Chem Biol Drug Des 2016; **87:** 905–917

Research Article

Synthesis and Antimicrobial Activity of 4-Chloro-3-Nitrophenylthiourea Derivatives Targeting Bacterial Type II Topoisomerases

Anna Bielenica[1,*], Karolina Stępień[2], Agnieszka Napiórkowska[3], Ewa Augustynowicz-Kopeć[3], Sylwester Krukowski[4], Marta Włodarczyk[5] and Marta Struga[5]

[1]*Chair and Department of Biochemistry, Medical University, 02-097 Warszawa, Poland*
[2]*Department of Pharmaceutical Microbiology, Medical University, 02-007 Warszawa, Poland*
[3]*Department of Microbiology, National Tuberculosis and Lung Diseases Research Institute, 01-138 Warszawa, Poland*
[4]*Department of Inorganic and Analytical Chemistry, Faculty of Pharmacy, Medical University, 02-097 Warszawa, Poland*
[5]*Department of Pharmacogenomics, Faculty of Pharmacy, Medical University, 02-097 Warszawa, Poland*
**Corresponding author: Anna Bielenica, abielenica@wum.edu.pl*

A series of novel 4-chloro-3-nitrophenylthiourea derivatives were synthesized and evaluated for their antimicrobial, antibiofilm and tuberculostatic activities. Most of compounds exhibited high antibacterial activity against both standard and hospital strains (MIC values 0.5–2 μg/mL), as compared to Ciprofloxacin. Derivatives with 3,4-dichlorophenyl (11) and 3-chloro-4-methylphenyl (13) substituents were the most promising towards Gram-positive pathogens. Both of them exhibited antibiofilm potency and effectively inhibited the formation of biofilms of methicillin-resistant and standard strains of *Staphylococcus epidermidis*. Two N-alkylthioureas (20, 21) showed twofold to fourfold increase in in vitro potency against isolates of *Mycobacterium tuberculosis*, as compared to Isoniazid. An action of 7, 10, 11, 13, 20 and 21 against activity of topoisomerases isolated from *Staphylococcus aureus* was studied. Synthesized compounds were found as non-genotoxic.

Key words: antimicrobial activity, antitubercular activity, biofilm, thiourea derivatives, topoisomerase

Received 27 November 2015, revised 7 January 2016 and accepted for publication 7 January 2016

Bacterial topoisomerases are an attractive target for the discovery and development of new antibacterial agents. The type II topoisomerase includes topoisomerase IV and DNA gyrase that share significant sequence similarity, however play different functions in DNA replication. Gyrase introduces the negative supercoiling into DNA, whereas topoisomerase IV preferentially binds and relaxes positive supercoils (1,2). Type II topoisomerases possess ATP binding site that could be the intracellular target for potent antibacterials (3). Topoisomerase poisons can also trap cleavage complexes by binding at the enzyme-DNA interface. Usually gyrase and topoisomerase IV inhibitors represent the class of dual-targeting inhibitors, *that is* both block ATP binding and interfere with DNA binding/strand passage (2). Several urea derivatives with high antistaphylococcal (4–6) activities were described as dual-targeting ATP-site inhibitors. On the other hand, antimycobacterial potency of this class of compounds is the result of DNA gyrase B inhibition (6).

Bacteria growing in slime-enclosed aggregates known as biofilms are dominant in chronic bacterial infections (7). In contrast to planktonic forms of bacteria responsible mainly for acute infections, biofilm-based diseases are extremely resistant to antibiotics and other conventional antimicrobial agents (8,9). What is more, they are insensitive to host immune response, allowing them to persist and promote continued infection despite intensive antibiotic therapy (10). One of the most common aetiologies of device-related infections is staphylococci, either coagulase-negative strains or *Staphylococcus aureus* (11). Up to now, only several chemical scaffolds have been described that can inhibit or disperse biofilm of these type of strains (12,13). Their action is based either on inhibition of intercellular communication (quorum sensing) and signalling pathways (14,15) or enzymatic degradation of the integrity of extracellular matrix required for the biofilm formation (16).

Antimicrobial activity of thiourea derivatives has been the subject of numerous investigations. Structure-activity relationship (SAR) study of both *N*-phenylurea and thiourea derivatives showed that these containing electron-withdrawing substituents exert higher activity as compared to other analogues (17,18). Moreover, several thiourea-containing compounds exhibit potent antimycobacterial (19,20), antiviral (21), antimalarial (22) and analgesic properties (23).

 doi: 10.1111/cbdd.12723

Taking into consideration significant antimicrobial activity of thioureas, to continue our work on the evaluation of the bioactivity of thiourea-containing compounds (24,25), we designed and synthesized a series of derivatives combining 4-chloro-3-nitrophenylthiourea moiety and various aromatic substituents.

