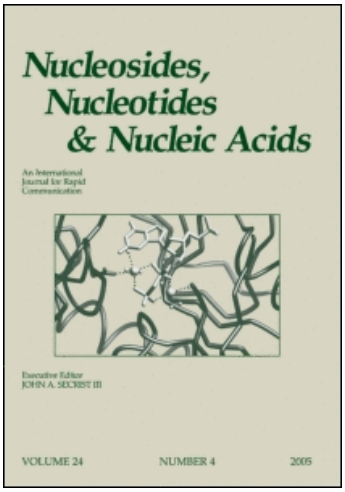

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Synthesis and Biological Evaluation of Inhibitors of Thymidine Monophosphate Kinase from *Bacillus Anthracis*

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SYNTHESIS AND BIOLOGICAL EVALUATION OF INHIBITORS OF THYMIDINE MONOPHOSPHATE KINASE FROM *BACILLUS ANTHRACIS*

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□ *Nineteen lipophilic thymidine phosphate-mimicking compounds were designed and synthesized as potential inhibitors of thymidine monophosphate kinase of Bacillus anthracis, a Gram-positive bacterium that causes anthrax. These thymidine analogues were substituted at the 5′-postion with sulfonamide-, amide-, (thio)urea-, or triazole groups, which served as lipophilic surrogates for phosphate. Three of the tested compounds produced inhibition of B. anthracis Sterne growth and/or thymidine monophosphate activity. Additional studies will be necessary to elucidate the potential of this type of B. anthracis thymidine monophosphate inhibitors as novel antibiotics in the treatment of anthrax.*

Keywords *Bacillus anthracis* thymidine monophosphate kinase (*Ba*TMPK); 5′-Modified thymidine-based *Ba*TMPK kinase inhibitors

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INTRODUCTION

Anthrax is an infectious disease that is caused by the Gram-positive bacterium *Bacillus anthracis*. The primary concern with this bacterium in the 21st century is its use as a biological weapon.[1] Ciprofloxacin, doxycycline, and penicillin G procaine are approved in the US for the treatment of anthrax and a vaccine (Biothrax) is available.[1,2] However, resistance could be developed towards these antibiotics and vaccination of large populations is difficult. This calls for the discovery of novel antibiotics for the treatment of unvaccinated individuals who may become exposed to anthrax or infected with antibiotic-resistant strains of *B. anthracis*.

Key enzymes involved in the salvage and de novo pathways of DNA synthesis, such as DNA polymerase, dihydrofolate reductase (DHFR), thymidylate synthetase (TS), ribonucleotide reductase (RR), and 2′-deoxynucleoside kinases (dNKs), are classic molecular targets for anticancer- and antiviral chemotherapy.[3–5] Prokaryotes rely on the same pathways for DNA synthesis.[6–9] With the exception of DHFR inhibitors such as trimethoprim,[10] antibiotics targeting other key enzymes of these pathway have not yet entered the clinical stage. Novel inhibitors of bacterial DHFR,[7] TS,[8] RR,[9] and thymidine monophosphate kinase (TMPK)[6] are currently under investigation. With respect to inhibiting growth of *B. anthracis*, inhibitors of the *de novo* pathway enzymes TS and RR have proven to be effective in biological studies.[9,11]

Thymidine monophosphate kinase of *B. anthracis* (*Ba*TMPK) could be an attractive target for the treatment of anthrax because thymidine monophosphate (dTMP), supplied by both salvage and de novo pathways of DNA synthesis, is diphosphorylated only by *Ba*TMPK. To our knowledge, there is no report on transport of thymidine diphosphate (dTDP) into bacteria and, therefore, it is likely that dTDP production in *B. anthracis* relies solely on the action of *Ba*TMPK. Unless *B. anthracis* possesses an unknown alternative mechanism to generate or transport dTDP, *Ba*TMPK provides an excellent molecular target for the discovery of novel antibiotics for the treatment of anthrax infections. For similar reasons, the inhibition of TMPK in *Mycobacterium tuberculosis* by bicyclic nuclesoside analogues has been explored for the treatment of multidrug-resistant tuberculosis.[6]

Based on the discussion in the aforementioned, our group has initiated studies on the design and synthesis of 5′-substituted thymidine (dThd) analogues that have the potential to function as inhibitors of *Ba*TMPK. These studies are the subject of this article, which also includes results from the preliminary biological evaluation of the prepared *Ba*TMPK inhibitors in enzyme- and in vitro growth inhibition assays.

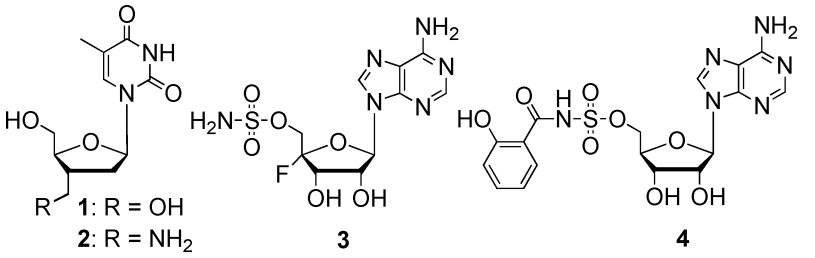


FIGURE 1 3′-modified dThd analogues: 1-[2,3-Dideoxy-3-(hydroxymethyl)-β-D-*erythro*-pentofuranosyl]thymine (**1**) and 1-[3-(aminomethyl)-2,3-dideoxy-β-D-*erythro*-pentofuranosyl]thymine (**2**); 5′-subsituted antimicrobial nucleoside analogues: Nucleocidin (**3**) and salicyl-AMS (**4**).

RESULTS AND DISCUSSION

Design Strategies

Initially, we tested the in vitro growth inhibition of *B. anthracis* Sterne by the dThd analogues 1-[2,3-dideoxy-3-(hydroxymethyl)-β-D-*erythro*-pentofuranosyl]thymine (**1**) and 1-[3-(aminomethyl)-2,3-dideoxy-β-D-*erythro*-pentofuranosyl]thymine (**2**) (Figure 1).[12] Both compounds proved to be effective inhibitors of *M. tuberculosis* TMPK (K_i's: 41 and 57 μM).[12] Unfortunately, neither compound produced any growth inhibition of *B. anthracis* Sterne (IC_{50}'s: > 1000 μM). Therefore, we decided to pursue a different strategy for the design of *Ba*TMPK inhibitors, which was based on the introduction of groups at the 5′-position that potentially could act as lipophilic replacements of phosphate in dTMP. Such inhibitors could compete with endogenous dTMP in the substrate-binding pocket of *Ba*TMPK and thereby decrease thymidine triphosphate (dTTP) production. The resulting imbalanced dNTP pool could affect DNA synthesis in *B. anthracis*, and thus, reduce its growth and spread.

