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New 5-arylamino-4-(5-nitrofuran-2-yl)pyrimidines as promising antibacterial agents

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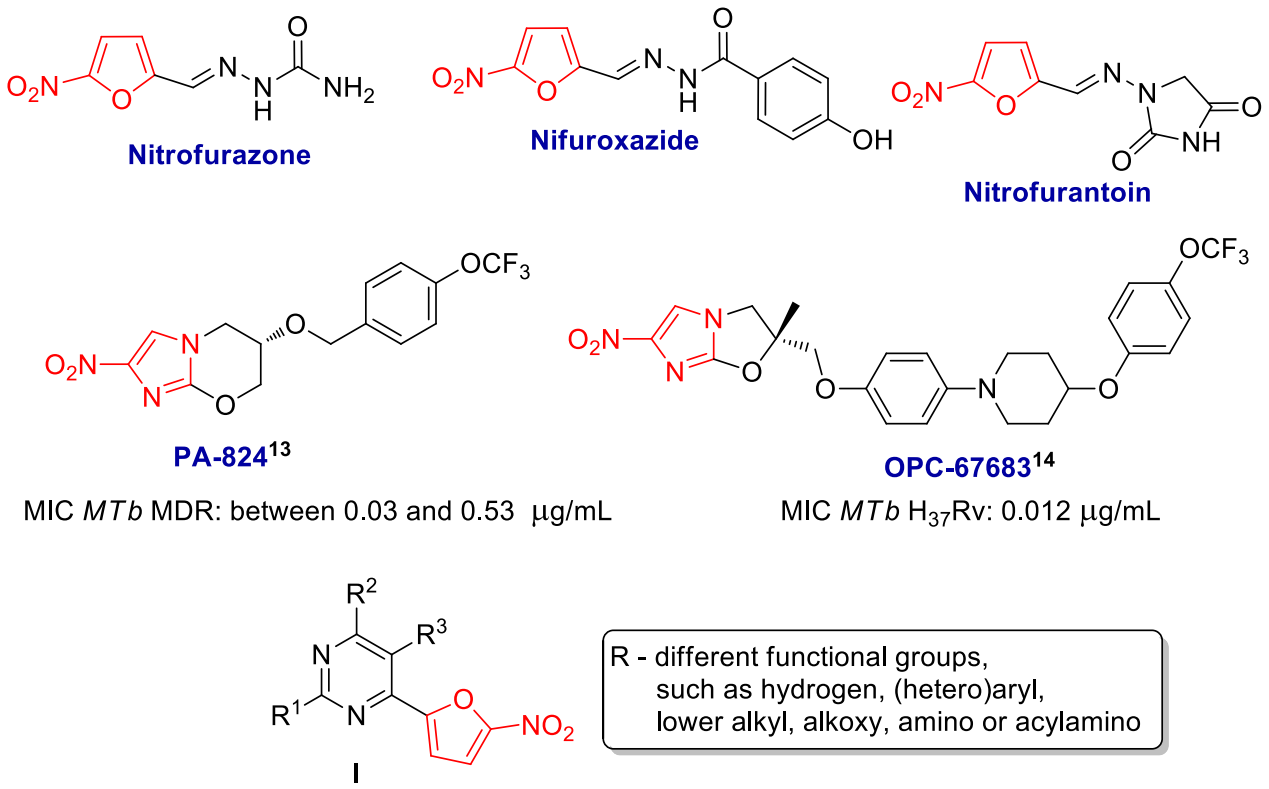

Figure S1. Classical antibacterial drugs of the nitrofuran family, examples of the recently reported nitroimidazoles and 4-(5-nitofuranyl)-pyrimidines with broad-spectrum antibacterial and antimycobacterial efficacy.

Table S1. *In vitro* antibacterial activity of *N*-aryl-4-(5-nitrofuran-2-yl)pyrimidin-5-amines (**3a-j**).

