Chem Biol Drug Des 2015; **86:** 1387–1396

Research Article

5-Arylaminouracil Derivatives: New Inhibitors of *Mycobacterium tuberculosis*

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Three series of 5-arylaminouracil derivatives, including 5-(phenylamino)uracils, 1-(4′-hydroxy-2′-cyclopenten-1′-yl)-5-(phenylamino)uracils, and 1,3-di-(4′-hydroxy-2′-cyclopenten-1′-yl)-5-(phenylamino)uracils, were synthesized and screened for potential antimicrobial activity. Most of compounds had a negative effect on the growth of the *Mycobacterium tuberculosis* H37Rv strain, with 100% inhibition observed at concentrations between 5 and 40 μg/mL. Of those, 1-(4′-hydroxy-2′-cyclopenten-1′-yl)-3-(4‴-hydroxy-2‴-cyclopenten-1‴-yl)-5-(4″-butyloxyphenylamino)uracil proved to be the most active among tested compounds against the *M. tuberculosis* multidrug-resistant strain MS-115 (MIC_{90} 5 μg/mL). In addition, the thymidylate kinase of *M. tuberculosis* was evaluated as a possible enzymatic target.

Key words: carbocyclic nucleosides, *Mycobacterium tuberculosis*, uracil derivatives

Received 11 February 2015, revised 8 April 2015 and accepted for publication 30 May 2015

Tuberculosis (TB) remains a global health issue in many countries, with the World Health Organization reporting approximately 9 M new cases in 2013, 1.5 M deaths, including 0.36 M coinfected with both HIV and TB. The rapid emergence and spread of drug-resistant strains of *Mycobacterium tuberculosis* requires new and more efficacious treatments and, in particular, new anti-TB agents. In that regard, several pyrimidine derivatives have proved to be highly effective as antiviral, anticancer, antifungal, and pertinent to this work, antibacterial agents (1). These reports have led to a new focus for exploring pyrimidine heterocyclic inhibitors of *M. tuberculosis*.

Notably, TB utilizes a number of enzymes in the pyrimidine salvage pathway which provide attractive targets for rational drug design. This pathway is vital for all bacterial cells and is composed of enzymes significantly different from those present in humans (2). Moreover, the enzymes involved in the pyrimidine salvage pathway have been proposed to have an important role in the mycobacterial latent state, as *M. tuberculosis* has to recycle heterobases and/or nucleosides to survive in the hostile environment imposed by the host (2). During last 10 years, a variety of pyrimidine analogues have been reported to inhibit TB (3). For example, bicyclic thymidine analogues **I** (Figure 1) were shown to inhibit *M. bovis* (4), while derivatives of 1-benzyluracil **II** lacking the ribose moiety have been patented as anti-TB compounds (5). The most active compounds showed inhibitory activity with MIC_{50}′s of 45–55 μg/mL (6).

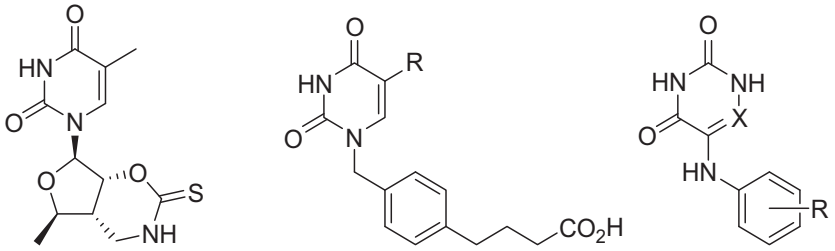
Based on those results, the design and synthesis of a series of 5-(phenylamino)uracils (**III**, Figure 1), which are structural analogues of **II**, was undertaken in an effort to pursue their potential anti-TB activity.

Methods and Materials

General

All reagents (highest grade available) were commercially available and used without further purification unless otherwise noted. Anhydrous DMF, isopropanol, and ethylene glycol were purchased from Sigma-Aldrich Co (Madison, WI, USA). NMR spectra were registered on a Bruker Avance 400 spectrometer (400 MHz for ^{1}H and 100 MHz for ^{13}C) in $CDCl_3$, DMSO-D_6, or CD_3CN with tetramethylsilane as an internal standard. High-resolution mass spectra were measured on Bruker micrOTOF II or maXis (Bruker,

 doi: 10.1111/cbdd.12603



I

II
R = Me, Cl

III
X = CH, N
R = Alkyl, Alkoxy

Figure 1: Pyrimidine containing inhibitors of *M. tuberculosis*.

Bremen, Germany) instruments using electrospray ionization (HRESIMS). The measurements were taken in a positive ion mode (interface capillary voltage −4500 V) in a mass range from m/z 50 to m/z 3000 Da; external or internal calibration was performed with Electrospray Calibrant Solution™ (Fluka, Buchs, Switzerland). A syringe injection was used for solutions in acetonitrile (flow rate 3 μL/min). Nitrogen was applied as a dry gas, and it was produced by Dominick Hunter LC-MS nitrogen generator; interface temperature was set at 180 °C. TLC was performed on Merck (Kenilworth, NJ, USA) TLC Silica gel 60 F_{254} plates and developed with iodine or using UV lamp VL-6.LC (Vilber-Lourmat Deutschland, Eberhardzell, Germany). Melting points were determined using a MEL-TEMP 3.0 (Laboratory Devices Inc., Placerville, CA, USA). Yields refer to spectroscopically (^{1}H and ^{13}C NMR) homogeneous materials. The purities of the compounds were determined using elemental analysis (C, H, N) on a CE1106 automatic analyzer (Italy) and agreed with the theoretical values within 0.4%.

General method for the synthesis of the 5-(phenylamino)uracils 1–8 and 5-(phenylamino)-6-azauracils 9–10

A suspension of 5-bromo-6-azauracil (2.5 g, 13.09 mmol) and aniline (40 mmol), 4-methylaniline (40 mmol), or 4-phenoxyaniline (40 mmol) was heated in 30 mL freshly distilled ethylene glycol, until a homogeneous solution was observed. The solution was then refluxed for 2 h, then cooled to room temperature at which point the amination product precipitated out. The reaction mixture then was poured into cold water (150 mL), the solid filtered off, washed with water, and reprecipitated from a 5% aqueous solution of NaOH. The product was then recrystallized from DMF.

5-(Phenylamino)uracil (1)

Yield 68%; mp: > 330 °C. $E_1(\lambda_{260.0})$ = 12 400. ^{1}H-NMR (DMSO-d_6): δ 11.27 (1H, s, N^3H), 10.59 (1H, s, N^1H), 7.29 (1H, s, H-6), 7.11–7.09 (2H, m, H-2″, H-6″), 6.83 (1H, s, 5-NH), 6.75–6.72 (2H, m, H-3″, H-5″), 6.68–6.64 (1H, m, H4″). ^{13}C-NMR (DMSO-d_6): δ 162.38, 150.43, 146.26, 132.45, 128.79 (C-3″, C5″), 117.90, 115.75, 114.26 (C-2″, C-6″). HRMS: found *m/z* 204.0771; calcd for $C_{10}H_9N_3O_2$ $[M + H]^+$ 204.0768. Anal. calcd for $C_{10}H_9N_3O_2$: C, 59.11; H, 4.46; N, 20.68; found: C, 59.18; H, 4.41; N, 20.75.