Bisubstrate inhibitors, consisting of adenosine and dThd units linked through 4-6 phosphates (Ade-$P_{4,5,6}$-dThd), proved to be powerful inhibitors of TMPK.[13,14] However, these compounds have high molecular weights (>900 Da) and strongly ionic characters. Presumably, they cannot cross cell membranes, which renders them unsuitable as therapeutics. Compounds containing lipophilic phosphate-mimicking groups have been explored extensively.[15–26] These mimics included, but were not limited to, sulfonamide-, sulfonate-, fluoride-, hydroxyl-, urea-, amide-, ester-, carboxylate-, thiomethyl-, pyrrolidinedione-, and thiazolidine groups. Aromatic moieties were frequently included in these structures to increase lipophilicity. Figure 2 shows four different design strategies for *Ba*TMPK inhibitors. The dThd scaffold was kept intact because it provides the element of selective affinity for *Ba*TMPK. Sulfonamide-, (thio)urea-, amide-, or triazole groups were attached to the 5′-position of dThd to generate

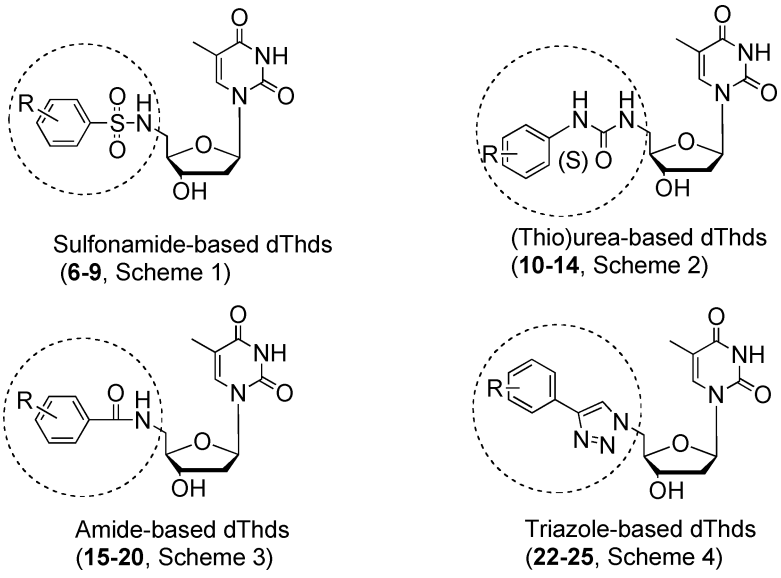


FIGURE 2 Design of dThd-based *Ba*TMPK inhibitors substituted with lipophilic phosphate-mimicking groups at the 5′-position.

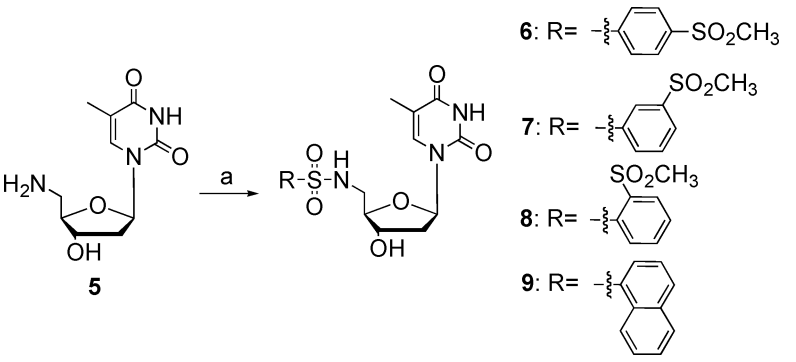
hydrogen-bond interactions with amino acid residues in the phosphate-loop (P-loop) region of *Ba*TMPK. To increase lipophilicity, aromatic moieties were bound to these groups.

Among many known nucleoside analogues with phosphate-mimicking groups at the 5′-postion, the natural product nucleocidin (**3**), an inhibitor of protein synthesis with broad antibacterial spectrum,[27–29] and salicyl-AMS (**4**), an inhibitor of the siderophore biosynthesis, are of special interest (Figure 1).[30,31] Both agents demonstrated antimicrobial activity. Therefore, it is reasonable to assume that *Ba*TMPK inhibitor containing stable lipophilic phosphate-mimics at the 5′-position should also be able to enter the interior of *B. anthracis*.

Chemistry

Compounds **5** (Schemes 1–3) and **21** (Scheme 4) were used as starting materials for all synthesized *Ba*TMPK inhibitors. Both compounds were synthesized according to previously reported procedures.[16,32,33] Sulfonamide-linked dThd inhibitors (**6–9**) were prepared in 65–80% yield by reacting **5** with the appropriate sulfonyl chlorides [4-(methylsulfonyl)benzenesulfonyl chloride for **6**, 3-(methylsulfonyl)benzenesulfonyl chloride for **7**, 2-(methylsulfonyl)benzenesulfonyl chloride for **8**, 1-naphthalenesulfonyl chloride for **9**] in the presence of (Scheme 1) triethylamine.

The thiourea-linked inhibitor **10** was prepared by reacting compound **5** with 2-(methythio)phenylisothiocyanate and a catalytic amount of DMAP

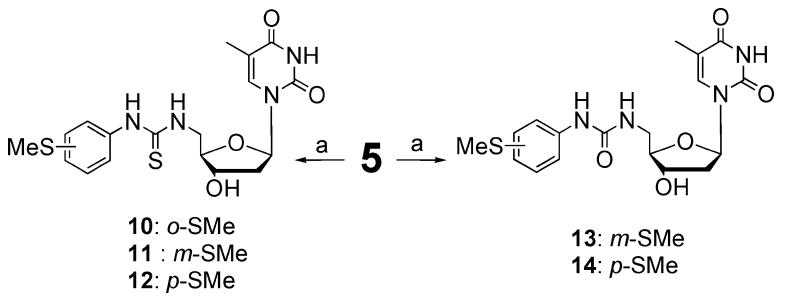


SCHEME 1 Reagents and conditions: (a) Sulfonyl chloride, TEA, DMF, rt, 6 hours.

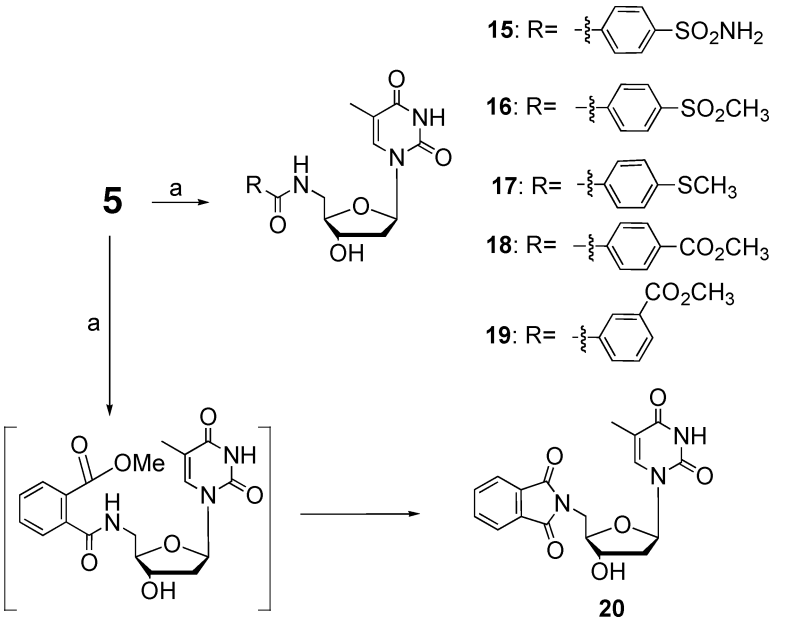
in pyridine in 76% yield (Scheme 2). The *meta-* (**11**) and *para*-isomers (**12**) of **10** were prepared in 73% and 83% yield by using 3-(methylthio)- and 4-(methylthio) phenylisothiocyanate, respectively. Identical reaction conditions were used for the synthesis of the urea-linked dThd inhibitors **13** and **14**. Treatment of 3-(methylthio)- and 4-(methylthio)phenylisocyanate with **5** afforded **13** (68% yield) and **14** (73% yield), respectively.