Experimental

Chemistry

All reagents and solvents were purchased from Alfa Aesar (Lancashire, UK), Sigma Aldrich (Saint Louis, MO, USA) or POCh (Polskie Odczynniki Chemiczne). The IR spectra were obtained on Perkin Elmer Spectrum 1000 spectrometer (Perkin Elmer, Waltham, MA, USA). The NMR spectra were recorded on Varian VNMRS 300 Oxford NMR spectrometer (Varian, Palo Alto, CA, USA), using TMS as the internal reference. Mass spectral ESI measurements were carried out on Waters ZQ Micro-mass instruments (Waters, Milford, MA, USA) with quadruple mass analyser, at a declustering potential of 40–60 V. Flash chromatography was performed on Merck silica gel 60 (200–400 mesh) (Merck, Darmstadt, Germany) using chloroform eluent.

General procedure for the synthesis of N-substituted (4-chloro-3-nitrophenyl)thiourea derivatives (1 21)

A solution of 4-chloro-3-nitroaniline (0.0029 mol) in dry acetonitrile (10 mL) was treated with appropriate isothiocyanate (0.0029 mol), and the mixture was stirred at room temperature for 12 h. Then, the solvent was evaporated and the solid residue was either crystallized from acetonitrile (chloroform) or purified by column chromatography (chloroform).

The structures of compounds along with atom numbering are presented in Supporting information section (Table S1).

1-(4-chloro -3-nitrophenyl)-3-(2-chlorophenyl) thiourea (1)

Yield 68%, m.p. 146–148 °C. FT-IR (KBr, cm-1): 3198.3, 3165.3, 2996.2, 1585.6, 1533.2, 1475.5, 1401.4, 1317.2, 1262.2, 1122.8, 1042.5, 879.2, 722.5. ^{1}H NMR (300 MHz, DMSO) δ: 10.31 (s, 1H, NH), 9.84 (s, 1H, NH), 8.41 (d, 1H, J = 2.7 Hz, H-2), 7.84 (dd, 1H, $J_1 = J_2$ = 2.7 Hz, H-6), 7.78–7.70 (m, 1H, H-5), 7.60–7.53 (m, 2H, H-3', H-5'), 7.40–7.28 (m, 2H, H-4', H-6'). ^{13}C NMR (75.4 MHz, DMSO) δ: 180.57 (C=S), 146.82, 139.54, 135.85, 131.37, 130.44, 129.89, 129.63, 128.37, 128.13, 127.45, 119.73, 119.65. ESI MS: m/z = 340.0 [M-2H]- (100%).

1-(4-chloro-3-nitrophenyl)-3-(3-chlorophenyl) thiourea (2)

Yield 30%, m.p. 140–142 °C. FT-IR (KBr, cm-1): 3247.0, 3189.7, 3089.0, 3031.3, 1587.4, 1549.5, 1476.5, 1404.5, 1338.7, 1255.7, 1126.8, 1047.6, 869.4, 695.2. ^{1}H NMR (300 MHz, DMSO) δ: 10.26 (s, 2H, NH), 8.33 (d, 1H, J = 2.4 Hz, H-2), 7.80 (dd, 1H, J_1 = 2.7 Hz, J_2 = 2.4 Hz, H-6), 7.72 (m, 1H, J = 9.0 Hz, H-5), 7.65 (t, 1H, J = 1.2 Hz, H-5'), 7.41–7.35 (m, 2H, H-2', H-4'), 7.26–7.19 (m, 1H, H6'). ^{13}C NMR (75.4 MHz, DMSO) δ: 179.81 (C=S), 146.89, 140.43, 139.51, 132.69, 131.36, 130.21, 128.51, 124.63, 123.34, 122.29, 119.92, 119.70. ESI MS: m/z = 340.0 [M-2H]- (100%).

1-(4-chloro-3-nitrophenyl)-3-(4-chlorophenyl) thiourea (3)

Yield 64%, m.p. 176–178 °C. FT-IR (KBr, cm-1): 3318.5, 3177.0, 3083.4, 1589.0, 1533.7, 1481.7, 1406.3, 1357.5, 1240.5, 1191.5, 1160.5, 1088.5, 866.0, 709.5. ^{1}H NMR (300 MHz, DMSO) δ: 10.21 (s, 2H, NH), 8.33 (d, 1H, J = 2.4 Hz, H-2), 7.80 (dd, 1H, $J_1 = J_2$ = 2.4 Hz, H-6), 7.71 (d, 1H, J = 8.7 Hz, H-5), 7.50 (d, 2H, J = 9.0 Hz, H3', H-5'), 7.43–7.40 (m, 2H, H2', H-6'). ^{13}C NMR (75.4 MHz, DMSO) δ: 179.79 (C=S), 146.85, 139.61, 137.83, 131.31, 128.94, 128.51, 128.42, 125.59, 119.80, 119.57. ESI MS: m/z = 340.0 [M-2H]- (100%).