5-[(3-Methylphenyl)amino]uracil (2)

Yield 66%; mp: 324–326 °C. $E_1(\lambda_{259.6})$ = 17 500, $E_2(\lambda_{328.2})$ = 6200. ^{1}H-NMR (DMSO-d_6): δ 11.25 (1H, s, N^3H), 10.62 (1H, d, J = 3.9 Hz, N^1H), 7.28 (1H, d, J = 5.9 Hz, H-6), 6.98 (1H, t, J = 7.6 Hz, H-5′), 6.47–6.53 (4H, m, H-2′, H-4′, H-6′, 5-NH), 2.17 (3H, s, CH_3). ^{13}C-NMR (DMSO-d_6): δ 162.8, 150.8, 146.6, 138.3, 132.9, 129.0, 119.2, 116.2, 115.1, 111.9, 21.6. HRMS: found *m/z* 218.0917; calcd for $C_{11}H_{11}N_3O_2$ $[M + H]^+$ 218.0924. Anal. calcd for $C_{11}H_{11}N_3O_2$: C, 60.82; H, 5.10; N, 19.34; found: C, 60.80; H, 5.04; N, 19.31.

5-[(4-Methylphenyl)amino]uracil (3)

Yield 82%; mp: dec > 330 °C. $E_1(\lambda_{260.4})$ = 15 100. ^{1}H-NMR (DMSO-d_6): δ 11.21 (1H, s, N^3H), 10.51 (1H, s, N^1H), 7.19 (1H, s, H-6), 6.94–6.92 (2H, m, H-3″, H-5″), 6.70–6.67 (3H, m, 5-NH, H-2″, H-6″), 2.17 (3H, s, CH_3). ^{13}C-NMR (DMSO-d_6): δ 162.28, 150.27, 143.33, 129.94, 129.24 (C-3″, C5″), 126.84, 116.62, 114.91 (C-2″, C-6″), 20.10. HRMS: found *m/z* 218.0927; calcd for $C_{11}H_{11}N_3O_2$ $[M + H]^+$ 218.0924. Anal. calcd. for $C_{11}H_{11}N_3O_2$: C, 60.82; H, 5.10; N, 19.34; found: C, 60.98; H, 5.09; N, 19.43.

5-[(2,3-Dimethylphenyl)amino]uracil (4)

Yield 74%; mp: 306.5–308 °C. $E_1(\lambda_{253})$ = 13 600, $E_2(\lambda_{320})$ = 5300. ^{1}H-NMR (DMSO-d_6): δ 11.28 (1H, s, N^3H), 10.53 (1H, d, J = 5.2 Hz, N^1H), 6.95 (1H, d, J = 5.6 Hz, H-6), 6.88 (1H, t, J = 7.8 Hz, H-5′), 6.64 (1H, d, J = 7.3 Hz, H-6′), 6.51 (1H, d, J = 8.0 Hz, H-4′), 6.09 (1H, br. s, NH-Ar), 2.20 (3H, s, CH_3), 2.05 (3H, s, CH_3). ^{13}C-NMR (DMSO-d_6): δ 162.5, 150.7, 143.6, 136.9, 129.8, 125.9, 124.2, 122.0, 118.0, 114.0, 20.6, 13.3. HRMS: found *m/z* 231.1008; calcd for $C_{12}H_{13}N_3O_2$ [M] 231.1002, found *m/z* 232.1078; calcd for $C_{12}H_{13}N_3O_2$ $[M + H]^+$ 232.1081. Anal. calcd for $C_{12}H_{13}N_3O_2$: C, 62.33; H, 5.67; N, 18.34; found: C, 62.44; H, 5.68; N, 18.17.

5-[(2,5-Dimethylphenyl)amino]uracil (5)

Yield 50%; mp: 269–270 °C. $E_1(\lambda_{261.2})$ = 14 000, $E_2(\lambda_{322.5})$ = 4700. ^{1}H-NMR (DMSO-d_6): δ 11.27 (1H, s, N^3H), 10.60 (1H, d, J = 4.4 Hz, N^1H), 7.13 (1H, d, J = 5.6 Hz, H-6), 6.91 (1H, d, J = 7.3 Hz, H-3′), 6.49 (1H, d, J = 7.6 Hz, H-4′), 6.39 (1H, s, H-6′), 6.10 (1H, br. s, 5-NH), 2.14 (3H, s, CH_3), 2.10 (3H, s, CH_3). ^{13}C-NMR (DMSO-d_6): δ 162.6, 150.9, 144.0, 142.5, 135.7, 132.1, 130.4, 121.6, 119.9, 116.9, 114.9, 21.3, 17.5. HRMS: found *m/z* 231.1005; calcd for $C_{12}H_{13}N_3O_2$ [M] 231.1002, found *m/z* 232.1081; calcd for $C_{12}H_{13}N_3O_2$ $[M + H]^+$ 232.1081. Anal. calcd for $C_{12}H_{13}N_3O_2$: C, 62.33; H 5.67; N, 18.21; found C, 62.54; H, 5.61; N, 18.17.

5-[(4-Butylphenyl)amino]uracil (6)

Yield 77%; mp: > 330 °C. $E(\lambda_{262})$ = 19200. ^{1}H-NMR (DMSO-d_6): δ 11.21 (1H, br. s, N^3H), 10.52 (1H, br. s, N^1H), 7.22 (1H, s, H-6), 6.94 (2H, d, J = 8.4 Hz, H-2′, H-6′), 6.68–6.70 (3H, m, 5-NH, H-2″, H-6″), 2.44 (2H, t, J = 7.6 Hz, CH_2), 1.48 (2H, m, CH_2), 1.29 (2H, m, CH_2), 0.88 (3H, t, J = 7.3 Hz, CH_3). ^{13}C-NMR (DMSO-d_6): δ 162.3, 150.2, 143.6, 131.9, 130.2, 128.5, 116.5, 114.7, 34.0, 33.4, 21.6, 13.7. HRMS: found *m/z* 259.1315; calcd for $C_{14}H_{17}N_3O_2$ [M] 259.1315, found *m/z* 260.1387; calcd for $C_{14}H_{17}N_3O_2$ $[M + H]^+$ 260.1394. Anal. calcd for $C_{14}H_{17}N_3O_2$: C, 64.85; H, 6.61; N, 16.20; found: C, 64.97; H, 6.68; N, 16.36.

5-(4-Butyloxyphenylamino)uracil (7)

Yield 74%; mp: > 330 °C. $E(\lambda_{259.3})$ = 18 950. ^{1}H-NMR (DMSO-d_6): δ 11.21 (1H, br. s, N^3H), 10.42 (1H, br. s, N^1H), 7.07 (1H, s, H-6), 6.76–6.78 (4H, m, aromatic H), 6.54 (1H, s, 5-NH), 3.86 (2H, t, J = 6.5 Hz, CH_2O), 1.65 (2H, m, CH_2), 1.42 (2H, m, CH_2), 0.92 (3H, t, J = 7.4 Hz, CH_3). ^{13}C-NMR (DMSO-d_6): δ 162.1, 152.0, 150.0, 138.6, 126.6, 117.8, 117.1, 115.1, 67.4, 30.8, 18.7, 13.6. HRMS: found *m/z* 275.1256; calcd for $C_{14}H_{17}N_3O_3$ [M] 275.1264. Anal. calcd. for $C_{14}H_{17}N_3O_3$: C, 61.08; H, 6.22; N, 15.26; found: C, 61.09; H, 6.31: N, 15.28.

5-[(4-Phenoxyphenyl)amino]uracil (8)

Yield 59%; mp: 329–331 °C. $E_1(\lambda_{260.6})$ = 28 400. ^{1}H-NMR (DMSO-d_6): δ 11.23 (1H, s, N^3H), 10.58 (1H, s, N^1H), 7.33–7.27 (3H, m, H-3′, H-5′, H-6), 7.04–7.00 (1H, m, H-4′), 6.91–6.77 (7H, m, 5-NH, H-2′, H-6′ H-2″, H-3″, H-5″, H-6″). ^{13}C-NMR (DMSO-d_6): 162.34, 158.41, 150.39, 147.55, 142.70, 131.57, 129.73 (C-3′, C-5′), 122.12 (C-6), 120.52 (C-2′, C-6′), 116.85 (C-3″, C5″), 116.26 (C-2″, C-6″), 115.77. HRMS: found *m/z* 295.0941; calcd for $C_{16}H_{13}N_3O_3$ [M] 295.0951. Anal. calcd for $C_{16}H_{13}N_3O_3$: C, 65.08; H, 4.44; N, 14.23; found: C, 65.05; H, 4.44; N, 14.33.