Various benzene carboxylic acids (see experimental section) were coupled with **5** using 1-hydroxybenzotriazole (HOBt) and 1,3-dicyclohexylcarbodiimide (DCC) to afford the amide-linked inhibitors **15–19** in yields ranging from 38% to 58% (Scheme 3). When methyl hydrogen phthalate was reacted with **5**, compound **20** was obtained in 28% yield. The formation of **20** was presumably facilitated by the close proximity of the CO_2Me- and the NH group in an intermediate amide, resulting in the formation of the phthalimide *via* intramolecular cyclization, as shown in Scheme 3.

The triazole-linked inhibitors **22–25** were prepared by reacting **21** with the appropriate alkynes in 8–15% (Scheme 4) yields. The formation of 1,4-substituted-1,2,3-triazoles from azides and alkynes has been described as "click chemistry."[34] No protective groups were required in this reaction.



SCHEME 2 Reagents and conditions: a) Phenylisothiocyanate or phenylisocyanate, DMAP, pyridine, rt, 12 hours.

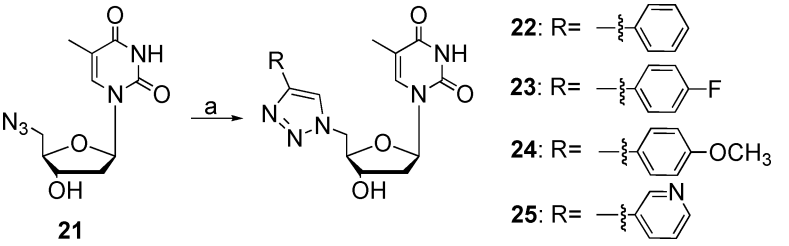


SCHEME 3 Reagents and conditions: a) Benzoic acid, DCC, HOBt, DMF, rt, 12 hours.

Copper (I) is the most powerful catalyst of this 1,3-dipolar cycloaddition of azides and alkynes.[35] We used the 'copper (II)/ascorbate system,' which generates the Cu (I) catalyst in situ by reducing $CuSO_4$ with sodium ascorbate.[36] To our knowledge, this is the first report on 5′-triazole-substituted dThds.

Biology

Initially, the in vitro growth inhibiting effects of all target compounds (**6–20** and **22–25**) on *B. anthracis* Sterne were tested. The results of these studies are shown in Table 1. Compounds with IC_{50} values <200 μM (**11**, **23**) and several representatives of compounds with IC_{50} values >200 μM (**6**,



SCHEME 4 Reagents and conditions: Alkyne, sodium ascorbate, $CuSO_4$, H_2O/t-BuOH (1:1), vigorous stirring, rt, 48 hours.

TABLE 1 Growth inhibition of *B. anthracis* Sterne and inhibition of *Ba*TMPK activity by compounds **6–20** and **22–25**

Compd.	Growth inhibition IC_{50} (μM)[a]	*Ba*TMPK inhibition[c,d]	Compd.	Growth inhibition IC_{50} (μM)[a]	*Ba*TMPK inhibition[c,d]
6	>200	94 ± 2	**16**	>200	—
7	>200	—	**17**	>200	—
8	>200	91 ± 3	**18**	>200	—
9	>200		**19**	>200	—
10	>200	84 ± 11	**20**	>200	—
11	85.1 (76.4–95.1)[b]	69 ± 6	**22**	>200	—
12	>200	69 ± 9	**23**	112.8 (78.2–194.2)[b]	87 ± 8
13	>200	—	**24**	>200	—
14	>200	—	**25**	>200	—
15	>200	—			

[a]IC_{50} after 6 hours incubation. Data are based at least on three measurements.

[b]Lower and upper limits of 95% confidence using EPA PROBIT analysis program. Compound concentrations ranged from 1 μM to 200 μM.

[c]Remaining enzyme activity (in %) as compared to enzyme activity without addition of inhibitor (100 %).

[d]Mean ± SD values are based on at least three separate determinations.

8, **10**, **12**) were evaluated in enzyme assays with *Ba*TMPK at concentrations of 100 μM.

Only compound **11**, with a thiourea linker and a SCH_3 group at the *meta*-position of the phenyl ring, and **23**, with a triazole linker and a fluorine atom at the *para*-position of the phenyl ring, showed moderate growth inhibition in vitro with IC_{50}'s of 85 μM and 113 μM, respectively. Compounds **11** and **23** were at least 10 times better inhibitors of *B. anthracis* Sterne growth than the dThd analogues **1** and **2**. Compounds **11** and **12** produced moderate (~30%) inhibition of *Ba*TMPK. Only compound **11** showed both inhibition of *B. anthracis* Sterne growth and *Ba*TMPK activity. The inhibitory capacities of compounds **6**, **8**, **10**, **11**, **12**, **23** on *B. anthracis* thymidine kinase (*Ba*TK)[11] activity were also tested and compounds **6**, **8**, **10**, and **11** produced >50% inhibition at a concentration of 100 μM (Usova, E., Byun, Y., Eriksson, S. and Tjarks, W., unpublished results). However, Gram-positive bacteria have low utilization levels for TKs, and thus, low dependence on salvage pathways for their DNA biosynthesis.[37,38] Therefore, it is unlikely that *Ba*TK inhibition played a significant role in the growth inhibitory effect observed for **11**. It is conceivable that the moderate growth inhibiting effect of **23** was caused by interaction with other intracellular kinases or even RR or DNA polymerase. The lack of growth inhibition seen with most of the tested compounds could have been related to poor transport into *B. anthracis* Sterne. Additional studies will be necessary to elucidate the general potential of these *Ba*TMPK inhibitors as

novel antibiotics in the treatment of anthrax. Such studies would include eukaryotic systems.

EXPERIMENTAL PROCEDURES

General

^{1}H and ^{13}C NMR spectra were obtained on Bruker 250 or 400 MHz (^{1}H resonance frequency) FT-NMR instruments. Chemical shifts are reported in parts per million (ppm). The coupling constants are reported in Hertz (Hz). High-resolution electrospray ionization (HR-ESI) mass spectra were recorded on a Micromass QTOF-Electrospray mass spectrometer and a 3-Tesla Finnigan FTMS-2000 Fourier Transform mass spectrometer at The Ohio State University Campus Chemical Instrumentation Center. Compound visualization on Silica Gel 60 F_{254} precoated TLC plates (0.25 mm layer thickness) (Merck, Darmstadt, Germany) was attained by UV light and $KMnO_4$ spray. Reagent grade solvents were used for column chromatography using Silica gel 60, particle size 0.040–0.063 mm (Merck, Whitehouse Station, NJ, USA). Reagent grade chemicals were obtained from commercial vendors and used as such. Anhydrous benzene and THF were obtained using distillation from sodium benzophenone ketyl prior to use. All reactions were carried out under argon atmosphere.

General procedure for the synthesis of sulfonamide-linked BaTMPK inhibitors (6–9)

To a solution of **5** (60 mg, 0.25 mmol) and the indicated sulfonylchloride (0.28 mmol) [see below] in DMF (5 mL) was added triethylamine (0.5 mL). The resulting solution was stirred for 6 hours at room temperature. The solution was cooled to 4°C, triethylamine hydrochloride was removed by filtration, and the filtrate was concentrated. The residue was triturated with methanol to give a crystalline solid, which was collected by filtration. Washing the solid with hexanes/ethyl acetate (1:1) afforded the following products.