1-(2-bromophenyl)-3-(4-chloro-3-nitrophenyl) thiourea (4)

Yield 63%, m.p. 147–148 °C. FT-IR (KBr, cm-1): 3205.8, 3166.4, 2991.9, 1582.6, 1533.5, 1475.7, 1403.2, 1323.5, 1296.2, 1125.5, 1043.6, 879.5, 725.2. ^{1}H NMR (300 MHz, DMSO) δ: 10.30 (s, 1H, NH), 9.81 (s, 1H, NH), 8.43 (d, 1H, J = 2.4 Hz, H-2), 7.88 (dd, 1H, $J_1 = J_2$ = 2.7 Hz, H-6), 7.73–7.68 (m, 2H, H-5, H-3'), 7.52 (dd, 1H, $J_1 = J_2$ = 1.5 Hz, H-5'), 7.44–7.39 (m, 1H, H-6'), 7.27–7.21 (m, 1H, H-4'). ^{13}C NMR (75.4 MHz, DMSO) δ: 180.50 (C=S), 146.81, 139.52, 137.32, 132.76, 131.37, 130.29, 128.53, 128.38, 128.06, 121.56, 119.74, 119.65. ESI MS: m/z = 384.9 [M-2H]- (100%).

1-(4-chloro-3-nitrophenyl)-3-(3-bromophenyl) thiourea (5)

Yield 45%, m.p. 136–138 °C. FT-IR (KBr, cm-1): 3248.0, 3086.4, 3012.5, 1585.5, 1548.9, 1475.7, 1403.9, 1335.6, 1254.9, 1128.3, 1049.4, 871.3, 700.0. ^{1}H NMR (300 MHz, DMSO) δ: 10.28–10.24 (m, 2H, NH), 8.32 (d, 1H, J = 2.4 Hz, H-2), 7.81–7.76 (m, 2H, H-6, H-4'), 7.72 (d, 1H, J = 8.7 Hz, H-5), 7.44 (dt, 1H, J_1 = 1.95 Hz, J_2 = 1.8 Hz, H-5'), 7.38–7.34 (m, 1H, H-2'), 7.30 (d, 1H, J = 7.5 Hz, H-6'). ^{13}C NMR (75.4 MHz, DMSO) δ: 179.82 (C=S), 146.89, 140.57, 139.50, 131.36, 130.48, 128.51, 127.52, 126.20, 122.74, 121.03, 119.91, 119.68. ESI MS: m/z = 384.9 [M-2H]- (100%).

1-(4-bromophenyl)-3-(4-chloro-3-nitrophenyl) thiourea (6)

Yield 52%, m.p. 182–184 °C. FT-IR (KBr, cm-1): 3319.9, 3185.5, 3074.8, 2978.6, 1585.9, 1534.2, 1479.3, 1403.8,

1355.4, 1296.4, 1191.8, 1157.2, 1068.2, 867.4, 710.0. ^{1}H NMR (300 MHz, DMSO) δ: 10.21 (s, 2H, NH), 8.32 (d, 1H, J = 2.4 Hz, H-2), 7.79 (dd, 1H, J_1 = 2.7 Hz, J_2 = 2.4 Hz, H-6), 7.71 (d, 1H, J = 8.7 Hz, H-5), 7.57–7.50 (m, 2H, H3', H-5'), 7.46–7.42 (m, 2H, H2', H-6'). ^{13}C NMR (75.4 MHz, DMSO) δ: 179.71 (C=S), 146.85, 139.59, 138.26, 131.43, 131.31, 128.41, 125.87, 119.79, 119.58, 117.10. ESI MS: m/z = 384.9 [M-2H]- (100%).

1-(4-chloro -3-nitrophenyl)-3-(2-fluorophenyl) thiourea (7)

Yield 60%, m.p. 151–153 °C. FT-IR (KBr, cm-1): 3323.6, 3152.7, 3089.6, 2985.4, 1593.0, 1543.4, 1483.4, 1405.3, 1340.5, 1250.0, 1134.2, 1105.3, 1046.6, 864.5, 709.7. ^{1}H NMR (300 MHz, DMSO) δ: 10.29 (s, 1H, NH), 9.87 (s, 1H, NH), 8.39 (d, 1H, J = 2.4 Hz, H-2), 7.84 (dd, 1H, J_1 = 2.4 Hz, J_2 = 2.7 Hz, H-6), 7.71 (d, 1H, J = 8.7 Hz, H-5), 7.58–7.53 (m, 1H, H-3'), 7.33–7.18 (m, 3H, H-4', H-5', H-6'). ^{13}C NMR (75.4 MHz, DMSO) δ: 180.78 (C=S), 156.42, 146.82, 139.61, 131.35, 128.72, 128.35, 127.92, 126.39, 124.35, 119.69, 119.63, 115.93. ESI MS: m/z = 324.0 [M-H]- (100%).