5-(Phenylamino)-6-azauracil (9)

Yield 68%; mp: > 330 °C. $E_1(\lambda_{246.9})$ = 22600, $E_2(\lambda_{325.8})$ = 11 900. ^{1}H-NMR (DMSO-d_6): δ 11.99 (1H, s, N^3H), 11.28 (1H, s, N^1H), 8.61 (1H, s, 5-NH), 7.73 (2H, d, J = 8.0 Hz, H-2′, H-6′), 7.26 (2H, t, J = 8.4 Hz, H-3′, H-5′), 6.94 (1H, t, J = 7.2 Hz, H-4′). ^{13}C-NMR (DMSO-d_6): δ 154.6, 148.4, 139.7, 139.6, 128. 4(C-3″, C-5″)128.4, 121.5, 118.5 (C-2″, C-6″). HRMS: found *m/z* 204.0647; calcd for $C_9H_8N_4O_2$ [M] 204.0642, found *m/z* 205.0725; calcd for $C_9H_8N_4O_2$ $[M + H]^+$ 205.0720. Anal. calcd. for $C_9H_8N_4O_2$: C, 52.94; H, 3.95; N, 27.44; found: C, 52.91; H, 3.95; N, 27.51.

5-[(4-Butylphenyl)amino]-6-azauracil (10)

Yield 47%; mp: 306–308 °C. $E(\lambda_{260.2})$ = 15 150. ^{1}H-MNR (DMSO-d_6): δ 11.96 (1H, br. s, N^3H), 11.23 (1H, br. s, N^1H), 8.51 (1H, s, 5-NH), 7.61 (2H, d, J = 8.4 Hz, H-2′, H-6′), 7.07 (2H, d, J = 8.8 Hz, H-3′, H-5′), 2.50 (2H, m, CH_2), 1.52 (2H, m, CH_2), 1.29 (2H, m, CH_2), 0.88 (3H, t, J = 7.4 Hz, CH_3). ^{13}C-NMR (DMSO-d_6): δ 154.7, 148.4, 139.7, 137.4, 135.5, 128.2, 118.6, 34.1, 33.2, 21.6, 13.7. HRMS: found *m/z* 260.1270; calcd for $C_{13}H_{16}N_4O_2$ [M] 260.1268, found *m/z* 261.1341; calcd for $C_{13}H_{16}N_4O_2$ $[M + H]^+$ 261.1346. Anal. calcd. for $C_{13}H_{16}N_4O_2$: C, 59.99; H, 6.20; N, 21.52; found: C, 60.11; H, 6.21: N, 21.51.

1-(4′-Hydroxy-2′-cyclopenten-1′-yl)-5-(phenylamino) uracil (11)

To a solution of **1** (200 mg, 0.7 mmol) in dry DMF (50 mL) epoxycyclopentene (63 mg, 0.77 mmol) in freshly distilled THF (15 mL) and $Pd(PPh_3)_4$ (40 mg, 0.035 mmol) was added. The reaction mixture was stirred for 4 h at rt. The solvents were concentrated under reduced pressure, and the residue purified by column chromatography on silica eluting with $CHCl_3$:MeOH (98:2) to give **11** as an off-white powder (56 mg, 0.16 mmol, 20%). Mp: decomposed at 214 °C. $E_1(\lambda_{263.4})$ = 21 500, $E_2(\lambda_{331.8})$ = 9700. ^{1}H-NMR (DMSO-d_6): δ 11.46 (1H, s, NH), 7.41 (1H, s, H-6), 7.12–7.08 (2H, m, H-2″, H-6″), 7.01 (1H, s, 5-NH), 6.80–6.78 (2H, m, H-3″, H-5″), 6.69–6.67 (1H, m, H-4″), 6.14–6.12 (1H, d, J = 5.52 Hz, H-2′), 5.85–5.83 (1H, d, J = 5.51 Hz, H-3′), 5.47–5.45 (1H, m, H-1′), 5.25 (1H, s OH), 4.64–4.62 (1H, m, H-4′), 2.76–2.72 (1H, m, H_a-5′), 1.46–1.43(1H, m, H_b-5′). ^{13}C-NMR (DMSO-d_6): δ 161.30, 149.63 (C-4, C-2), 145.48 (C-1″), 140, 130.97 (C-2′, C-3′), 130.36 (C-6), 128.78 (C-3″, C-5″), 118.10 (C-5), 116.99 (C-4″), 114.41 (C-2″, C-6″), 73.13 (C-1′), 58.16 (C-4′), 40.91(C-5′). HRMS: found *m/z* 308.1001; calcd for $C_{15}H_{15}N_3O_3$ $[M + Na]^+$ 308.1006. Anal. calcd for $C_{15}H_{15}N_3O_3$: C, 63.15; H, 5.30; N, 14.73; found: C, 63.22; H, 5.38; N, 14.77.

1-(4′-Hydroxy-2′-cyclopenten-1′-yl)-5-[(4″-methylphenyl)amino]uracil (12)

In an analogous manner as was used to synthesize (**11**), compound (**12**) was obtained as a pale yellow powder (61 mg, 0.2 mmol, 21%); mp: 212–213 °C. $E_1(\lambda_{264.2})$ = 20 300, $E_2(\lambda_{334.8})$ = 8400. ^{1}H-NMR (DMSO-d_6): δ 11.44 (1H, s, NH), 7.35 (1H, s, H-6), 6.94–6.92 (2H, m, H-2″,

H-6″), 6.85 (1H, s, 5-NH), 6.75–6.73 (2H, m, H-3″, H-5″), 6.13–6.11 (1H, d, J = 5.52 Hz, H-2′), 5.83–5.81(1H, d, J = 5.52 Hz, H-3′), 5.47–5.44 (1H, m, H-1′), 5.26 (1H, s OH), 4.63–4.61 (1H, m, H-4′), 2.75–2.67 (1H, m, H_a-5′), 2.17 (3H, s, CH_3), 1.45–1.39 (1H, m, H_b-5′). ^{13}C-NMR (DMSO-d_6): δ 161.30, 149.63 (C-4, C-2), 145.48 (C-1″), 139.96, 131.00 (C-2′, C-3′), 129.24 (C-3″, C5″), 127.67, 126.99 (C-6, C-5), 117.79 (C-4″), 115.02 (C-2″, C-6″), 73.13 (C-1′) 58.12 (C-4′), 41.11(C-5′), 20.07 (CH_3). HRMS: found m/z 300.1342; calcd for $C_{16}H_{17}N_3O_3$ $[M + H]^+$ 300.1343; found m/z 322.1159; calcd for $C_{16}H_{17}N_3O_3$ $[M + Na]^+$ 322.1162. Anal. calcd. for $C_{16}H_{17}N_3O_3$: C, 64.20; H, 5.72; N, 14.07; found: C, 64.12; H, 5.78; N, 14.04.

