F. thanks the Spanish Ministerio de Educación y Ciencia for a predoctoral fellowship. We thank Andrea Von Groll for performing the REMA assays on the M. tuberculosis isolates and Gilles Marchal and Pascal Chavarot for the assays on M. bovis BCG. Some antimycobacterial data have been provided by the Tuberculosis Antimicrobial Acquisition and Coordinating Facility (TAACF) through a research and development contract with the U.S. National Institute of Allergy and Infectious Diseases and is gratefully acknowledged.This work has been supported by grants from the Spanish MEC (SAF2003-07219-C02-01; SAF2006-12713-C02-01), Comunidad de Madrid (BIPEDD-CM, S-Bio-214-2006), the Pasteur Institute (GPH tuberculose) and INSERM.

Keywords: acyclic nucleosides, thymine, thymidylate kinase, naphtholactam

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Received: ((will be filled in by the editorial staff))
Published online: ((will be filled in by the editorial staff))

DOI: 10.1002/cmdc.200((Please insert last 6 DOI digits))

Supporting Information

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

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Contents

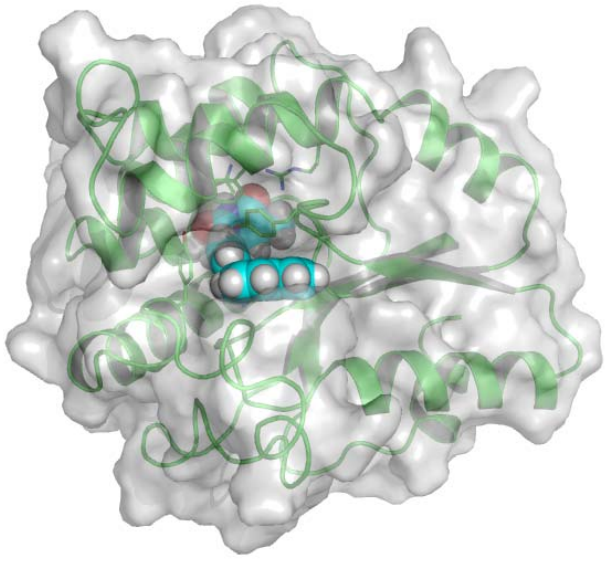

Figure S1. View of the proposed complex formed between TMPKmt (C atoms in grey and surrounded by a solvent accessible surface) and inhibitor **34** (spheres, with C atoms in cyan). The same side chains displayed in Figure 1 and discussed in the text are shown as sticks.

Analytical Data

		Calculated (%)			Found (%)		
Comp.	Formula	C	H	N	C	H	N
4	$C_{21}H_{19}N_3O_4$	66.83	5.07	11.13	66.58	5.31	11.24
16	$C_{21}H_{17}N_3O_4.0.5H_2O$	65.62	4.72	10.93	65.60	4.75	10.93
17	$C_{21}H_{17}N_3O_4$	67.19	4.56	11.19	67.59	4.98	10.81
18	$C_{21}H_{15}N_3O_4$	67.56	4.05	11.25	67.40	4.25	10.98
19	$C_{25}H_{19}N_3O_4.2H_2O$	65.07	5.02	9.11	65.43	4.78	9.30
20	$C_{22}H_{19}N_3O_4$	67.86	4.92	10.79	67.59	5.10	10.81
23	$C_{20}H_{14}BrN_3O_4$	54.56	3.21	9.54	54.68	3.45	9.34

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Supporting information for this article is available on the WWW under http://www.chemeurj.org/ or from the author.

24	$C_{20}H_{14}IN_3O_4$	49.30	2.90	8.62	49.23	3.24	8.90	.
25	$C_{21}H_{19}N_3O_4.0.5H_2O$	65.28	5.22	10.87	65.32	4.90	11.12	
26	$C_{22}H_{21}N_3O_4.H_2O$	64.54	5.66	10.26	64.63	5.28	10.60	
27	$C_{21}H_{19}N_3O_3$	69.79	5.30	11.63	69.61	5.23	11.60	
34	$C_{20}H_{17}N_3O_3.H_20$	65.74	5.24	11.50	65.96	4.88	11.79	
35	$C_{20}H_{16}N_4O_5$	61.22	4.11	14.28	60.98	3.99	14.31	
36	$C_{22}H_{20}N_4O_4$	65.34	4.98	13.85	65.21	4.59	13.61	
37	$C_{20}H_{18}N_4O_3.3H_2O$	57.68	5.81	13.45	57.40	5.45	13.30	
40	$C_{19}H_{17}N_3O_4S$	59.52	4.47	10.96	59.21	4.55	10.98	

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