1-(4-chloro-3-nitrophenyl)-3-(3-fluorophenyl) thiourea (8)

Yield 45%, m.p. 162–164 °C. FT-IR (KBr, cm-1): 3250.5, 3152.2, 3009.4, 1593.4, 1549.7, 1486.1, 1404.5, 1337.7, 1256.5, 1141.4, 1131.5, 1049.5, 870.1, 705.7. ^{1}H NMR (300 MHz, DMSO) δ: 10.28–10.26 (m, 2H, NH), 8.34 (d, 1H, J = 2.4 Hz, H-2), 7.80 (dd, 1H, J_1 = J_2 = 2.4 Hz, H-6), 7.72 (d, 1H, J = 8.4 Hz, H-5), 7.48 (dt, 1H, J_1 = 2.1 Hz, J_2 = 2.25 Hz, H-5'), 7.43–7.36 (m, 1H, H-4'), 7.27–7.24 (m, 1H, H-2'), 7.03–6.97 (m, 1H, H-6'). ^{13}C NMR (75.4 MHz, DMSO) δ: 179.70 (C=S), 161.72, 146.89, 140.66, 139.53, 131.34, 130.19, 128.53, 119.93, 119.68, 119.37, 111.39, 110.40. ESI MS: m/z = 324.0 [M-H]- (100%).

1-(4-chloro-3-nitrophenyl)-3-(4-fluorophenyl) thiourea (9)

Yield 45%, m.p. 172–173 °C. FT-IR (KBr, cm-1): 3319.7, 3181.4, 2996.7, 1587.3, 1536.4, 1504.5, 1404.4, 1352.3, 1310.0, 1152.6, 1138.2, 1043.1, 866.5, 703.3. ^{1}H NMR (300 MHz, DMSO) δ: 10.12 (s, 2H, NH), 8.34 (d, 1H, J = 2.1 Hz, H-2), 7.80 (dd, 1H, J_1 = J_2 = 2.4 Hz, H-6), 7.71 (d, 1H, J = 8.7 Hz, H-5), 7.48–7.43 (m, 2H, H-3', H-5'), 7.20 (t, 2H, J = 8.7 Hz, H-2', H-6'). ^{13}C NMR (75.4 MHz, DMSO) δ: 180.07 (C=S), 159.48, 146.85, 139.73, 135.12, 131.28, 128.42, 126.50, 119.79, 119.45, 115.31. ESI MS: m/z = 324.0 [M-H]- (100%).

1-(3-chloro-4-fluorophenyl)-3-(4-chloro-3-nitrophenyl)thiourea (10)

Yield 70%, m.p. 142–144 °C. FT-IR (KBr, cm-1): 3240.3, 3146.2, 3026.8, 1593.6, 1551.5, 1480.6, 1402.8, 1337.5, 1249.3, 1126.6, 1051.8, 872.6, 703.1. ^{1}H NMR (300 MHz, DMSO) δ: 10.24–10.19 (m, 2H, NH), 8.31 (d, 1H, J = 2.4 Hz, H-2), 7.79 (dd, 1H, J_1 = 2.4 Hz, J_2 = 2.1 Hz, H-6), 7.75–7.70 (m, 2H, H-5, H-5'), 7.42–7.40 (m, 2H, H-2', H-6'). ^{13}C NMR (75.4 MHz, DMSO) δ: 180.17 (C=S), 154.18, 146.92, 139.47, 136.06, 131.37, 128.67, 126.28, 125.04, 120.04, 119.76, 118.93, 116.67. ESI MS: m/z = 358.0 [M-2H]- (100%).

1-(4-chloro-3-nitrophenyl)-3-(3,4-dichlorophenyl) thiourea (11)

Yield 85%, m.p. 173–175 °C. FT-IR (KBr, cm-1): 3242.4, 3177.5, 3080.9, 3038.4, 1581.0, 1545.2, 1470.5, 1335.4, 1255.6, 1129.4, 1048.0, 868.4, 702.8. ^{1}H NMR (300 MHz, DMSO) δ: 10.31 (s, 2H, NH), 8.31 (d, 1H, J = 2.4 Hz, H-2), 7.84 (d, 1H, J = 2.4 Hz, H-5'), 7.80 (dd, 1H, J_1 = J_2 = 2.4 Hz, H-6), 7.72 (d, 1H, J = 8.7 Hz, H-5), 7.61 (m, 1H, J = 8.7 Hz, H-2'), 7.44 (dd, 1H, J_1 = 2.1 Hz, J_2 = 2.7 Hz, H-6'). ^{13}C NMR (75.4 MHz, DMSO) δ: 179.88 (C=S), 146.93, 139.38, 139.12, 131.41, 130.65, 130.38, 128.62, 126.70, 125.27, 123.97, 120.05, 119.86. ESI MS: m/z = 373.9 [M-3H]- (100%).