5′-Deoxy-5′-[4-(methylsulfonyl)benzenesulfonamido]thymidine (6). Sulfonylchloride: 4-(Methylsulfonyl)benzenesulfonyl chloride; yield: 92 mg (80%); R_f 0.10 (dichloromethane/methanol, 10:1); ^{1}H NMR (DMSO-d_6) δ 1.78 (s, 3H, CH_3), 2.00–2.15 (m, 2H, H-2′), 2.98 (dd, 1H, H-5′, J = 13.6, 6.7 Hz), 3.11 (dd, 1H, H-5′, J = 13.6, 4.1 Hz), 3.29 (s, 3H, SO_2CH_3), 3.68-3.72 (m, 1H, H-4′), 4.12–4.15 (m, 1H, H-3′), 5.33 (d, 1H, OH, J = 4.3 Hz), 6.11 (t, 1H, H-1′, J = 6.8 Hz), 7.49 (s, 1H, H-6), 8.04 (d, 2H, ArH, J = 8.4 Hz), 8.13 (d, 2H, ArH, J = 8.4 Hz), 8.21 (s, 1H, NH), 11.25 (s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ 12.10 (CH_3), 38.19 (C-2′), 43.14 (C-5′), 44.71 (SO_2CH_3), 70.84 (C-3′), 83.86 (C-1′), 84.69 (C-4′), 109.70 (C-5), 127.48

(ArC), 128.12 (ArC), 136.28 (C-6), 144.00 (ArC), 145.15 (ArC), 150.44 (C-2), 163.72 (C-4); MS (HR-ESI) $C_{17}H_{21}N_3O_8S_2$ $(M+Na)^+$ calcd 482.0668, found 482.0672.

5′-Deoxy-5′-[3-(methylsulfonyl)benzenesulfonamido]thymidine (7). Sulfonylchloride: 3-(Methylsulfonyl)benzenesulfonyl chloride; yield: 89 mg (78%); R_f 0.16 (dichloromethane/methanol, 10:1); ^{1}H NMR (MeOH-d_4) δ 1.89 (d, 3H, CH_3, J = 1.1 Hz), 2.00–2.23 (m, 2H, H-2′), 3.10–3.25 (m, 2H, H-5′), 3.18 (s, 1H, SO_2CH_3), 3.78–3.84 (m, 1H, H-4′), 4.27–4.33 (m, 1H, H-3′), 6.14 (t, 1H, H-1′, J = 6.8 Hz), 7.52 (d, 1H, H-6, J = 1.1 Hz), 7.82 (t, 1H, ArH, J = 7.9 Hz), 8.17 (d, 1H, ArH, J = 7.9 Hz), 8.18 (d, 1H, ArH, J = 7.9 Hz), 8.40 (t, 1H, ArH, J = 1.7 Hz); ^{13}C NMR (MeOH-d_4) δ 12.38 (CH_3), 40.18 (C-2′), 44.17 (C-5′), 45.77 (SO_2CH_3), 72.47 (C-3′), 86.23 (C-1′), 86.61 (C-4′), 111.88 (C-5), 126.86 (ArC), 131.79 (ArC), 132.22 (ArC), 132.83 (ArC), 138.30 (C-6), 143.46 (ArC), 143.88 (ArC), 152.28 (C-2), 166.39 (C-4); MS (HR-ESI) $C_{17}H_{21}N_3O_8S_2$ $(M+Na)^+$ calcd 482.0668, found 482.0666.

5′-Deoxy-5′-[2-(methylsulfonyl)benzenesulfonamido]thymidine (8). Sulfonylchloride: 2-(Methylsulfonyl)benzenesulfonyl chloride; yield: 83 mg (72%); R_f 0.16 (dichloromethane/methanol, 10:1); ^{1}H NMR (MeOH-d_4) δ 1.88 (d, 3H, CH_3, J = 1.1 Hz), 2.15–2.25 (m, 2H, H-2′), 3.17 (dd, 1H, H-5′, J = 13.4, 6.1 Hz), 3.24 (dd, 1H, H-5′, J = 13.4, 3.9 Hz), 3.40 (s, 1H, SO_2CH_3), 3.82–3.86 (m, 1H, H-4′), 4.26–4.30 (m, 1H, H-3′), 6.11 (t, 1H, H-1′, J = 6.9 Hz), 7.42 (d, 1H, H-6, J = 1.1 Hz), 7.86–7.90 (m, 2H, ArH), 8.21–8.24 (m, 1H, ArH), 8.27–8.31 (m, 1H, ArH); ^{13}C NMR (MeOH-d_4) δ 12.46 (CH_3), 40.10 (C-2′), 44.54 (C-5′), 45.90 (SO_2CH_3), 72.45 (C-3′), 85.85 (C-1′), 86.41 (C-4′), 111.94 (C-5), 132.73 (ArC), 133.82 (ArC), 134.92 (ArC), 135.57 (ArC), 138.03 (C-6), 139.68 (ArC), 140.20 (ArC), 152.17 (C-2), 166.39 (C-4); MS (HR-ESI) $C_{17}H_{21}N_3O_8S_2$ $(M+Na)^+$ calcd 482.0668, found 482.0673.

5′-Deoxy-5′-(1-naphthalenesulfonamido)thymidine (9). Sulfonylchloride: 1-Naphthalenesulfonyl chloride; yield: 70 mg (65%); R_f 0.15 (dichloromethane/methanol, 10:1); ^{1}H NMR (MeOH-d_4) δ 1.85 (d, 3H, CH_3, J = 1.1 Hz), 2.12–2.19 (m, 2H, H-2′), 3.08 (dd, 1H, H-5′, J = 14.0, 6.0 Hz), 3.24 (dd, 1H, H-5′, J = 14.0, 4.1 Hz), 3.75–3.81 (m, 1H, H-4′), 4.23–4.27 (m, 1H, H-3′), 6.05 (t, 1H, H-1′, J = 6.9 Hz), 7.46 (d, 1H, H-6, J =1.1 Hz), 7.53–7.70 (m, 3H, ArH), 8.00 (dd, 1H, ArH, J = 7.8, 1.5 Hz), 8.12 (d, 1H, ArH, J = 7.8 Hz), 8.20 (dd, 1H, ArH, J = 7.8, 1.5 Hz), 8.69 (dd, 1H, ArH, J = 7.8, 1.5 Hz); ^{13}C NMR (MeOH-d_4) δ 12.33 (CH_3), 40.08 (C-2′), 45.73 (C-5′), 72.56 (C-3′), 86.30 (C-1′), 86.84 (C-4′), 111.70 (C-5), 125.25 (ArC), 125.87 (ArC), 127.95 (ArC), 129.01 (ArC), 129.47 (ArC), 130.07 (ArC), 130.14 (ArC), 135.19 (ArC), 135.81 (ArC), 136.71 (ArC), 138.45 (C-6), 152.21 (C-2), 166.39 (C-4); MS (HR-ESI) $C_{20}H_{21}N_3O_6S$ $(M+Na)^+$ calcd 454.1049, found 454.1031.