Entry	Compound	Antibacterial activity (MIC), μg·ml^{-1}														
		***Mbt* $H_{37}Rv$**	***N. gonorrhoeae* NCTC 8375/ ATCC19424**	***N. gonorrhoeae* NCTC12700 /ATCC49226**	***N. gonorrhoeae* AZMR**	***N. gonorrhoeae* AZMS**	***E. coli***	***C. braakii***	***S. flexneri***	***P. vulgaris***	***S. marcescens***	***K. pneumoniae***	***P. aeruginosa***	***S. pyogenes***	***S. aureus***	***S. aureus MRSA***
1	**3a**	1.5	1.9	0.9	1.9	7.8	31.2	125	125	>250	250	62.5	>250	62.5	3.9	1.9
2	**3b**	n.d.	7.8	15.6	31.2	62.5	>1000	>1000	>1000	>1000	>1000	>1000	>1000	0.9	7.8	0.9
3	**3c**	12.5	1.9	0.9	3.9	>250	>250	>250	>250	>250	>250	>250	>250	7.8	15.6	125
4	**3d**	n.d.	7.8	0.45	125	125	>1000	>1000	>1000	>1000	>1000	>1000	>1000	0.9	7.8	3.9
5	**3e**	12.5	3.9	1.9	7.8	>250	>250	>250	>250	>250	>250	>250	>250	62.5	125	125
6	**3f**	12.5	3.9	1.9	3.9	1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	3.9	3.9	15.6
7	**3g**	12.5	0.9	0.45	3.9	125	125	125	125	>250	>250	62.5	>250	15.6	62.5	7.8
8	**3h**	n.d.	500	7.8	500	250	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000
9	**3i**	n.d.	7.8	15.6	125	125	>250	>250	>250	>250	>250	250	>250	>250	250	250
10	**3j**	n.d.	62.5	62.5	125	125	>250	>250	>250	>250	>250	250	>250	>250	62.5	62.5
11	**INH**	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12	**PZA**	12.5	-	-	-	-	-	-	-	-	-	-	-		-	-
13	**SPEC**	-	15.6	15.6	4.0	15.6	15.6	31.25	7.8	125	15.6	15.6	250	15.6	31.25	>250

n.d. – not determined; n.a. – data not available; INH – Isoniazid; PZA – Pyrazinamide; SPEC – Spectinomycin;
ATCC – American Type Culture Collection; RCPM – Russian Collection of Pathogenic Microorganisms.
Mbt $H_{37}Rv$ – *Mycobacterium tuberculosis* $H_{37}Rv$;
N. gonorrhoeae NCTC 8375/ATCC19424 – *Neisseria gonorrhoeae* NCTC 8375/ATCC19424;
N. gonorrhoeae NCTC12700/ATCC49226 – *Neisseria gonorrhoeae* NCTC12700/ATCC49226;
N. gonorrhoeae AZMR – Spectinomycin susceptible, but Azithromycin resistant *Neisseria gonorrhoeae* strain isolated from gonorrhea patients in Ural region (Russia);
N. gonorrhoeae AZMS – Azithromycin susceptible, but Ciprofloxacin and Cefepime resistant *Neisseria gonorrhoeae* strain isolated from gonorrhea patients in Ural region (Russia);
E. coli – *Escherichia coli* ATCC 8739;
C. braakii – *Citrobacter braakii* ATCC 101/57;
S. flexneri – *Shigella flexneri* RCPM 1a8516;
P. vulgaris – *Proteus vulgaris* RCPM 160125 (222);
S. marcescens – *Serratia marcescens* ATCC 13880;
K. pneumoniae – *Klebsiella pneumoniae* ATCC 13883;
P. aeruginosa – *Pseudomonas aeruginosa* ATCC 9027;
S. pyogenes – *Streptococcus pyogenes* ATCC 19615;
S. aureus MRSA– Methicillin-resistant *Staphylococcus aureus* NCTC 12493;
S. aureus – *Staphylococcus aureus* NCTC 12981 (F-49) / ATCC 25923.

Experimental Section

General Information. All reagents and solvents were obtained from commercial sources and dried by using the standard procedures before use. 5-Bromo-4-(5-nitrofuran-2-yl)pyrimidine **1** was synthesized as described previously.[1] 1,4-Dioxane for the Buchwald–Hartwig cross-coupling reaction was deoxygenated by bubbling argon for 1h.