1-(4-chloro-3-nitrophenyl)-3-(2,4-dichlorophenyl) thiourea (12)

Yield 53%, m.p. 154–155 °C. FT-IR (KBr, cm-1): 3361.7, 3338.3, 3100.7, 1576.8, 1512.5, 1475.6, 1322.5, 1251.9, 1144.6, 1127.9, 1054.0, 822.5, 689.3. ^{1}H NMR (300 MHz, DMSO) δ: 10.34 (br. s, 1H, NH), 9.87 (br. s, 1H, NH), 8.39 (d, 1H, J = 2.4 Hz, H-2), 7.84 (dd, 1H, J_1 = 2.7 Hz, J_2 = 2.4 Hz, H-6), 7.73–7.71 (m, 2H, H-5, H-3'), 7.58 (d, J = 8.4 Hz, 1H, H-5'), 7.45 (dd, 1H, J_1 = J_2 = 2.4 Hz, H-6'). ^{13}C NMR (75.4 MHz, DMSO) δ: 180.57 (C=S), 146.83, 139.39, 135.14, 131.62, 131.53, 131.40, 131.09, 129.11, 128.45, 127.61, 119.83. ESI MS: m/z = 373.9 [M-3H]- (100%).

1-(3-chloro-4-methylphenyl)-3-(4-chloro-3-nitrophenyl)thiourea (13)

Yield 65%, m.p. 239–240 °C. FT-IR (KBr, cm-1): 3363.1, 3180.0, 3108.5, 1601.0, 1536.8, 1483.5, 1394.8, 1346.9, 1298.5, 1130.0, 1045.2, 875.6, 706.6. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 9.29 (br. s, 1H, NH), 8.99 (br. s, 1H, NH), 8.29–8.29 (m, 1H, H-2), 7.68–7.61 (m, 3H, H-5, H-6, H-5'), 7.28–7.20 (m, 2H, H-2', H-6'), 2.27 (s, 3H, H-4'a). ^{13}C NMR (75.4 MHz, DMSO) δ: 180.17 (C=S), 147.46, 139.70, 138.25, 133.08, 131.77, 131.18, 128.89, 123.21, 118.59, 117.41, 116.80, 114.40, 18.80. ESI MS: m/z = 354.0 [M-2H]- (100%).

1-(5-chloro-2-methylphenyl)-3-(4-chloro-3-nitrophenyl)thiourea (14)

Yield 61%, m.p. 148–150 °C. FT-IR (KBr, cm-1): 3220.4, 3115.5, 3076.9, 2971.5, 1582.5, 1537.4, 1479.6, 1445.6,

1404.6, 1334.0, 1256.2, 1126.3, 1047.5, 862.4, 709.8. ^{1}H NMR (300 MHz, DMSO) δ: 10.17 (s, 1H, NH), 9.77 (s, 1H, NH), 8.36 (d, 1H, J = 2.4 Hz, H-2), 7.83 (dd, 1H, $J_1 = J_2$ = 2.4 Hz, H-6), 7.71 (d, 1H, J = 9.0 Hz, H-5), 7.38–7.36 (m, 1H, H-3'), 7.32–7.29 (m, 1H, H-4'), 7.28–7.23 (m, 1H, H-6'), 2.21 (s, 3H, H-2'a). ^{13}C NMR (75.4 MHz, DMSO) δ: 180.48 (C=S), 146.83, 139.63, 138.60, 133.95, 131.90, 131.30, 129.89, 128.54, 127.60, 126.64, 119.94, 119.61, 17.25. ESI MS: m/z = 354.0 [M-2H]- (100%).

1-(4-chloro-3-nitrophenyl)-3-[3-(trifluoromethyl) phenyl]thiourea (15)

Yield 42%, m.p. 147–149 °C. FT-IR (KBr, cm-1): 3255.6, 3160.6, 3098.8, 3047.9, 1591.2, 1557.5, 1481.1, 1407.9, 1331.5, 1259.4, 1173.5, 1122.8, 1071.6, 882.4, 698.2. ^{1}H NMR (300 MHz, DMSO) δ: 10.35 (s, 2H, NH), 8.32 (d, 1H, J = 2.4 Hz, H-2), 7.91 (m, 1H, H-5'), 7.79 (dd, 1H, J_1 = 2.7 Hz, J_2 = 2.4 Hz, H-6), 7.76–7.71 (m, 2H, H-5, H-4'), 7.60–7.52 (m, 2H, H-2', H-6'). ^{13}C NMR (75.4 MHz, DMSO) δ: 180.03 (C=S), 146.97, 139.88, 139.40, 131.43, 129.71, 129.11, 128.53, 127.68, 123.87, 121.20, 120.14, 119.94, 119.77. ESI MS: m/z = 374.0 [M-H]- (100%).