General procedure for the synthesis of (thio)urea-linked BaTMPK inhibitors (10–14)

To a solution of **5** (60 mg, 0.25 mmol) and the indicated phenylisocyanate or phenylisothiocyanate (0.28 mmol) [see below] in pyridine (10 mL) was added 4-(dimethylamino)pyridine (6 mg, 0.05 mmol). The resulting solution was stirred for 12 h at room temperature. The solvent was evaporated and the residue was purified by silica gel column chromatography using ethyl acetate/methanol (25:1) as the eluent to give the following products.

5′-Deoxy-5′-{[2-(methylthio)anilinothiocarbonyl]amino}thymidine (10). Phenylisothiocyanate: 2-(Methylthio)phenyl isothiocyanate; yield: 80 mg (76%); R_f 0.30 (ethyl acetate/methanol, 25:1); ^{1}H NMR (MeOH-d$_4$) δ 1.88 (s, 3H, CH_3), 2.22–2.25 (m, 2H, H-2′), 2.40 (s, 3H, SCH_3), 3.83–3.98 (m, 2H, H-5′), 4.02–4.06 (m, 1H, H-4′), 4.34–4.38 (m, 1H, H-3′), 6.19 (t, 1H, H-1′, J = 6.9 Hz), 7.18 (dd, 1H, ArH, J = 7.8, 7.4 Hz), 7.23 (dd, 1H, ArH, J = 7.8, 7.4 Hz), 7.34 (d, 2H, ArH, J = 7.8 Hz), 7.52 (s, 1H, H-6); ^{13}C NMR (MeOH-d$_4$) δ 12.47 (CH_3), 15.42 (CH_3), 39.96 (C-2′), 49.65 (C-5′), 73.09 (C-3′), 86.34 (C-1′), 87.00 (C-4′), 111.81 (C-5), 126.74 (ArC), 128.07 (ArC), 129.09 (ArC), 129.45 (ArC), 126.25 (ArC), 138.12 (C-6), 141.03 (ArC), 152.28 (C-2), 166.43 (C-4), 182.27 (C=S); MS (HR-ESI) $C_{18}H_{22}N_4O_4S_2Na$ $(M+Na)^+$ calcd 445.0980, found 445.0995.

5′-Deoxy-5′-{[3-(methylthio)anilinothiocarbonyl]amino}thymidine (11). Phenylisothiocyanate: 3-(Methylthio)phenyl isothiocyanate; yield: 77 mg (73%); R_f 0.31 (ethyl acetate/methanol, 25:1); ^{1}H NMR (MeOH-d$_4$) δ 1.86 (d, 3H, CH_3, J = 1.0 Hz), 2.22–2.30 (m, 2H, H-2′), 2.45 (s, 3H, SCH_3), 3.77–3.98 (m, 2H, H-5′), 4.04–4.10 (m, 1H, H-4′), 4.32–4.38 (m, 1H, H-3′), 6.21 (t, 1H, H-1′, J = 6.9 Hz), 7.02–7.04 (m, 1H, ArH), 7.06 (t, 1H, ArH, J = 6.9 Hz) 7.23 (d, 1H, ArH, J = 7.9 Hz), 7.27–7.30 (m, 1H, ArH), 7.50 (d, 1H, H-6, J = 1.0 Hz); ^{13}C NMR (MeOH-d$_4$) δ 12.40 (CH_3), 15.43 (CH_3), 38.85 (C-2′), 50.31 (C-5′), 73.12 (C-3′), 86.10 (C-1′), 87.11 (C-4′), 111.97 (C-5), 121.61 (ArC), 122.71 (ArC), 124.50 (ArC), 130.51 (ArC), 138.16 (C-6), 140.15 (ArC), 141.43 (ArC), 152.30 (C-2), 166.36 (C-4), 182.66 (C=S); MS (HR-ESI) $C_{18}H_{22}N_4O_4S_2Na$ $(M+Na)^+$ calcd 445.0980, found 445.0995.

5′-Deoxy-5′-{[4-(methylthio)anilinothiocarbonyl]amino}thymidine (12). Phenylisothiocyanate: 4-(Methylthio)phenyl isothiocyanate; yield: 88 mg (83%); R_f 0.26 (ethyl acetate/methanol, 25:1); ^{1}H NMR (MeOH-d$_4$) δ 1.87 (s, 3H, CH_3), 2.22–2.28 (m, 2H, H-2′), 2.45 (s, 3H, SCH_3), 3.82–3.96 (m, 2H, H-5′), 4.04–4.07 (m, 1H, H-4′), 4.34–4.38 (m, 1H, H-3′), 6.20 (t, 1H, H-1′, J = 6.9 Hz), 7.22–7.27 (m, 4H, ArH), 7.52 (s, 1H, H-6); ^{13}C NMR (MeOH-d$_4$) δ 12.41 (CH_3), 16.00 (CH_3), 39.84 (C-2′), 49.71 (C-5′), 73.08 (C-3′), 86.17 (C-1′), 87.08 (C-4′), 111.87 (C-5), 126.34 (ArC), 128.43 (ArC), 138.17 (C-6), 152.29 (C-2), 166.38 (C-4), 182.80 (C=S); MS (HR-ESI) $C_{18}H_{22}N_4O_4S_2Na$ $(M+Na)^+$ calcd 445.0980, found 445.1007.

5′-Deoxy-5′-{[3-(methylthio)anilinocarbonyl]amino}thymidine (13). Phenylisocyanate: 3-(Methylthio)phenyl isocyanate; yield: 69 mg (68%); R_f 0.36 (ethyl acetate/methanol, 15:1); ^{1}H NMR (DMSO-d$_6$) δ 1.77 (s, 3H, CH$_3$), 2.04–2.19 (m, 2H, H-2′), 2.42 (s, 3H, SCH$_3$), 3.19–3.48 (m, 2H, H-5′), 3.76–3.79 (m, 1H, H-4′), 4.15–4.16 (m, 1H, H-3′), 5.33 (d, 1H, OH, J = 4.2 Hz), 6.18 (t, 1H, H-1′, J = 7.4 Hz), 6.32 (t, 1H, NH, J = 5.8 Hz), 6.78 (d, 1H, ArH, J = 7.8 Hz), 7.05 (d, 1H, ArH, J = 7.8 Hz), 7.15 (t, 1H, ArH, J = 7.8 Hz) 7.43 (s, 1H, ArH), 7.52 (s, 1H, H-6), 8.58 (s, 1H, NH), 11.32 (s, 1H, NH); ^{13}C NMR (DMSO-d$_6$) δ 12.06 (CH$_3$), 14.57 (CH$_3$), 38.39 (C-2′), 41.43 (C-5′), 71.13 (C-3′), 83.74 (C-1′), 85.30 (C-4′), 109.77 (C-5), 114.18 (ArC), 114.60 (ArC), 118.46 (ArC), 129.14 (ArC), 136.07 (C-6), 138.38 (ArC), 140.91 (ArC), 150.48 (C-2), 155.12 (C=O), 163.70 (C-4); MS (HR-ESI) $C_{18}H_{22}N_4O_5S$ (M+Na)$^+$ calcd 429.1209, found 429.1214.