The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AVANCE-500 instrument using Me_4Si as an internal standard. Elemental analysis was carried on a Eurovector EA 3000 automated analyzer. Melting points were determined on Boetius combined heating stages and were not corrected.

The GC-MS analysis of all samples was carried out using an Agilent GC 7890A MS 5975C Inert XL EI/CI GC-MS spectrometer with a quadrupole mass-spectrometric detector with electron ionization (70 eV) and scan over the total ionic current in the range m/z 20–1000 and a quartz capillary column HP-5MS (30 m × 0.25 mm, film thickness 0.25 mm). Helium served as a carrier gas, the split ratio of the flow was 1 : 50, and the consumption through the column was 1.0 ml min^{-1}; the initial temperature of the column was 40 °C (storage 3 min), programming rate was 10 deg min^{-1} to 290 °C (storage 20 min), the temperature of the evaporator was 250 °C, the temperature of the source was 230 °C, the temperature of the quadrupole was 150 °C, and the temperature of the transition chamber was 280 °C. Solutions of the samples with a concentration of 3-4 mg ml^{-1} were prepared in THF. Samples of 1 mL of the obtained solutions were analyzed.

Column chromatography was carried out using Alfa Aesar silica gel 0.040-0.063 mm (230–400 mesh). The progress of reactions and the purity of compounds were checked by TLC on Sorbfil plates (Russia), in which the spots were visualized with UV light (λ 254 or 365 nm).

X-ray diffraction analysis was performed on an automated X-ray diffractometer "Xcalibur E" on standard procedure. CCDC 1587557 (for **3a**) and 1587558 (for **3g**) contain the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via *www.ccdc.cam.ac.uk/data_request/cif*.

General procedure for the synthesis of *N*-aryl-4-(5-nitrofuran-2-yl)-pyrimidin-5-amines (3a-j).

A stirred mixture of 5-bromo-4-(5-nitrofuran-2-yl)-pyrimidine **1,** (270 mg, 1.0 mmol), corresponding aniline **2** (1.2 mmol), 1,1′-ferrocenediyl-bis(diphenylphosphine) (222 mg, 40 mol%), $Pd(OAc)_2$ (45 mg, 20 mol%) and K_3PO_4 (531 mg, 2.5 mmol) in deoxygenated 1,4-dioxane (25 ml) was heated at 85 °C under nitrogen for 15 h in Schlenk tube. The reaction mixture was cooled, filtered, and dissolved with a mixture of AcOEt and water 1:1 (50 ml) and the organic layer was separated. The aqueous layer was

[1] Verbitskiy, E. V.; Baskakova, S. A.; Gerasimova, N. A.; Evstigneeva, N. P.; Zil'berberg, N. V.; Kungurov, N. V.; Kravchenko, M. A.; Skornyakov, S. N.; Pervova, M. G.; Rusinov, G. L.; Chupakhin, O. N.; Charushin, V. N. *Bioorg. Med. Chem. Lett.* **2017**, *27*, 3003.

extracted with AcOEt (2×25 ml). The combined organic extracts were dried with $MgSO_4$ and the solvents evaporated. The residue was purified by flash column chromatography (hexane/ethyl acetate, 1:3) to afford the desired cross-coupling products (**3a-j**).

4-(5-Nitrofuran-2-yl)-*N*-phenylpyrimidin-5-amine (3a). Yield 56%, dark red crystals, mp 123-124 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ = 8.89 (s, 1H), 8.82 (s, 1H), 8.30 (s, 1H), 7.83 (d, *J* = 4.0 Hz, 1H), 7.47 (d, *J* = 4.0 Hz, 1H), 7.28 (m, 2H), 7.05 (d, *J* = 7.7 Hz, 2H), 6.95 (m, 1H) ppm. ^{13}C NMR (126 MHz, DMSO-d_6): δ = 151.8, 151.6, 151.1, 150.5, 141.8, 141.5, 135.0, 129.4, 121.7, 117.9, 116.2, 114.2 ppm. GC t_R 26.40 min; MS m/z (rel intensity) 282 (M^+, 100). Anal. Calcd for $C_{14}H_{10}N_4O_3$ (282.26): C 59.57, H 3.57, N 19.85. Found: C 59.40, H 3.48, N 19.82. HRMS (ESI): m/z calcd for $C_{14}H_{11}N_4O_3$: 283.0826 $[M+H]^+$; found: 283.0822.