1-(4′-Hydroxy-2′-cyclopenten-1′-yl)-5-(4″-butylphenylamino)uracil (13)

In an analogous manner as was used to synthesize (**11**), compound (**13**) was obtained as off-white syrup (42 mg, 0.12 mmol, 17%). E($\lambda_{261.4}$) = 22 400. ^{1}H-NMR (DMSO-d_6): δ 11.45 (1H, s, NH), 7.37 (1H, s, H-6), 6.95–6.93 (2H, m, H-2″, H-6″), 6.87 (1H, s, 5-NH), 6.76–6.74 (2H, m, H-3″, H-5″), 6.14–6.12 (1H, m, H-2′), 5.84–5.83 (1H, m, H-3′), 5.48–5.45 (1H, m, H-1′), 5.27 (1H, s OH), 4.63–4.62 (1H, m, H-4′), 2.74–2.70 (1H, m, H_a-5′), 2.46–2.42 (2H, m, CH_2), 1.49–1.47 (3H, m, H_b-5′, CH_2), 1.31–1.29 (2H, m, CH_2), 0.90–0.88 (3H, m, CH_3). ^{13}C-NMR (DMSO-d_6): δ 161.22, 149.46 (C-4, C-2), 142.82 (C-4″), 139.96 (C-2′), 132.08 (C-1″),130.97 (C-3′), 128.55 (C-3″, C5″),128.28 (C-6), 117.61 (C-5), 114.74 (C-2″, C-6″), 73.14 (C-1′), 58.11 (C-4′), 39.09 (C-5′), 33.93 (CH_2), 33.33 (CH_2), 21.62 (CH_2), 13.70 (CH_3). HRMS: found m/z 342.1805; calcd for $C_{19}H_{23}N_3O_3$ $[M + H]^+$ 342.1812; found m/z 364.1629; calcd for $C_{19}H_{23}N_3O_3$ $[M + Na]^+$ 364.1632. Anal. calcd. for $C_{19}H_{23}N_3O_3$: C, 66.84; H, 6.79; N, 12.31; found: C, 66.74; H, 6.78; N, 12.34.

1-(4′-Hydroxy-2′-cyclopenten-1′-yl)-5-(4″-butyloxyphenylamino)uracil (14)

In an analogous manner as was used to synthesize (**11**), compound (**14**) was obtained as yellow syrup (54 mg, 0.15 mmol, 22%). E($\lambda_{262.2}$) = 21 100. ^{1}H-NMR (DMSO-d_6): δ 11.44 (1H, s, NH), 7.28 (1H, s, H-6), 6.84–6.82 (2H, m, H-2″, H-6″), 6.75–6.72 (3H, m, 5-NH, H-3″, H-5″), 6.13–6.11 (1H, d, J = 5.52 Hz, H-2′), 5.83–5.80 (1H, d, J = 5.51 Hz, H-3′), 5.48–5.44 (1H, m, H-1′), 5.26 (1H, s OH), 4.63–4.61 (1H, m, H-4′), 3.88–3.85 (2H, m, CH_2), 2.74–2.66 (1H, m, H_a-5′), 1.65–1.63 (2H, m, CH_2), 1.44–1.41 (3H, m, CH_2, H_b-5′), 0.94–0.90 (3H, m, CH_3). ^{13}C-NMR (DMSO-d_6): δ 161.10, 151.97 (C-4, C-2), 149.29 (C-4″), 139.92 (C-2′), 137.87 (C-1″), 131.05 (C-3′), 124.99 (C-6), 116.96 (C-3″, C5″), 115.06 (C-2″, C-6″), 73.13, 73.03 (C-1′, C-4′), 67.43 (C-5), 58.11 (C-5′), 39.12 (CH_2), 30.87 (CH_2), 18.71 (CH_2), 13.65 (CH_3). HRMS: found m/z 380.1570; calcd for $C_{19}H_{23}N_3O_4$ $[M + Na]^+$ 380.1581. Anal. calcd. for $C_{19}H_{23}N_3O_4$ C, 63.85; H, 6.49; N, 11.76; found: C, 63.84; H, 6.58; N, 11.77.

1-(4′-Hydroxy-2′-cyclopenten-1′-yl)-5-[(4″-phenoxyphenyl)amino]uracil (15)

In an analogous manner as was used to synthesize (**11**), compound (**15**) was obtained as a pale yellow powder (45 mg, 0.12 mmol, 18%). Mp: decomposes at 200 °C. E($\lambda_{265.3}$) = 26 750, E($\lambda_{328.8}$) = 9320. ^{1}H-NMR (DMSO-d_6): δ 11.45 (1H, s, NH), 7.40 (1H, s, H-6), 7.32–7.30 (2H, m, H-3″, H-5″), 7.04 (2H, m, H-2′ and H-6′), 6.89–6.88 (2H, m, H-3′, H-5′), 6.84 (4H, m, H-2″, H-4″, H-6″, 5-NH), 6.14–6.13 (1H, d, J = 5.50 Hz, H-2′), 5.84–5.83(1H, d, J = 5.51 Hz, H-3′), 5.48–5.46 (1H, m, H-1′), 5.28–5.27 (1H, m, H-4′) 4.63–4.62 (1H, s OH), 2.71–2.67 (1H, m, H_a-5′), 1.44–1.41(1H, m, H_b-5′). ^{13}C-NMR (DMSO-d_6): δ 161.24 (C-4), 158.24 (C-1Ph), 149.57 (C-2), 147.73 (C-1″), 141.85, 139.97 (C-2′, C-3′), 131.01(C-6), 129.71 (C-3Ph, C-5Ph), 129.51 (C-5), 122.17 (C-4Ph), 120.34 (C-2Ph, C-6Ph), 117.46 (C-4″), 117.46(C-3″, C5″), 115.88 (C-2″, C-6″), 73.15 (C-1′) 58.17 (C-4′), 41.21 (C-5′). HRMS: found m/z 400.1258; calcd for $C_{21}H_{19}N_3O_4$ $[M + Na]^+$ 400.1268. Anal. calcd. for $C_{21}H_{19}N_3O_4$: C, 66.83; H, 5.07; N, 11.13; found: C, 66.84; H, 5.08; N, 11.17.

1-(4′-Hydroxy-2′-cyclopenten-1′-yl)-3-(4‴-hydroxy-2‴-cyclopent-en-1‴-yl)-5-(phenylamino)uracil (16)

The title compound was obtained as side product in the synthesis of (**11**). The purification described for compound (**11**) gave product (**16**) as a yellow syrup (140 mg, 0.38 mmol, 39%). E($\lambda_{264.7}$) = 15 360, E($\lambda\lambda_{336.7}$) = 6000. ^{1}H-NMR ($CDCl_3$): δ 7.37 (1H, s, H-6), 7.24–7.21 (3H, m, H-3″, H-5″, 5-NH), 6.95–6.94 (2H, m, H-2″, H-6″), 6.90–6.86 (1H, m, H-4″), 6.20–6.19 (1H, d, J = 5.51 Hz, H-2′), 6.14–6.13 (1H, d, J = 5.50 Hz, H-2‴), 5.99–5.97 (2H, m, H-1′, H-1‴), 5.82–5.81 (1H, d, J = 5.52 Hz, H-3′), 5.77–5.75 (1H, d, J = 5.49 Hz, H-3‴), 5.65–5.62 (1H, m, H-4′), 4.85–4.83 (1H, m, H-4‴), 4.71 (1H, s, OH′), 4.24 (1H, s, OH‴), 2.92–2.82 (2H, m, H_a-5′, H_a-5‴), 2.0 (1H, m, H_b-5′), 1.96(1H, m, H_b-5‴). ^{13}C-NMR (DMSO-d_6): δ 160.83, 149.36 (C-4, C-2), 142.08 (C-1″), 139.42, 137.38 (C-2′, C-2‴), 132.23, 130.76 (C-3′, C-3‴), 129.54 (C-3″, C5″), 128.67 (C-4″), 121.20 (C-6), 118.76 (C-5), 117.20 (C-2″, C-6″), 76.31 (C-1′), 74.71 (C-1‴), 60.38 (C-4′), 56.94 (C-4‴), 39.89 (C-5′), 37.56 (C-5‴). HRMS: found m/z 390.1417; calcd for $C_{20}H_{21}N_3O_4$ $[M + Na]^+$ 390.1424. Anal. calcd. for $C_{20}H_{21}N_3O_4$: C, 65.38; H, 5.76; N, 11.44; found: C, 65.35; H, 5.83; N, 11.43.