5′-Deoxy-5′-{[4-(methylthio)anilinocarbonyl]amino}thymidine (14). Phenylisocyanate: 4-(Methylthio)phenyl isocyanate, yield: 74 mg (73%); R_f 0.34 (ethyl acetate/methanol, 15:1); ^{1}H NMR (DMSO-d$_6$) δ 1.77 (s, 3H, CH$_3$), 2.04–2.17 (m, 2H, H-2′), 2.40 (s, 3H, SCH$_3$), 3.17–3.43 (m, 2H, H-5′), 3.75–3.78 (m, 1H, H-4′), 4.17–4.21 (m, 1H, H-3′), 5.37 (d, 1H, OH, J = 4.2 Hz), 6.15 (t, 1H, H-1′, J = 7.4 Hz), 6.88 (t, 1H, NH, J = 5.8 Hz), 7.14 (d, 2H, ArH, J = 8.6 Hz), 7.37 (d, 2H, ArH, J = 8.6 Hz), 7.66 (s, 1H, H-6), 9.38 (s, 1H, NH), 11.28 (s, 1H, NH); ^{13}C NMR (DMSO-d$_6$) δ 11.90 (CH$_3$), 16.26 (CH$_3$), 38.50 (C-2′), 40.50 (C-5′), 70.82 (C-3′), 83.39 (C-1′), 85.21 (C-4′), 109.80 (C-5), 118.06 (ArC), 127.96 (ArC), 128.47 (ArC), 136.13 (C-6), 138.60 (ArC), 150.45 (C-2), 155.49 (C=O), 163.71 (C-4); MS (HR-ESI) $C_{18}H_{22}N_4O_5S$ (M+Na)$^+$ calcd 429.1209, found 429.1212.

General procedure for the synthesis of amide-linked BaTMPK inhibitors (15–20)

To a solution of **5** (60 mg, 0.25 mmol) and the indicated benzoic acid (0.30 mmol) [See below] in DMF (8 mL) was added 1-hydroxybenzotriazole (HOBt, 41 mg, 0.30 mmol). A solution of 1,3-dicyclohexylcarbodiimide (DCC, 62 mg, 0.30 mmol) in DMF (2 mL) was added slowly and the resulting reaction mixture was stirred for 12 hours at room temperature. The solvent was removed under reduced pressure and the residue was purified by silica gel column chromatography using ethyl acetate/methanol (20:1) as the eluent to give the following products.

5′-Deoxy-5′-[4-(sulfamoyl)benzamido]thymidine (15). Benzoic acid: 4-(Sulfamoyl)benzoic acid; yield: 51 mg (48%); R_f 0.22 (ethyl acetate/methanol, 15:1); ^{1}H NMR (DMSO-d$_6$) δ 1.77 (s, 3H, CH$_3$), 2.07–2.18 (m, 2H, H-2′), 3.52–3.55 (m, 2H, H-5′), 3.88–3.90 (m, 1H, H-4′), 4.24–4.26 (m, 1H, H-3′), 5.33 (d, 1H, OH, J = 4.4 Hz), 6.15 (t, 1H, H-1′, J = 6.7 Hz), 7.48 (s, 2H, NH$_2$), 7.52 (s, 1H, H-6), 7.90 (d, 2H, ArH, J = 8.3 Hz), 8.01 (d, 2H, ArH, J = 8.3 Hz), 8.82 (t, 1H, NH, J = 5.6 Hz), 11.30 (s, 1H, NH); ^{13}C NMR

(DMSO-d_6) δ 11.96 (CH_3), 38.50 (C-2′), 41.67 (C-5′), 71.26 (C-3′), 84.03 (C-1′), 84.71 (C-4′), 109.62 (C-5), 125.60 (ArC), 127.88 (ArC), 136.17 (ArC), 137.11 (C-6), 146.30 (ArC), 150.41 (C-2), 163.68 (C-4), 165.47 (C=O); MS (HR-ESI) $C_{17}H_{20}N_4O_7S$ $(M+Na)^+$ calcd 447.0950, found 447.0953.

5′-Deoxy-5′-[4-(methylsulfonyl)benzamido]thymidine (16). Benzoic acid: 4-(Methylsulfonyl)benzoic acid; yield: 56 mg (53%); R_f 0.27 (ethyl acetate/methanol, 15:1); ^{1}H NMR (DMSO-d_6) δ 1.76 (s, 3H, CH_3), 2.07–2.17 (m, 2H, H-2′), 3.28 (s, 3H, SO_2CH_3), 3.54–3.58 (m, 2H, H-5′), 3.90–3.94 (m, 1H, H-4′), 4.25–4.29 (m, 1H, H-3′), 5.40 (d, 1H, OH, J = 4.3 Hz), 6.15 (t, 1H, H-1′, J = 6.8 Hz), 7.53 (s, 1H, H-6), 8.02–8.18 (m, 4H, ArH), 9.02 (s, 1H, NH), 11.38 (s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ 12.10 (CH_3), 38.11 (C-2′), 42.14 (C-5′), 44.19 (SO_2CH_3), 70.89 (C-3′), 83.81 (C-1′), 84.56 (C-4′), 110.11 (C-5), 127.40 (ArC), 128.64 (ArC), 139.50 (C-6), 143.33 (ArC), 150.64 (C-2), 164.02 (C-4), 165.62 (ArC); MS (HR-ESI) $C_{18}H_{21}N_3O_7S$ $(M+Na)^+$ calcd 446.0998, found 446.1002.

5′-Deoxy-5′-[4-(methylthio)benzamido]thymidine (17). Benzoic acid: 4(Methylthio)benzoic acid; yield: 57 mg (58%); R_f 0.37 (ethyl acetate/methanol, 15:1); ^{1}H NMR (DMSO-d_6) δ 1.76 (s, 3H, CH_3), 2.07–2.12 (m, 2H, H-2′), 2.51 (s, 3H, SCH_3), 3.49–3.52 (m, 2H, H-5′), 3.88–3.90 (m, 1H, H-4′), 4.22–4.26 (m, 1H, H-3′), 5.32 (d, 1H, OH, J = 4.3 Hz), 6.14 (t, 1H, H-1′, J = 6.8 Hz), 7.32 (d, 2H, ArH, J = 8.4 Hz), 7.51 (s, 1H, H-6), 7.81 (d, 2H, ArH, J = 8.4 Hz), 8.58 (t, 1H, NH, J = 5.6 Hz), 11.30 (s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ 12.45 (CH_3), 14.59 (CH_3), 41.90 (C-2′), 49.08 (C-5′), 71.76 (C-3′), 84.84 (C-1′), 85.21 (C-4′), 110.47 (C-5), 125.48 (ArC), 128.32 (ArC), 130.47 (ArC), 136.88 (C-6), 143.51 (ArC), 150.99 (C-2), 164.59 (C-4), 167.14 (C=O); MS (HR-ESI) $C_{18}H_{21}N_3O_5S$ $(M+Na)^+$ calcd 414.1100, found 414.1104.