4-(5-Nitrofuran-2-yl)-*N*-(*m*-tolyl)pyrimidin-5-amine (3b). Yield 47%, dark red crystals, mp 133 °C. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.80 (s, 1H), 8.71 (s, 1H), 7.53–7.50 (m, 2H), 7.39 (s, 1H), 7.31 (m, 1H), 7.08–7.06 (m, 2H), 7.03 (d, *J* = 7.6 Hz, 1H), 2.38 (s, 3H) ppm. ^{13}C NMR (126 MHz, $CDCl_3$): δ = 154.6, 151.7, 148.5, 146.0, 140.2, 138.6, 136.2, 135.6, 129.8, 126.1, 122.8, 119.2, 114.0, 113.1, 21.4 ppm. GC t_R 27.02 min; MS m/z (rel intensity) 296 (M^+, 100). Anal. Calcd for $C_{15}H_{12}N_4O_3$ (296.29): C 60.81, H 4.08, N 18.91. Found: C 60.94, H 4.24, N 19.08. HRMS (ESI): m/z calcd for $C_{15}H_{13}N_4O_3$: 297.0982 $[M+H]^+$; found: 297.0985.

4-(5-Nitrofuran-2-yl)-*N*-(*p*-tolyl)pyrimidin-5-amine (3c). Yield 67%, dark orange powder, mp 174-176 °C. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.73 (s, 1H), 8.68 (s, 1H), 7.52 (d, *J* = 3.9 Hz, 1H), 7.50 (d, *J* = 3.9 Hz, 1H), 7.36 (s, 1H), 7.23 (d, *J* = 8.1 Hz, 2H), 7.17 (d, *J* = 8.1 Hz, 2H), 2.38 (s, 3H) ppm. ^{13}C NMR (126 MHz, $CDCl_3$): δ = 154.9, 151.6, 148.6, 145.9, 135.9, 135.5, 135.3, 130.6, 122.9, 113.7, 113.2, 20.9 ppm. GC t_R 27.23 min; MS m/z (rel intensity) 296 (M^+, 100). Anal. Calcd for $C_{15}H_{12}N_4O_3$ (296.29): C 60.81, H 4.08, N 18.91. Found: C 60.69, H 4.26, N 19.07. HRMS (ESI): m/z calcd for $C_{15}H_{13}N_4O_3$: 297.0982 $[M+H]^+$; found: 297.0980.

***N*-(3-Methoxyphenyl)-4-(5-nitrofuran-2-yl)pyrimidin-5-amine (3d)**. Yield 35%, red powder, mp 155 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ = 8.90 (s, 1H), 8.84 (s, 1H), 8.28 (s, 1H), 7.82 (d, *J* = 3.9 Hz, 1H), 7.46 (d, *J* = 4.0 Hz, 1H), 7.17 (m, 1H), 6.60 (m, 2H), 6.53 (dd, *J* = 7.7, 1.6 Hz, 1H), 3.71 (s, 3H) ppm. ^{13}C NMR (126 MHz, DMSO-d_6): δ = 160.3, 151.7, 151.3, 151.2, 143.3, 141.9, 134.8, 130.2, 116.2, 114.1, 109.9, 107.3, 103.4, 99.5, 55.0 ppm. GC t_R 28.15 min; MS m/z (rel intensity) 312 (M^+, 100). Anal. Calcd for $C_{15}H_{12}N_4O_4$ (312.09): C 57.69, H 3.87, N 17.94. Found: C 57.43, H 3.96, N 17.88. HRMS (ESI): m/z calcd for $C_{15}H_{13}N_4O_4$: 313.0931 $[M+H]^+$; found: 313.0929.