1-(4′-Hydroxy-2′-cyclopenten-1′-yl)-3-(4‴-hydroxy-2‴-cyclopent-en-1‴-yl)-5-[(4″-methylphenyl)amino]uracil (17)

The title compound was obtained as side product in the synthesis of (**12**). The purification described for compound (**12**) gave product (**17**) as a yellow syrup (122 mg, 0.32 mmol, 35%). E($\lambda_{265.4}$) = 19 000. ^{1}H-NMR ($CDCl_3$): δ 7.27 (1H, s, H-6), 7.05–7.03 (3H, m, H-3″, H-5″, 5-NH), 6.87–6.85 (2H, m, H-2″, H-6″), 6.19–6.17 (1H, d, J = 5.52 Hz, H-2′), 6.14–6.13 (1H, d, J = 5.51 Hz, H-2‴),

6.00–6.99 (1H, m, H-1′), 5.97 (1H, m, H-1‴), 5.82–5.81 (1H, d, J = 5.50 Hz, H-3′), 5.77–5.75 (1H, d, J = 5.51 Hz, H-3‴), 5.75–5.63 (1H, m, H-4′), 4.85–4.84 (1H, m, H-4‴), 4.71 (1H, s, OH′), 4.27 (1H, s, OH‴), 2.82–2.76 (2H, m, H_a-5′, H_a-5‴), 2.25 (3H, s, CH_3), 2.0 (1H, m, H_b-5′), 1.65 (1H, m, H_b-5‴). ^{13}C-NMR (DMSO-d_6): δ 160.78, 149.30 (C-4, C-2), 139.29 (C-1″), 137.33 (C-2′, C-2‴), 132.28 (C-3′, C-3‴), 130.79 (C-6), 130.05 (C-3″, C5″), 120.32 (C-4″), 117.87 (C-2″, C-6″), 117.24 (C-5), 76.31 (C-1′), 74.76 (C-1‴), 60.42 (C-4′), 56.90 (C-4‴), 39.90 (C-5′), 37.72 (C-5‴), 20.67 (CH_3). HRMS: found m/z 404.1570; calcd for $C_{21}H_{23}N_3O_4$ $[M + Na]^+$ 404.1581. Anal. calcd. for $C_{21}H_{23}N_3O_4$: C, 66.13; H, 6.08; N, 11.02; found: C, 66.09; H, 6.03; N, 11.03.

1-(4′-Hydroxy-2′-cyclopenten-1′-yl)-3-(4‴-hydroxy-2‴-cyclopent-en-1‴-yl)-5-(4″-butylphenylamino) uracil (18)

The title compound was obtained as side product in the synthesis of (**13**). The purification described for compound (**13**) gave product (**18**) as a yellow syrup (96 mg, 0.22 mmol, 31%). E($\lambda_{336.2}$) = 8300. ^{1}H-NMR ($CDCl_3$): δ 7.98 (1H, s, 5-NH), 7.29 (1H, m, H-6), 7.06–7.04 (2H, m, H-3″, H-5″), 6.89–6.87 (2H, m, H-2″, H-6″), 6.20–6.18 (1H, m, H-2′), 6.15–6.14 (1H, m, H-2‴), 6.00–5.97 (1H, d, H-3′), 5.94 (1H, m, H-1′), 5.82–5.81 (1H, m, H-3‴), 5.77–5.76 (1H, m, H-1‴), 5.64–5.62 (1H, m, H-4′), 4.85–4.84 (1H, m, H-4‴), 4.72 (1H, s, OH′), 4.25 (1H, s, OH‴), 2.85–2.80 (2H, m, H_a-5′, H_a-5‴), 2.54–2.50 (2H, m, CH_2), 2.01–1.97 (1H, m, H_b-5′), 1.68–1.67 (1H, m, H_b-5‴), 1.55–1.53 (2H, m, CH_2), 1.32–1.30 (2H, m, CH_2), 0.92–0.91 (3H, m, CH_3). ^{13}C-NMR ($CDCl_3$): δ 160.89, 149.89 (C-4, C-2), 139.43, 137.46 (C-2′, C-2‴), 136.13 (C-4″), 134.0 (C-1″), 132.34, 130.86 (C-3′, C-3‴), 129.50 (C-3″, C5″), 128.68 (C-6), 120.30 (C-5), 117.70, 117.56 (C-2″, C-6″), 76.41 (C-1′), 74.86 (C-1‴), 60.53 (C-4′), 57.0 (C-4‴), 40.0 (C-5′), 37.68 (C-5‴), 36.61 (CH_2), 35.0 (CH_2), 22.48 (CH_2), 14.09 (CH_3). HRMS: found m/z 423.2141; calcd for $C_{24}H_{29}N_3O_4$ $[M]^+$ 423.2153; found m/z 446.2040; calcd for $C_{24}H_{29}N_3O_4$ $[M + Na]^+$ 446.2050. Anal. calcd. for $C_{24}H_{29}N_3O_4$: C, 68.06; H, 6.90; N, 9.92; found: C, 68.15; H, 6.83; N, 9.93.

1-(4′-Hydroxy-2′-cyclopenten-1′-yl)-3-(4‴-hydroxy-2‴-cyclopenten-1‴-yl)-5-(4″-butyloxyphenylamino) uracil (19)

The title compound was obtained as side product in the synthesis of (**14**). The purification described for compound (**14**) gave product (**19**) as a yellow syrup (115 mg, 0.26 mmol, 38%). E($\lambda_{262.4}$) = 11 300. ^{1}H-NMR ($CDCl_3$): δ 7.98 (1H, s, 5-NH), 7.12 (1H, m, H-6), 6.93–6.91 (2H, m, H-3″, H-5″), 6.82–6.79 (2H, m, H-2″, H-6″), 6.16–6.13 (2H, m, H-2′, H-2‴), 6.00–5.98 (1H, d, H-3′), 5.80–5.77 (3H, m, H-3‴, H-1′, H-1‴), 5.60–5.57 (1H, m, H-4′), 4.83–4.81 (1H, m, H-4‴), 4.71 (1H, s, OH′), 4.30 (1H, s, OH‴), 3.92–3.90 (2H, m, CH_2), 2.80–2.79 (2H, m, H_a-5′, H_a-5‴), 2.0–1.97 (1H, m, H_b-5′), 1.65–1.64 (2H, m, CH_2), 1.63–1.61 (1H, m, H_b-5‴), 1.47–1.46 (2H, m, CH_2), 0.97–0.94 (3H, m, CH_3). ^{13}C-NMR ($CDCl_3$): δ 160.80, 154.71 (C-4, C-2), 149.35 (C-4″), 139.32 (C-1″), 137.44 (C-2′, C-2‴), 134.81 (C-6), 132.33, 130.88 (C-3′, C-3‴), 121.60 (C-5), 120.64 (C-3″, C5″), 115.80, 115.70 (C-2″, C-6″), 76.41 (C-1′), 74.85 (C-1‴), 68.32 (C-4′), 60.57 (C-4‴), 56.95 (C-5′), 39.96 (C-5‴), 37.67 (CH_2), 31.57 (CH_2), 19.41 (CH_2), 14.00 (CH_3). HRMS: found m/z 462.1991; calcd for $C_{24}H_{29}N_3O_5$ $[M + Na]^+$ 462.1999. Anal. calcd. for $C_{24}H_{29}N_3O_5$: C, 65.59; H, 6.65; N, 9.56; found: C, 65.55; H, 6.63; N, 9.59.