1-(4-chloro-3-nitrophenyl)-3-[4-(trifluoromethyl) phenyl]thiourea (16)

Yield 40%, m.p. 172–173 °C. FT-IR (KBr, cm-1): 3246.5, 3203.4, 3094.1, 3039.2, 1593.6, 1552.2, 1478.5, 1404.9, 1329.6, 1255.6, 1162.2, 1114.5, 1053.7, 868.6, 684.6. ^{1}H NMR (300 MHz, DMSO) δ: 10.41 (br. s, 2H, NH), 8.34 (d, 1H, J = 2.7 Hz, H-2), 7.81 (dd, 1H, J_1 = 2.7 Hz, J_2 = 2.4 Hz, H-6), 7.75 (m, 1H, H-5), 7.72 (m, 4H, H-2', H-3', H-5', H-6'). ^{13}C NMR (75.4 MHz, DMSO) δ: 179.80 (C=S), 146.92, 142.76, 139.44, 131.41, 129.64, 128.48, 125.74, 124.50, 124.26, 123.27, 119.87, 119.79. ESI MS: m/z = 374.0 [M-H]- (100%).

1-(4-chloro-3-nitrophenyl)-3-(4-iodophenyl)thiourea (17)

Yield 40%, m.p. 184–186 °C. FT-IR (KBr, cm-1): 3363.1, 3317.5, 3177.5, 2980.6, 1581.8, 1537.2, 1478.9, 1400.7, 1353.5, 1299.4, 1196.8, 1147.8, 1053.6, 869.2, 712.4. ^{1}H NMR (300 MHz, DMSO) δ: 10.20 (s, 2H, NH), 8.33 (d, 1H, J = 2.7 Hz, H-2), 7.80 (dd, 1H, $J_1 = J_2$ = 2.7 Hz, H-6), 7.72–7.68 (m, 3H, H-5, H-3', H-5'), 7.32–7.28 (m, 2H, H-2', H-6'). ^{13}C NMR (75.4 MHz, DMSO) δ: 179.59 (C=S), 146.85, 139.62, 138.75, 137.30, 131.31, 128.42, 125.97, 119.80, 119.56, 89.40. ESI MS: m/z = 431.9 [M-2H]- (100%).

1-(4-chloro-3-nitrophenyl)-3-(4-nitrophenyl) thiourea (18)

Yield 59%, m.p. 160–162 °C. FT-IR (KBr, cm-1): 3329.9, 3239.2, 3084.8, 3025.0, 1587.6, 1533.7, 1477.4, 1402.6, 1334.6, 1250.1, 1178.9, 1114.2, 1046.3, 849.5, 708.6. ^{1}H NMR (300 MHz, DMSO) δ: 10.66–10.54 (m, 2H, NH), 8.33 (dd, 1H, $J_1 = J_2$ = 2.4 Hz, H-2), 8.26–8.21 (m, 2H, H-3', H-5'), 7.87–7.73 (m, 4H, H-5, H-6, H-2', H-6'). ^{13}C NMR (75.4 MHz, DMSO) δ: 179.62 (C=S), 146.70, 145.30, 142.53, 138.95, 131.23, 128.44, 124.17, 121.90, 119.88, 119.66. ESI MS: m/z = 351.0 [M-H]- (100%).

1-(4-chloro-3-nitrophenyl)-3-(4-cyanophenyl) thiourea (19)

Yield 57%, m.p. 173–175 °C. FT-IR (KBr, cm-1): 3202.2, 3067.2, 2996.8, 2237.6, 1591.2, 1540.5, 1483.2, 1363.4, 1309.3, 1193.5, 1141.2, 1045.2, 871.5, 689.9. ^{1}H NMR (300 MHz, DMSO) δ: 10.47 (s, 2H, NH), 8.33 (d, 1H, J = 2.4 Hz, H-2), 7.81–7.71 (m, 6H, H-5, H-6, H-2', H-3', H-5', H-6'). ^{13}C NMR (75.4 MHz, DMSO) δ: 179.35 (C=S), 146.94, 143.71, 132.82, 131.41, 128.43, 122.69, 119.80, 119.69, 118.95, 105.78. ESI MS: m/z = 331.0 [M-H]- (100%).

1-benzyl-3-(4-chloro-3-nitrophenyl)thiourea (20)

Yield 55%, m.p. 150–152 °C. FT-IR (KBr, cm-1): 3228.2, 3184.5, 3028.5, 2955.9, 2907.8, 1583.3, 1543.5, 1480.4, 1454.7, 1409.4, 1339.2, 1253.1, 1155.6, 1129.4, 1048.5, 877.3, 695.8. ^{1}H NMR (300 MHz, DMSO) δ: 10.02 (br. s, 1H, NH), 8.55 (br. s, 1H, NH), 8.42 (d, 1H, J = 2.1 Hz, H-2), 7.76 (dd, 1H, $J_1 = J_2$ = 2.7 Hz, H-6), 7.68 (d, 1H, J = 8.7 Hz, H-5), 7.35 (d, 4H, J = 4.5 Hz, H-3', H-4', H-6', H-7'), 7.30–7.24 (m, 1H, H-5'), 4.76 (d, 2H, J = 5.4 Hz, H-1'). ^{13}C NMR (75.4 MHz, DMSO) δ: 180.76 (C=S), 146.84, 139.79, 138.35, 131.35, 128.32, 127.49, 127.36, 127.03, 118.80, 118.62, 47.13. ESI MS: m/z = 320.0 [M-H]- (100%).