***N*-(4-Methoxyphenyl)-4-(5-nitrofuran-2-yl)pyrimidin-5-amine (3e)**. Yield 45%, dark red crystals, mp 177-178 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ = 8.74 (s, 1H), 8.58 (s, 1H), 8.00 (s, 1H), 7.87 (d, *J* = 3.9 Hz, 1H), 7.49 (d, *J* = 3.9 Hz, 1H), 7.13 (d, *J* = 8.7 Hz, 2H), 6.94 (d, *J* = 8.7 Hz, 2H), 3.74 (s, 3H) ppm. ^{13}C NMR (126 MHz, DMSO-d_6): δ = 155.6, 152.3, 151.5, 149.3, 147.2, 138.5, 136.7, 133.5, 122.5, 115.5,

114.8, 114.3, 55.3 ppm. GC t_R 28.41 min; MS m/z (rel intensity) 312 (M^+, 100). Anal. Calcd for $C_{15}H_{12}N_4O_4$ (312.09): C 57.69, H 3.87, N 17.94. Found: C 57.70, H 3.76, N 18.05. HRMS (ESI): m/z calcd for $C_{15}H_{13}N_4O_4$: 313.0931 $[M+H]^+$; found: 313.0933.

***N*-(3,4-Dimethoxyphenyl)-4-(5-nitrofuran-2-yl)pyrimidin-5-amine (3f)**. Yield 55%, burgundy powder, mp 181-182 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ = 8.76 (s, 1H), 8.64 (s, 1H), 8.02 (s, 1H), 7.87 (d, J = 4.0 Hz, 1H), 7.49 (d, J = 4.0 Hz, 1H), 6.92 (d, J = 8.6 Hz, 1H), 6.82 (d, J = 2.4 Hz, 1H), 6.68 (dd, J = 8.6, 2.4 Hz, 1H), 3.73 (s, 3H), 3.72 (s, 3H) ppm. ^{13}C NMR (126 MHz, DMSO-d_6): δ = 152.3, 151.5, 149.6, 149.4, 147.7, 145.1, 138.7, 136.6, 134.1, 115.5, 114.3, 112.8, 112.4, 105.9, 55.8, 55.5 ppm. GC t_R 29.77 min; MS m/z (rel intensity) 342 (M^+, 100). Anal. Calcd for $C_{16}H_{14}N_4O_5$ (342.31): C 56.14, H 4.12, N 16.37. Found: C 56.00, H 4.13, N 16.26. HRMS (ESI): m/z calcd for $C_{16}H_{15}N_4O_5$: 343.1037 $[M+H]^+$; found: 343.1044.

4-(5-Nitrofuran-2-yl)-*N*-(3,4,5-trimethoxyphenyl)pyrimidin-5-amine (3g). Yield 50%, red crystals, mp 180-182 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ = 8.83 (s, 1H), 8.80 (s, 1H), 8.19 (s, 1H), 7.85 (d, J = 4.0 Hz, 1H), 7.48 (d, J = 4.0 Hz, 1H), 6.40 (s, 2H), 3.71 (s, 6H), 3.60 (s, 3H) ppm. ^{13}C NMR (126 MHz, DMSO-d_6): δ = 153.5, 152.1, 151.6, 150.3, 149.6, 140.1, 137.4, 135.7, 132.9, 115.8, 114.2, 96.9, 60.1, 55.7 ppm. GC t_R 30.93 min; MS m/z (rel intensity) 372 (M^+, 100). Anal. Calcd for $C_{17}H_{16}N_4O_6$ (372.34): C 54.84, H 4.33, N 15.05. Found: C 54.68, H 4.52, N 15.16. HRMS (ESI): m/z calcd for $C_{17}H_{17}N_4O_6$: 373.1143 $[M+H]^+$; found: 373.1140.