1-(4′-Hydroxy-2′-cyclopenten-1′-yl)-3-(4‴-hydroxy-2‴-cyclopent-en-1‴-yl)-5-[(4″-phenoxyphenyl) amino]uracil (20)

The title compound was obtained as side product in the synthesis of (**15**). The purification described for compound (**15**) gave product (**20**) as a yellow syrup (123 mg, 0.27 mmol, 40%). E$_1$($\lambda_{265.0}$) = 21 870, E$_2$($\lambda_{333.0}$) = 6700. ^{1}H-NMR ($CDCl_3$): δ 7.29 (2H, m, H-3Ph, H-5Ph), 7.24 (1H, s, H-6), 7.04–7.02 (1H, m, H-4Ph), 6.93 (7H, m, H-2Ph, H-6Ph, H-2″, H-3″, H-5″, H-6″, 5-NH), 6.19–6.18 (1H, d, J = 5.52 Hz, H-2′), 6.14–6.13 (1H, d, J = 5.51 Hz, H-2‴), 6.00 (1H, m, H-1′), 5.98 (1H, m, H-1‴), 5.83–5.81 (1H, d, J = 5.50 Hz, H-3′), 5.77–5.76 (1H, d, J = 5.51 Hz, H-3‴), 5.65–5.62 (1H, m, H-4′), 4.84–4.83 (1H, m, H-4‴), 4.73 (1H, s, OH′), 4.28 (1H, s, OH‴), 2.83–2.79 (2H, m, H_a-5′, H_a-5‴), 2.01–1.97 (1H, m, H_b-5′), 1.67–1.63 (1H, m, H_b-5′). ^{13}C-NMR (DMSO-d_6): δ 160.78 (C-4), 158.09 (C-1Ph), 151.26 (C-2), 149.32 (C-4″), 139.33 (C-2′), 137.80 (C-1″), 137.41 (C-2‴), 132.31 (C-3′), 130.67 (C-3‴), 129.74 (C-3Ph, C-5Ph), 122.84 (C-6), 120.61 (C-2Ph, C-6Ph), 120.34 (C-4Ph), 119.11 (C-3″, C5″), 118.12 (C-2″, C-6″), 117.24 (C-5), 76.32 (C-1′), 74.72 (C-1‴), 60.42 (C-4′), 56.95 (C-4‴), 39.79 (C-5′), 37.58 (C-5‴). HRMS: found m/z 482.1680; calcd for $C_{26}H_{25}N_3O_5$ $[M + Na]^+$ 482.1686. Anal. calcd. for $C_{26}H_{25}N_3O_5$: C, 67.96; H, 5.48; N, 9.14; found: C, 67.95; H, 5.43; N, 9.13.

Cytotoxicity

Vero cells (green monkey kidney epithelial cells, ATCC No CRL-1586), *A549 cells* (lung carcinoma cell line, ATCC No CCL-185), and *Huh7 cells* (hepatocellular carcinoma cells) were provided by Laboratory of cell cultures, Ivanovskii Institute of virology (Moscow) and Engelhardt Institute of Molecular Biology RAS (Moscow). The cell cultures were grown in Dulbecco's modified Eagle's minimal essential medium (DMEM) with 10% fetal calf serum (HyClone, GE Healthcare Life Sciences, HyClone Laboratories, South Logan, UT, USA) at 37 °C in a 96-well plate in the atmosphere of 5% CO_2. The tested compounds were dissolved in DMSO at a concentration of 40 mg/mL (stock solutions). Cytotoxicity (CD_{50}) was estimated by MTT-assay in the presence of tested compounds at the concentrations of 0–100 μg/mL after 72 h of incubation with uninfected cells and calculated as compound concentration, at which

50% cells died (7). DMSO (molecular biology grade; Sigma-Aldrich) was additionally purified by double distillation. The final concentration of DMSO in all tests was ≤ 1%. It has been shown in control experiments that 1% of DMSO does not affect the cell cultures we use in our experiments. At the same time, DMSO at final concentration of 10% was used as positive control (≥ 50% of cell died).

Antituberculosis tests

Mycobacterial strains

The laboratory *M. tuberculosis* H37Rv strain susceptible to anti-TB drugs and a *M. tuberculosis* MS-115 clinical strain resistant to five-first line anti-TB drugs (rifampicin, isoniazid, streptomycin, ethambutol, and pyrazinamide) were used. The mycobacteria were transformed into a suspension of single cells at the same growth phase and normalized by CFU (8). Enriched Dubois medium (Difco, Leeuwarden, The Netherlands) was used.

Antibacterial activity

The influence of the tested compounds on the growth of the bacterial strains was studied using the automated BACTEC MGIT 960 system (BD, Becton Dickinson and Company, Franklin Lakes, NJ, USA). The tested compounds were dissolved in DMSO at a concentration of 40 mg/mL (stock solutions). Water and Tween-80 were added to the stock solutions to give a 5: 0.5: 4.5 ratio of DMSO: Tween-80: H_2O. The mycobacterial suspension (500 μL) was inoculated in the culture medium (7.9 mL) up to the final concentration of 106 CFU/mL. Each sample including the control samples lacking the tested compound were studied in triplicate. It should be noted that this solvent mixture had no effect on *M. tuberculosis* growth. The antibacterial activity was evaluated by the proportion method with the TB Exist software (9). The method is based on the comparison of the bacterial growth in the samples containing the tested compounds and the control samples diluted 100× if compared with the initially inoculated culture. The culture is susceptible (that is, the compound is active) if the optical density of the control sample is 400 RFU (relative fluorescence units.) in and that of the tested sample is < 100 RFU. In addition, using the absolute concentrations, a delay in the bacterial growth of the tested sample was measured in comparison with the control samples, which reflected a negative effect of the tested compounds at the concentrations lower than MIC on the bacterial viability. The measurements were taken automatically every 1 h and registered with the Epicenter software (BD).

Kinase assays

Compounds **6**, **7** and **19** were tested as potential inhibitors of thymidylate kinase from *M. tuberculosis*. The enzyme was obtained by heterologous expression, using pMCSG7-tmk (clone ID: APC105701.102; DNASU) (10) in *E. coli* strain BL-21(DE3), under standard conditions (0.2 mM IPTG, 20 °C for 16 h). The crude cell lysate was washed over Ni-NTA agarose resin (Qiagen, Valencia, CA, USA) and the target enzyme eluted with 250 mM imidazole buffer. The purified enzyme was stored in buffer containing 50 mM Tris–HCl (pH 8), 300 mM NaCl, and 5 mM $MgCl_2$. Thymidylate kinase was > 95% pure as determined by SDS–PAGE. Enzyme concentration was determined by UV absorbance at 280 nm (ε = 32 430 M^{-1} cm^{-1}) (11).

Inhibition of thymidylate kinase activity was evaluated using an established coupled spectroscopic assay (12). Initial reaction rates were determined at 37 °C in reaction buffer containing 50 mM Tris–HCl (pH 8), 100 mM KCl, 5 mM $MgCl_2$, 1 mM ATP, 0.2 mM PEP, and 0.2 mM NADH, as well as five units of pyruvate kinase and lactate dehydrogenase, respectively (Roche Biochemicals, Indianapolis, IN, USA). Enzyme concentration was constant at 0.05 μM, while TMP ranged from 0 to 128 μM and inhibitor was varied from 0 to 200 μM. Phosphorylation of TMP was monitored via NADH oxidation at 340 nm (ε = 6220 M^{-1} cm^{-1}). None of the investigated inhibitors had significant absorbance at that wavelength. All experiments were performed in the presence of 0.4% DMSO (v/v), maximizing inhibitor solubility while minimizing detrimental effects on enzyme activity. Initial reaction rates at varying substrate/inhibitor concentrations were fit to a competitive inhibition model.