5′-Deoxy-5′-[4-(methoxycarbonyl)benzamido]thymidine (18). Benzoic acid: *mono*-Methyl terephthalate; yield: 45 mg (43%); R_f 0.37 (ethyl acetate/methanol, 15:1); ^{1}H NMR (DMSO-d_6) δ 1.76 (s, 3H, CH_3), 2.08–2.22 (m, 2H, H-2′), 3.51–3.56 (m, 2H, H-5′), 3.88 (s, 3H, OCH_3), 3.88–3.92 (m, 1H, H-4′), 4.24–4.26 (m, 1H, H-3′), 5.31 (d, 1H, OH, J = 4.4 Hz), 6.14 (t, 1H, H-1′, J = 6.7 Hz), 7.51 (s, 1H, H-6), 7.97 (d, 2H, ArH, J = 7.3 Hz), 8.04 (d, 2H, ArH, J = 7.3 Hz), 8.80 (t, 1H, NH, J = 5.0 Hz), 11.28 (s, 1H, NH); ^{13}C NMR (MeOH-d_4) δ 11.93 (CH_3), 38.36 (C-2′), 41.67 (C-5′), 52.33 (OCH_3), 71.28 (C-3′), 84.06 (C-1′), 87.73 (C-4′), 109.60 (C-5), 127.62 (ArC), 129.11 (ArC), 131.79 (ArC), 136.15 (ArC), 138.34 (C-6), 150.41 (C-2), 163.68 (C-4), 165.65 (C=O), 165.68 (C=O), 169.36 (C=O); MS (HR-ESI) $C_{19}H_{21}N_3O_7$ $(M+Na)^+$ calcd 426.1277, found 426.1285.

5′-Deoxy-5′-[3-(methoxycarbonyl)benzamido]thymidine (19). Benzoic acid: Methyl hydrogen isophthalate; yield: 38 mg (38%); R_f 0.36 (ethyl acetate/methanol, 15:1); ^{1}H NMR (MeOH-d_4) δ 1.82 (s, 3H, CH_3), 2.25–2.30 (m, 2H, H-2′), 3.63–3.78 (m, 2H, H-5′), 3.93 (s, 3H, OCH_3), 4.02–4.06 (m, 1H, H-4′), 4.34–4.38 (m, 1H, H-3′), 6.22 (t, 1H, H-1′, J = 7.4 Hz),

7.50 (s, 1H, H-6), 7.59 (t, 1H, ArH, J = 7.8 Hz), 8.09 (dt, 1H, ArH, J = 7.8, 1.2 Hz), 8.17 (dd, 1H, ArH, J = 7.8, 1.2 Hz), 8.50 (t, 1H, ArH, J = 1.2 Hz); ^{13}C NMR (MeOH-d_4) δ 12.29 (CH_3), 40.11 (C-2′), 42.88 (C-5′), 52.86 (OCH_3), 73.06 (C-3′), 86.43 (C-1′), 87.03 (C-4′), 111.75 (C-5), 129.42 (ArC), 130.02 (ArC), 131.88 (ArC), 132.87 (ArC), 133.45 (ArC), 136.04 (ArC), 138.20 (C-6), 152.32 (C-2), 163.36 (C-4), 167.73 (C=O), 169.36 (C=O); MS (HR-ESI) $C_{19}H_{21}N_3O_7$ $(M+Na)^+$ calcd 426.1277, found 426.1289.

5′-Deoxy-5′-(phthalimido)thymidine (20). Benzoic acid: Methyl hydrogen phthalate; yield: 26 mg (28%); R_f 0.57 (ethyl acetate/methanol, 15:1); ^{1}H NMR (DMSO-d_6) δ 1.82 (s, 3H, CH_3), 2.07–2.26 (m, 2H, H-2′), 3.78 (dd, 1H, H-5′, J = 14.2, 7.7 Hz), 3.85 (dd, 1H, H-5′, J = 14.2, 5.6 Hz), 3.99–4.04 (m, 1H, H-4′), 4.22–4.26 (m, 1H, H-3′), 5.37 (d, 1H, OH, J = 4.1 Hz), 6.14 (t, 1H, H-1′, J = 6.8 Hz), 7.56 (s, 1H, H-6), 7.83–7.92 (m, 4H, ArH), 11.25 (s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ 12.86 (CH_3), 38.84 (C-2′), 47.37 (C-5′), 72.44 (C-3′), 83.19 (C-1′), 84.18 (C-4′), 109.55 (C-5), 123.14 (ArC), 131.43 (ArC), 134.54 (ArC), 136.12(C-6), 150.36 (C-2), 163.65 (C-4), 167.86 (C=O); MS (HR-ESI) $C_{18}H_{17}N_3O_6$ $(M+Na)^+$ calcd 394.1015, found 394.1024.

General procedure for the synthesis of triazole-linked BaTMPK inhibitors (22–25)

Compound **21** (65 mg, 0.25 mmol) and the indicated alkyne (0.25 mmol) [see below] were dissolved in a mixture of water and *tert*-BuOH (6 mL, 1:1). Freshly prepared sodium ascorbate solution (0.05 mL, 1 M solution in water) was added, followed by the addition of freshly prepared copper (II) sulfate pentahydrate (0.01 mL, 0.5 M solution in water). During vigorous stirring of the reaction mixture at room temperature for 48 hours, a precipitate formed. The reaction mixture was diluted with 20 mL of water and cooled to 0°C. A precipitate was collected by filtration and dried in vacuum to give the following products.

5′-Deoxy-5′-[4-phenyl-(1,2,3)triazol-1-yl]thymidine (22). Alkyne: Ethynylbenzene; yield: 14 mg (15%); R_f 0.18 (dichloromethane/methanol, 10:1); ^{1}H NMR (DMSO-d_6) δ 1.68 (s, 3H, CH_3), 2.07–2.21 (m, 2H, H-2′), 3.78 (s, 3H, OCH_3), 4.11–4.14 (m, 1H, H-4′), 4.29–4.33 (m, 1H, H-3′), 4.67 (dd, 1H, H-5′, J = 14.3, 6.8 Hz), 4.76 (dd, 1H, H-5′, J = 14.3, 4.5 Hz), 5.53 (d, 1H, OH, J = 4.4 Hz), 6.19 (t, 1H, H-1′, J = 6.8 Hz), 7.24 (s, 1H, H-6), 7.31-7.44 (m, 3H, ArH), 7.84 (d, 2H, ArH, J = 7.5 Hz), 8.57 (s, 1H, ArH), 11.31 (s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ 11.96 (CH_3), 37.85 (C-2′), 51.09 (C-5′), 71.38 (C-3′), 83.72 (C-1′), 84.65 (C-4′), 115.16 (C-5), 122.19 (ArC-triazole), 125.10 (ArC), 127.87 (ArC), 128.87 (ArC), 130.59 (ArC), 138.20 (C-6), 146.35 (ArC-triazole), 152.32 (C-2), 163.36 (C-4); MS (HR-ESI) $C_{18}H_{19}N_5O_4$ $(M+Na)^+$ calcd 392.1335, found 392.1332.

5′-Deoxy-5′-[4-(4-fluorophenyl)-(1,2,3)triazol-1-yl]thymidine (23). Alkyne: 1-Ethynyl-4-fluorobenzene; yield: 13 mg (13%); R_f 0.14 (dichloromethane/methanol, 10:1); ^{1}H NMR (DMSO-d_6) δ 1.69 (s, 3H, CH_3), 2.11–2.21 (m, 2H, H-2′), 4.09–4.14 (m, 1H, H-4′), 4.28–4.32 (m, 1H, H-3′), 4.67 (dd, 1H, H-5′, J = 14.5, 6.3 Hz), 4.76 (dd, 1H, H-5′, J = 14.5, 4.7 Hz), 5.52 (d, 1H, OH, J = 4.4 Hz), 6.19 (t, 1H, H-1′, J = 6.8 Hz), 7.23 (s, 1H, H-6), 7.28 (t, 2H, ArH, J = 8.7 Hz), 7.88 (dd, 2H, ArH, J = 8.3, 5.7 Hz), 8.55 (s, 1H, ArH), 11.28 (s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ 12.00 (CH_3), 37.88 (C-2′), 51.12 (C-5′), 70.52 (C-3′), 83.72 (C-1′), 83.98 (C-4′), 109.82 (C-5), 115.71 (ArC), 115.93 (ArC), 122.12 (ArC-triazole), 127.11 (ArC), 127.19 (ArC), 135.95 (C-6), 145.51 (ArC-triazole), 150.44 (C-2), 160.55 (ArC), 162.98 (ArC), 163.57 (C-4); MS (HR-ESI) $C_{18}H_{18}FN_5O_4$ $(M+Na)^+$ calcd 410.1241, found 410.1241.