4-(5-Nitrofuran-2-yl)-*N*-(2-nitrophenyl)pyrimidin-5-amine (3h). Yield 29%, dark yellow crystals, mp 172-173 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ = 9.86 (s, 1H), 9.06 (s, 1H), 8.99 (s, 1H), 8.30 (dd, J = 8.5, 1.4 Hz, 1H), 7.55 (d, J = 3.9 Hz, 1H), 7.50–7.46 (m, 1H), 7.44 (d, J = 3.9 Hz, 1H), 7.17–7.09 (m, 1H), 7.06–6.97 (m, 1H) ppm. ^{13}C NMR (126 MHz, DMSO-d_6): δ = 154.3, 154.2, 152.6, 151.9, 145.0, 139.7, 136.1, 135.7, 130.6, 127.1, 120.6, 116.5, 115.9, 112.4 ppm. GC t_R 29.26 min; MS m/z (rel intensity) 327 (M^+, 100). Anal. Calcd for $C_{14}H_9N_5O_5$ (327.26): C 51.38, H 2.77, N 21.40. Found: C 51.19, H 2.86, N 21.36. HRMS (ESI): m/z calcd for $C_{14}H_{10}N_5O_5$: 328.0676 $[M+H]^+$; found: 328.0673.

4-(5-Nitrofuran-2-yl)-*N*-(3-nitrophenyl)pyrimidin-5-amine (3i). Yield 53%, orange powder, mp 217-218 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ = 9.05 (s, 1H), 8.97 (s, 1H), 8.87 (s, 1H), 7.82 (d, J = 4.0 Hz, 1H), 7.75 (m, 1H), 7.69 (dd, J = 8.1, 1.6 Hz, 1H), 7.53 (d, J = 4.0 Hz, 1H), 7.49 (m, 1H), 7.36 (dd, J = 8.1, 1.8 Hz, 1H) ppm. ^{13}C NMR (126 MHz, DMSO-d_6): δ = 153.4, 153.1, 151.8, 151.0, 148.7, 144.5, 144.1, 133.3, 130.6, 122.0, 116.9, 114.8, 114.1, 110.2 ppm. GC t_R 30.32 min; MS m/z (rel intensity) 327 (M^+, 100). Anal. Calcd for $C_{14}H_9N_5O_5$ (327.26): C 51.38, H 2.77, N 21.40. Found: C 51.52, H 2.96, N 21.38. HRMS (ESI): m/z calcd for $C_{14}H_{10}N_5O_5$: 328.0676 $[M+H]^+$; found: 328.0673.

4-(5-Nitrofuran-2-yl)-*N*-(4-nitrophenyl)pyrimidin-5-amine (3j). Yield 48%, yellow powder, mp 245-246 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ = 9.37 (s, 1H), 9.17 (s, 1H), 9.02 (s, 1H), 8.10 (d, J = 9.1 Hz, 2H), 7.82 (d, J = 3.9 Hz, 1H), 7.49 (d, J = 3.9 Hz, 1H), 6.98 (d, J = 9.1 Hz, 2H) ppm. ^{13}C NMR (126

MHz, DMSO-d_6): δ = 156.3, 154.7, 152.0, 150.6, 150.5, 146.5, 143.7, 139.2, 131.7, 126.0, 117.4, 114.0 ppm. GC t_R 31.66 min; MS m/z (rel intensity) 327 (M^+, 100). Anal. Calcd for $C_{14}H_9N_5O_5$ (327.26): C 51.38, H 2.77, N 21.40. Found: C 51.44, H 2.67, N 21.39. HRMS (ESI): m/z calcd for $C_{14}H_{10}N_5O_5$: 328.0676 $[M+H]^+$; found: 328.0674.

Antimycobacterial assay.

To evaluate the inhibitory efficiency of molecules on *Mycobacterium tuberculosis* (MTB), *M. tuberculosis* $H_{37}Rv$, which is susceptible to all classical antituberculosis drugs, was used. The minimal inhibitory concentration (MIC) for *M. tuberculosis* $H_{37}Rv$ for each compound was determined by a micro broth dilution method. All molecules tested were dissolved in dimethylsulfoxide and their 1/2 dilutions were prepared in 5 mL tubes using Löwenstein-Jensen medium. A few colonies from freshly grown *M. tuberculosis* $H_{37}Rv$ were suspended in Löwenstein-Jensen medium to obtain 1.0 McFarland turbidity and diluted ten times using the same medium and the tubes were incubated at 37 °C medium with a different concentration of the tested molecule and to a positive control tube containing only clear growth medium. After 24 hours the tubes were placed in a vertical position and the free edge of the buried 0.3 mL of the substance in the test compounds concentrations: 12.5, 6.2, 3.1, 1.5, 0.7, 0.37, 0.15 μg mL^{-1}. The tubes were then placed in an thermostat at a temperature of 37 °C and incubated for 10 days. Growth estimate for the MTB were determined by standard methods, where the appearance of zones of growth retardation MTB (over 10 mm) indicated the presence of tuberculostatic properties in concentration of the compounds under study. Penetration size stunting MTB (in mm) is proportional to the degree of tuberculostatic activity. Growth delay of 100 mm or more is considered as a complete growth inhibition MTB.