Results

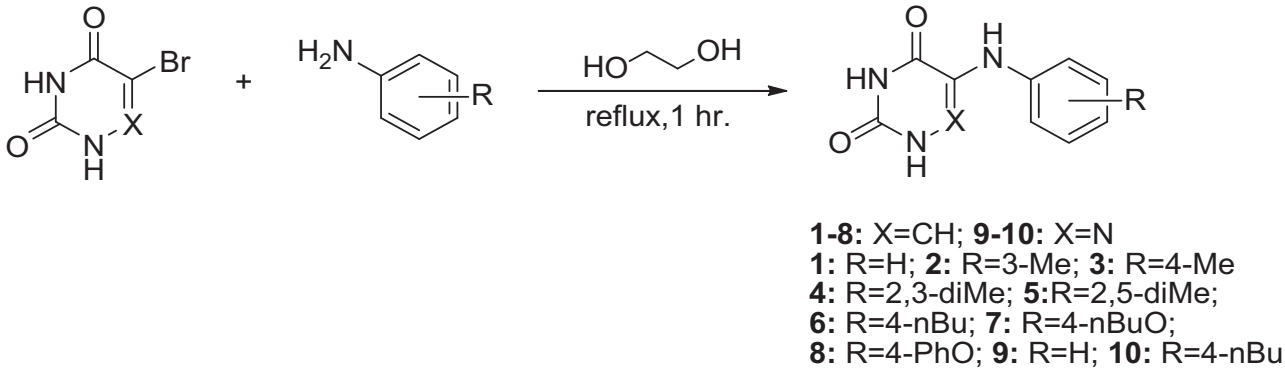
Synthesis of the 5-(phenylamino)uracils **1**–**10** was carried out as previously described (13) by treating 5-bromouracil with a threefold molar excess of the appropriate aniline in boiling ethylene glycol, as shown in Scheme 1.

This procedure gave 5-(phenylamino)uracils **1**–**8** with yields ranging between 50% and 82%. The moderate yields observed are likely due to the tendency of anilines to undergo oxidation. It should also be noted that the lowest yields were observed for anilines possessing a methyl group in the ortho-position (compounds **4** and **5**).

5-(Phenylamino)-6-azauracils **9** and **10** were prepared under similar conditions by the condensation of 5-bromo-6-azauracil (14) with a threefold molar excess of aniline or 4-*n*-butylaniline. The yield of compound **9** was high, while compound **10**, in which the butyl group is in the *p*-position, was obtained in only a 47% yield.

Once in hand, the compounds were screened for their inhibitory properties against TB. While several of the compounds showed promising activity (see Table 1), most proved to be too insoluble in the requisite medium (see Table 1), thus a solution to this problem was sought.

In that regard, we recently reported a series of 5′-nor carbocyclic uracil derivatives, which displayed moderate anti-TB



Scheme 1: Synthesis of 5-arylaminouracil derivatives.

Table 1: 100% inhibition of *Mycobacterium tuberculosis* growth, strain H37Rv, and MIC_{99}

	Concentration, μg/mL					
Compound	40	20	10	5	1	MIC_{99}[a]
1	I	I	I	–	–	–
2	I	+	–	–	–	20
3	I	I	+	–	–	10
4	I	–	–	–	–	–
5	I	–	–	–	–	–
6	I	I	I	+	–	10
7	I	I	I	+	–	5
8	I	I	I	I	I	–
9	I	I	I	–	–	–
10	I	+	–	–	–	20
11	–	–	–	–	–	–
12	–	–	–	–	–	–
13	–	–	–	–	–	–
14	I	–	–	–	–	–
15	+	–	–	–	–	40
16	+	–	–	–	–	40
17	+	–	–	–	–	40
18	–	–	–	–	–	–
19	+	+	–	–	–	20
20	–	–	–	–	–	–
Rifampicin	+	+	+	+	+	1

I: insoluble at this concentration.
[a]Compound concentration in μg/mL at which growth is 99% inhibited.

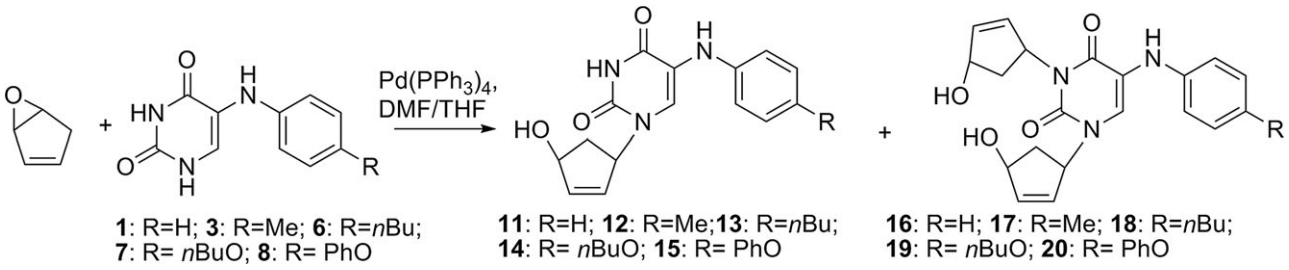
activity (15). The 5′-nor carbocyclic nucleosides in general possess several key advantages due to their increased stability, increased solubility, and lack of 5′-phosphorylation (16). This latter feature is responsible for a decrease in cytotoxicity that proved problematic for the triphosphates of aristeromycin and neplanocin A, two naturally occurring carbocyclic nucleosides that exhibit potent therapeutic properties against viruses, parasites, and various diseases including TB (17,18). The target 5-alkynyl uracil derivatives were found to have an MIC_{99} of 10–40 μg/mL against the H37Rv laboratory strain of *M. tuberculosis* with a corresponding increase in activity noted as the length of the side chain for the alkynyl derivatives increased (15).

Related to this, similar activity has been observed for a series of 6-aryl purine analogues. These nucleoside displayed selective antimycobacterial activity (19,20), with the flexible versions exhibiting potent inhibitory activity against *M. tuberculosis* (20). Likewise, acyclic thymidine analogues were found to inhibit *M. tuberculosis* monophosphate kinase (TMPKmt) (21). Given the increased solubility generally observed for carbocyclic nucleosides, and the promising inhibitory properties described above, combining the carbocyclic moiety with the uracil scaffold was predicted to improve their solubility and subsequently improve their biological activity.

In that regard, 5-phenylaminouracils **1**, **3**, **6**–**8** were condensed with epoxycyclopentene in the presence of a palladium catalyst using Trost methodology (22). High regio- and stereoselectivity of the reaction is achieved due to the unique structure of the π-allyl intermediate complex formed with the allylic acetate, which leads solely to cis-products via retention of configuration at the 1′-position (23) (Scheme 2). Due to their poor solubility in DMF, the heterogeneous mixture containing the 5-substituted uracils was vigorously stirred while heating to 50 °C. After isolation and purification, the yields of racemic N^1-monosubstituted products **11**–**15** were disappointingly low, ranging between 18% and 25%, while the N^1, N^3-disubstituted uracil analogues (**16**–**20**) were obtained in somewhat higher yields (35–40%) as diastereoisomeric mixtures.

Compounds **1**–**20** were then screened for potential antimicrobial activity. The laboratory *M. tuberculosis* H37Rv strain, which is a virulent strain susceptible to all antituberculosis drugs, was chosen for the initial screening. The compounds were tested at concentrations of 1, 5, 10, 20 and 40 μg/mL. The minimal inhibitory concentrations (MIC_{99}) of compounds **1**–**20** were determined using the proportion method in the BACTEC MGIT 960 system (9) in the TB Exist mode (24).

Compounds **2**, **3**, **6**, **7**, **10**, **15**–**17**, and **19** all had a negative effect on the growth of mycobacterial cells, with 99% inhibition observed at concentrations between 5 and 40 μg/mL. Compounds **2**, **3**, **6**, **7**, **10**, **15**–**17**, and **19**



Scheme 2: Synthesis of 5′-norcarbocyclic 5-arylaminouracil derivatives.

Table 2: *Mycobacterium tuberculosis* (strain MS-115) % growth inhibition

	Concentration, μg/mL				
Compound	20	10	5	1	0.1
2	NA	NA	NA	NA	NA
3	I	NA	NA	NA	NA
6	I	I	75%	NA	NA
7	I	I	75%	NA	NA
10	NA	NA	NA	NA	NA
15	NA	NA	NA	NA	NA
16	NA	NA	NA	NA	NA
17	90%	NA	NA	NA	NA
19	100%	99%	90%	NA	NA

I: insoluble at this concentration; NA: not active.

were also tested at concentrations ranging between 0.1 and 20 μg/mL against *M. tuberculosis* multidrug-resistant strain (MS-115), which is resistant to isoniazid, rifampicin, streptomycin, ethambutol, and pyrazinamide. The results are shown in Table 2.