1-(4-chloro-3-nitrophenyl)-3-(1-phenylethyl) thiourea (21)

Yield 30%, m.p. 145–147 °C. FT-IR (KBr, cm-1): 3406.5, 3215.6, 3092.9, 3033.4, 1592.4, 1543.5, 1480.3, 1448.2, 1409.4, 1345.6, 1306.3, 1126.5, 1051.7, 871.2, 695.2. ^{1}H NMR (300 MHz, DMSO) δ: 9.83 (br. s, 1H, NH), 8.56 (d, 1H, J = 7.2 Hz, H-2), 8.42 (br. s, 1H, NH), 7.74 (dd, 1H, J_1 = 2.4 Hz, J_2 = 2.7 Hz, H-6), 7.66 (d, 1H, J = 8.7 Hz, H-5), 7.39–7.32 (m, 3H, H-3', H-5', H-7'), 7.30–7.23 (m, 2H, H-4', H-6'), 5.92 (br.s, 1H, H-1'), 1.48 (d, 3H, J = 6.9 Hz, H-1'a). ^{13}C NMR (75.4 MHz, DMSO) δ: 179.59 (C=S), 146.78, 143.36, 139.86, 131.30, 128.34, 126.93, 126.23, 118.56, 118.36, 108.98, 52.70, 21.74. ESI MS: m/z = 334.0 [M-H]- (100%).

In vitro *evaluation of antimicrobial activity*

The antimicrobial activity of the compounds was tested on Gram-positive bacteria (*Staphylococcus aureus* NCTC 4163, *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* ATCC 6538, *Staphylococcus aureus* ATCC 29213,

Staphylococcus epidermidis ATCC 12228, *Staphylococcus epidermidis* ATCC 35984, *Enterococcus faecalis* ATCC 29212, *Bacillus subtilis* ATCC 6633, *Bacillus cereus* ATCC 11778, *Micrococcus luteus* ATCC 9341, *Micrococcus luteus* ATCC 10240), Gram-negative rods (*Escherichia coli* ATCC 10538, *Escherichia coli* ATCC 25922, *Escherichia coli* NCTC 8196, *Proteus vulgaris* NCTC 4635, *Pseudomonas aeruginosa* ATCC 15442, *Pseudomonas aeruginosa* NCTC 6749, *Pseudomonas aeruginosa* ATCC 27863, *Bordetella bronchiseptica* ATCC 4617) and yeasts (*Candida albicans* ATCC 10231, *Candida albicans* ATCC 90028, *Candida parapsilosis* ATCC 22019). Other micro-organisms used were obtained from the collection of the Department of Pharmaceutical Microbiology, Medical University of Warsaw, Poland.

Antibacterial activity was examined by the disc-diffusion method under standard conditions using Mueller-Hinton II agar medium (Becton Dickinson and Co, Sparks, MD, USA) according to CLSI (previously NCCLS) guidelines (26). Antifungal activities were assessed using Mueller-Hinton agar +2% glucose and 0.5 μg/mL Methylene Blue Dye Medium (27). Sterile filter paper discs (9 mm diameter, Whatman No 3 chromatography paper) were dripped with tested compound solutions (in DMSO) to load 400 μg of a given compound per disc. Dry discs were placed on the surface of appropriate agar medium. The results (diameter of the growth inhibition zone) were read after 18 h of incubation at 35 °C. Minimal inhibitory concentration (MIC) was tested by the twofold serial microdilution method (in 96-well microtitre plates) using Mueller-Hinton Broth medium (Beckton Dickinson) for bacteria or RPMI-1640 medium for *Candida* species according to CLSI guidelines (28,29). The stock solution of tested agent was prepared in DMSO and diluted in sterile water. Concentrations of tested agents ranged from 0.125 to 512 μg/mL. The final inoculum of all studied micro-organisms was 10^5 CFU/mL^{-1} (colony-forming units per mL). Minimal inhibitory concentrations (the lowest concentration of an tested agent that prevents visible growth of a micro-organism) were read after 18 h (bacteria) or 24 h (yeasts) of incubation at 35 °C.