Antibacterial activity assay.

The two fold serial dilution technique recommended by Clinical and Laboratory Standards Institute (CLSI)[2] was used to evaluate the inhibitory efficiency of molecules on *N. gonorrhoeae, E. coli, C. braakii, S. flexneri, P. vulgaris, S. marcescens, K. pneumoniae, P. aeruginosa, S. pyogenes* and *S. aureus*. The medium for testing anti-gonorrhea activity consists of GC agar to which a 1% defined growth supplement. The Muller-Hinton medium (Broth) was used for testing other antibacterial activities. Adjust the density of the suspension to contain 1.5×10^8 CFU ml^{-1} by comparison with a 0.5 McFarland turbidity standard. For suspension using colonies from an overnight (20- to 24-hour) agar plate incubated in 5% CO_2 36±1°C. Dilute this suspension 1:100 in Muller-Hinton to give 10^6 CFU ml^{-1}. The test compounds

[2] Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fourth Informational Supplement*; Clinical and Laboratory Standards Institute: Wayne, PA, **2014**. (http://www.cdc.gov/std/gonorrhea/arg/b88-feb-2005.pdf).

concentrations: 1000, 500, 250, 125, 62.5, 31.25, 15.6, 7.8, 3.9, 1.9, 0.97 μg mL^{-1} (solvent – DMSO, diluent – H_2O and GC agar base or Muller-Hinton Broth). The multidrug-resistant *Neisseria gonorrhoeae* strains has been isolated from gonorrhea patients in Ural Research Institute for Dermatology, Venereology and Immunopathology (Russia).

Cytotoxicity assay

Cytotoxicity of each compound was determined with a standard tetrazolium assay[3] using mouse fibroblast-like cells McCoy B (ATCC CRL 1696). The cell culture reagents were purchased from Russian Collection of cell cultures of vertebrates (Institute of Cytology of the Russian Academy of Sciences, Saint Petersburg). Cells were grown in DMEM/F12 media and salt L-glutamine HEPES (Biolot, Russia) supplemented with 10% fetal bovine serum (FBS, GE Healthcare, Austria). Cells were seeded at 3.0×10^4 cells per well in a 96-well microtiter plate (Eppendorf) and allowed to adhere overnight. Medium was carefully aspirated and replaced with 195 μl fresh medium, and compound solutions in DMSO (5 μl) were added to give a final concentration ranging from 500 to 0.97 μl. All concentrations were tested in triplicate. Plates were incubated for 72 h at 37 ºC and 5.0% CO_2 in a humidified chamber. The solutions were carefully removed and solution containing 1.0 mg ml^{-1} 2,2'-bis(4-nitrophenyl)-5,5'-diphenyl-3,3'-(3,3'-dimethoxy-4,4'-diphenylene)ditetrazolium chloride (*p*-Nitro-Blue tetrazolium chloride) was added (200 μl) and incubated for 3 h, after which the *p*-Nitro-Blue tetrazolium chloride medium was carefully removed. Isopropyl alcohol (200 μl) was added to dissolve the precipitated purple formazan crystals and the plates were read at 490 nm using a plate reader (Lazurit, Dynex, Technologies Inc., USA); plate background (690 nm) was subtracted and cell viability was estimated as the percentage absorbance relative to the DMSO control. Dose response curves were generated using trial GraphPad Prism 5 software and used to determine the IC_{50} concentrations (minimal concentration that inhibits 50% of growth).

[3] Mosmann, T. *J. Immunol. Methods* **1983**, *65*, 55.