It is important for compounds that suppress bacterial or viral replication to also be tested for toxicity, as, for example, what appears to be an antibacterial effect against *M. tuberculosis* could, in fact, be the result of cell depletion. As a result, the cytotoxicity of the compounds was evaluated in *Vero, A_{549}*, and *Huh7* cells with none of the compounds displaying cytotoxicity up to concentrations of 50 μg/mL.

Discussion

Although the precise mechanism of action for these compounds against TB is not known, speculation that it might be a result of inhibition of the thymidylate kinase of *M. tuberculosis* (TMPKmt, EC 2.7.4.9, ATP: phosphotransferase TMF) seemed most likely. TMPKmt belongs to the family of nucleoside monophosphate kinases and catalyzes the reversible phosphorylation of 2′-deoxythymidine phosphate to 2′-deoxythymidine diphosphate in the presence of ATP (25,26). TMPKmt is required for DNA synthesis, and, consequently, for the growth and reproduction of mycobacteria. In contrast to other known TMPKs, phosphate transfer in TMPKmt requires the transient binding of a magnesium ion co-ordinating the phosphate acceptor. Moreover, the unique configuration of the active site and catalytic mechanism of TMPKmt renders it a promising target for the development of highly active and selective anti-TB drugs (25,26). In addition, the crystallographic structure of TMPKmt was published (25,27) therefore providing a means for exploring potential inhibitors using computer-mediated design (28).

To evaluate TMPKmt as the possible target enzyme for the compounds anti-TB activity, they were docked in the active site of the enzyme using AUTODOCK VINA 1.1.2. (29) A crystallographic model of thymidylate kinase in *M. tuberculosis* PDB: 1G3U, (27) previously used for modeling of nucleoside inhibitors of the enzyme, was employed and the results are shown below.

Docking resulted in two distinctive conformations for compounds studied. The first one represents mode of binding that is analogous to previously described inhibitors of TMPKmt (6) (Figure 2).

The second, or 'reversed' binding pose, is shown in Figure 3. The Mg^{2+} is displaced by the inhibitor, as is typical for these types of inhibitors, and the predicted bonding interactions with the enzyme appeared encouraging. The locations of the aromatic rings of **6**, **7** and **19** were identical to previously reported inhibitors and are likely due to π–π stacking between the substituent at the 5th-position and residues Phe70 and Tyr103, as well as the π-cation interaction with the side chain of Arg95. The carbonyl function at the 2nd-position of the pyrimidine ring formed a hydrogen bond with the guanidine nitrogen Arg95, and analogous interactions could be expected for inhibitors containing the pyrrolidine-2-one moiety (21). Hence, a set of key features, which are essential for inhibitory properties, was established (26,30). Finally, the 4′-hydroxyls of the cyclopentene appear to interact with polar amino acids at the entrance to the active site (the blue area shown in Figure 3B).

Faced with these conflicting results, we decided to evaluate TMPKmt as the enzymatic target for compounds **6**, **7** and **19**. Heterologously expressed kinase, purified to homogeneity via its C-terminal polyHis tag, was used for these *in vi-*

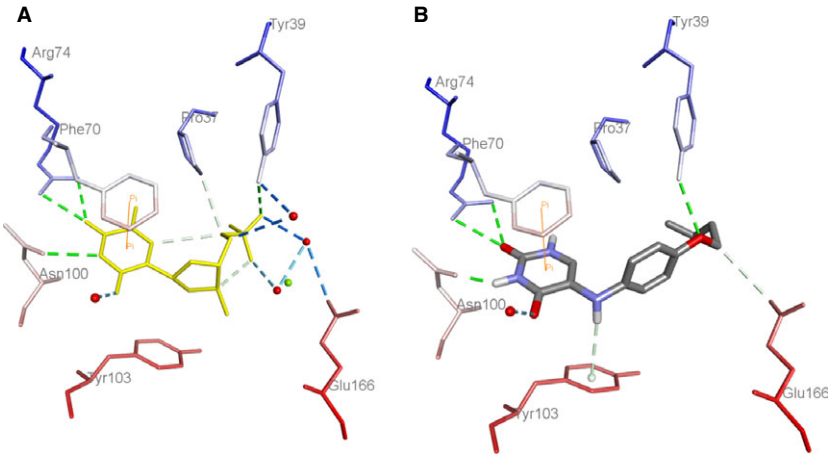


Figure 2: (A) dTMP in the active site of TMPKmt. (B) Predicted binding mode for compound **7**—note the analogous with dTMP ligand–protein interactions.

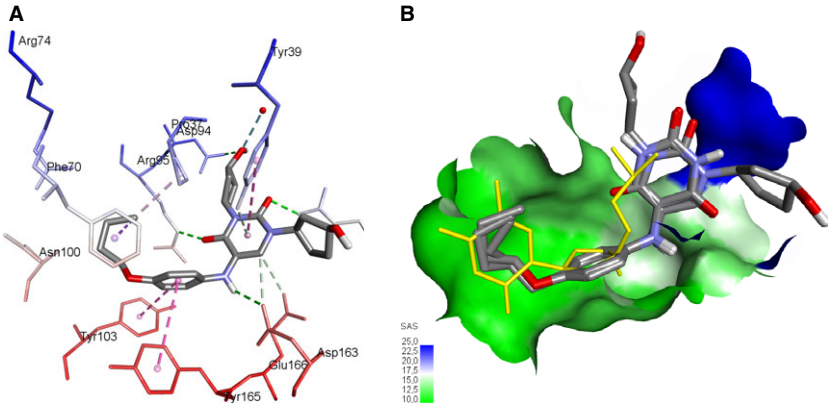


Figure 3: (A) Predicted 'reversed' binding for compound **19**. (B) Predicted 'reversed' binding modes for compounds **6**, **7** and **19** overlaid with dTMP (shown in yellow) inside the active center cavity. The blue surface area indicates the entrance region, while the green is the surface of the bottom of the pocket.

tro experiments. Enzyme activity was initially tested with native substrate (thymidine monophosphate; TMP), using a spectrophotometric coupled-enzyme assay. The enzyme showed activity and its steady-state parameters for TMP were consistent with the literature data (30). Given the limited solubility of the inhibitors in buffer, the kinase's tolerance for DMSO was tested. It was determined that the catalytic activity was not significantly affected at concentrations up to 0.4%. All inhibition studies were hence carried out in reaction buffer (50 mM Tris–HCl pH 8, 100 mM KCl, 5 mM $MgCl_2$, five units of PK and LDH, 1 mM ATP, 0.2 mM PEP, and 0.2 mM NADH) supplemented with 0.4% DMSO at 37 °C. Substrate phosphorylation was determined via the coupled oxidation of NADH, and corresponding absorbance loss at 340 nm (reduced NADH: $\varepsilon 340 = 6220\ M^{-1}\ cm^{-1}$). The three inhibitors were tested at concentrations from 0 to 200 μM in the presence of TMP at concentrations ranging from 0 to 128 μM. Unfortunately, at higher inhibitor concentrations, solubility once again became problematic. Based on the activity measurements, K_i values of > 200 μM must be assumed for all three compounds; however, the low affinity of these inhibitors is consistent with previous data for the corresponding thymine analogues (6).

In summary, while the anti-TB activity observed is promising, the mechanism of action remains elusive and further studies are needed. In addition, as solubility remains problematic, the development of prodrugs that could potentially increase solubility and allow the use of lower inhibitor concentrations will now be considered.

Acknowledgments

The research was supported by the Russian Science Foundation, project no. 14-50-00060.

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