5′-Deoxy-5′-[4-(4-methoxyphenyl)-(1,2,3)triazol-1-yl]thymidine (24). Alkyne: 4-Ethynylanisole; yield: 11 mg (10%); R_f 0.18 (dichloromethane/methanol, 10:1); ^{1}H NMR (DMSO-d_6) δ 1.69 (s, 3H, CH_3), 2.07–2.21 (m, 2H, H-2′), 3.78 (s, 3H, OCH_3), 4.08–4.12 (m, 1H, H-4′), 4.28–4.32 (m, 1H, H-3′), 4.65 (dd, 1H, H-5′, J = 14.2, 6.7 Hz), 4.73 (dd, 1H, H-5′, J = 14.2, 4.5 Hz), 5.53 (d, 1H, OH, J = 4.3 Hz), 6.19 (t, 1H, H-1′, J = 6.8 Hz), 7.00 (d, 2H, ArH, J = 8.7 Hz), 7.24 (s, 1H, H-6), 7.76 (d, 2H, ArH, J = 8.7 Hz), 8.45 (s, 1H, ArH), 11.31 (s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ 12.04 (CH_3), 38.75 (C-2′), 51.94 (C-5′), 56.00 (OCH_3), 71.44 (C-3′), 83.34 (C-1′), 84.65 (C-4′), 109.78 (C-5), 115.16 (ArC), 124.11 (ArC-triazole), 127.36 (ArC), 134.93 (ArC), 135.91 (C-6), 147.18 (ArC-triazole), 152.32 (C-2), 159.88 (ArC), 163.53 (C-4); MS (HR-ESI) $C_{19}H_{21}N_5O_5$ $(M+Na)^+$ calcd 422.1440, found 422.1452.

5′-Deoxy-5′-[4-(pyridine-3-yl)-(1,2,3)triazol-1-yl]thymidine (25). Alkyne: 3-Ethynylpyridine; yield: 7 mg (8%); R_f 0.06 (dichloromethane/methanol, 8:1); ^{1}H NMR (DMSO-d_6) δ 1.68 (s, 3H, CH_3), 2.08–2.20 (m, 2H, H-2′), 4.09–4.15 (m, 1H, H-4′), 4.27–4.33 (m, 1H, H-3′), 4.67 (dd, 1H, H-5′, J = 14.3, 6.5 Hz), 4.79 (dd, 1H, H-5′, J = 14.3, 4.9 Hz), 5.46 (d, 1H, OH, J = 4.3 Hz), 6.19 (t, 1H, H-1′, J = 6.8 Hz), 7.24 (s, 1H, H-6), 7.39–7.66 (m, 2H, ArH), 8.23 (d, 1H, ArH, J = 7.4 Hz), 8.69 (s, 1H, ArH), 8.86–8.89 (m, 1H, ArH), 11.29 (s, 1H, NH); MS (HR-ESI) $C_{17}H_{18}N_6O_4$ $(M+Na)^+$ calcd 393.1287, found 393.1310.

Biological Studies

Cloning and Expression of Ba TMPK

In order to clone and express *Ba*TMPK, the *B. anthracis* Sterne strain (34F2) was grown on nutrient agar plates for 12–18 hours at 37 °C. The bacteria were pelleted and genomic DNA was extracted according to Sambrook & Champ; Russell (*Molecular Cloning, A Laboratory Manual*, 2001). The *B. anthracis* Sterne strain (34F2) *tmpk* gene (BAS0029) was amplified with

the PCR primers (forward) 5′-CCACCATATGAAGGGATTATTTGTAACA-3′ and (reverse) 5′-CGGATCCTTATAACAATTTATCTTCTATAACCTG-3′. The primers contained an *Nde I* and a *BamH I* site, respectively, to facilitate subcloning into the pET-14b expression vector (Novagen). The PCR product was cloned into the pCR®4-TOPO vector (Invitrogen, Carlsbad, CA, USA) and sequenced by the automated dye terminator cycle sequencing method (3700 DNA Analyzer, Applied Biosystems, Foster City, CA). The *tmpk* gene was subcloned into the pET-14b expression vector and transformed into the chemical competent *E. coli* strain, *BL21 (DE3) pLysS* (Novagen). The bacteria were cultured as previously described[11,39] at 37°C to an OD_{600} of 0.6. Recombinant *Ba*TMPK was expressed by induction for 6 h with 0.4 mM IPTG at 25°C. Purification of recombinant *Ba*TMPK was carried out as previously described[11,39] and the enzyme was verified with SDS-PAGE.[40]

In Vitro Toxicity Studies

In vitro toxicity studies were carried out as described previously by us.[11] *B. anthracis* Sterne was cultured in M9 minimal media with amino acids for 12–18 hours at 37°C with constant shaking. An aliquot of the overnight culture was diluted with fresh M9 minimal media with amino acids to an optical density in the range of 0.050 to 0.100 at 600 nm. Compounds **6–20** and **22–25** were dissolved in DMSO to produce stock solutions with 500 μM concentrations. Low concentrations (200, 100, 10, and 1 μM of all tested compounds in the first experiment and 200, 100, 50, 25, 12.5, and 6.25 μM of **11** and **23** for next two experiments) were prepared by diluting the initial stock solutions with the M9 minimal media. Each stock solution was freshly prepared before the experiment. The stock solution (50 μL) and the culture media (5 mL) were mixed and incubated at 37°C with constant shaking. An aliquot (0.5 mL) of the culture was removed at 6 hours and the optical density at 600 nm was determined. The percentage of growth inhibition was determined using the formula $\%\mathrm{I} = \frac{\mathrm{test}(\mathrm{A}_{600\,\mathrm{nm}})}{\mathrm{control}(\mathrm{A}_{600\,\mathrm{nm}})} \times 100$. The minimal inhibitory concentration (IC_{50}) was determined by linear regression analysis using Pharmacokinetic compartment model 107 (inhibitory effect sigmoid E_{max} model) implemented in WinNonlin Professional (version 5.0.1, Cary, NC, USA).

Enzymatic Studies

Inhibition of *Ba*TMPK activity by compounds **6**, **8**, **10**, **11**, **12**, and **23** was followed by measuring ADP production in a coupled enzyme system with pyruvate kinase and lactate dehydrogenase.[41] The reaction mixture contained 0.6 μg *Ba*TMPK, 1 mM ATP, 10 μM dTMP, 50 mM Tris-HCl (pH 7.6), 2 mM $MgCl_2$, 5 mM DTT, 1 mM phosphoenolpyruvate, 2 units/mL

pyruvate kinase, 2 units/mL lactate dehydrogenase, 100 mM NADPH, and 100 μM inhibitor. The reaction was performed at 37°C with a Cary 3 spectrophotometer (Varian Techtron, Mulgrave, Australia).

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