Antitubercular activity

The synthesized compounds were examined *in vitro* for their tuberculostatic activity against the *M. tuberculosis* H_{37}Rv strain (ATCC 25618) and two 'wild-type' strains isolated from patients with tuberculosis: one (Spec. 210) resistant to *p*-aminosalicylic acid (PAS), isonicotinic acid hydrazide (Isoniazid, INH), etambutol (ETB) and rifampicin (RMP) and the another (Spec. 192) fully sensitive to the administrated tuberculostatics, as well as atypical strains (*M. bovis, M.scrofulaceum, M. fortuitum, M. avium, M. kansasii, M. xenopi, M. intracellulare*). Investigations were performed by a classical test-tube method of successive dilution in Youmans' modification of the Proskauer and Beck liquid medium containing 10% of bovine serum (30,31). Bacterial suspensions were prepared from 14 days old cultures of slowly growing strains. Solutions of compounds in DMSO were tested. Stock solutions contained 10 mg of compounds in 1 mL. Dilutions (in geometric progression) were prepared in Youmans' medium. The medium containing no investigated substances and containing Isoniazid (INH) as the reference drug were used for comparison. Incubation was performed at 37 °C. The MIC values were determined as minimum concentration inhibiting the growth of tested tuberculous strains in relation to the probe with no tested compound. The influence of the compound on the growth of bacteria at a certain concentration 3.1, 6.25, 12.5, 25, 50 and 100 μg/mL was evaluated.

Genotoxicity studies

DNA-damaging activity of compounds was tested by *rec*-assay using two genetically modified *Bacillus subtilis* strains: M45 (rec$^-$) and H17 (rec$^+$) (32,33). Tested compounds were dissolved in DMSO, and 10 μL of each solution was dripped onto sterile cotton discs (Rotilabo Carl Roth GmbH & Co. KG Karlsruhe, Germany) to load 256 μg of a given compound per 9 mm disc. Discs were placed on the surface Nutrient agar plates (Difco, Detroit, MI, USA) inoculated with 100 μL of bacterial overnight culture and incubated 24 h at 35 °C. After incubation, the growth inhibition zones were measured. 4-Nitroquinoline N-oxide (NOQ) was used as reference genotoxin (concentration 2 μg per disc). Results of the genotoxicity test were estimated after 18 h of incubation at 35 °C by comparing the diameter of the inhibition zone on the *B. subtilis* M45 (rec$^-$) strain with that on the *B. subtilis* H17 (rec$^+$) strain.

Inhibition of bacterial topoisomerase: Decatenation assay

The assay was performed using *S. aureus* topoisomerase IV (topoIV) decantation kit (Inspiralis Ltd., Norwich, UK). Kinetoplast DNA (kDNA) was the substrate for topoIV. 1 U of topoIV decatenated 200 ng of kDNA in the dedicated decantation assay buffer supplied by the manufacturer. Enzyme activity was detected by incubation for 30 min at 37 °C in a total reaction volume of 30 μL in the presence of tested compounds and the reference Ciprofloxacin at concentrations 0.25, 0.5, 1, 2, 4, 8 and 32 μg/mL. The reactions were terminated by adding of an equal volume of STEB buffer (40% sucrose, 100 mM Tris-HCl pH 8, 1 mM EDTA, 0.5 mg/mL bromophenol blue), followed by extraction with 1 volume of chloroform/isoamyl alcohol (24:1).

Then, 20 μL of the aqueous phase of each sample was loaded onto a 1% agarose gel. Electrophoresis was conducted in Tris-acetate-EDTA buffer for 1 h at 120 V. Gels were stained with ethidium bromide and visualized under UV light in a transilluminator (ChemiDoc MP, Bio-Rad, Hercules, CA, USA).

The assay was also performed using *S. aureus* Gyrase Supercoiling Assay Kit (Inspiralis). Supercoiled pBR322 plasmid DNA (500 ng) was incubated with 1 unit of gyrase in the 1× assay buffer in the presence of varying concentrations of the tested compounds. The reaction was carried out at 37 °C for 1 h and then terminated by the addition of equal volume of STOP buffer (40% sucrose, 100 mM Tris-HCL pH 8, 1 mM EDTA and 0.5 mg/mL bromophenol blue) and chloroform/izoamyl alcohol. Samples were vortexed, centrifuged and run through a 1% agarose gel in TAE buffer for 3 h at 50V. Gel was stained with ethidium bromide and visualized under UV. The IC_{50} values of the studied compounds were determined by plotting the results obtained from the densitometric analyses of the gel images using IMAGE LAB software (Bio-Rad). Means and SEM were calculated using STATISTICA 10 software (StatSoft, Cracow, Poland).

Biofilm inhibitory and biofilm eradication assay

Four hospital isolates of methicillin-resistant and two standard strains of *Staphylococcus epidermidis* (ATCC 12228, ATCC 35984) were cultured overnight in Tryptone Soy Broth supplemented with 0.25% glucose. The solution of tested compounds in TSB-glucose medium was mixed (1:1) with the bacterial inoculums (10^6 CFU/mL) in sterile 96-well polystyrene microtitre plates (Karell – Medlab, Italy) and incubated at 37 °C for 24 h. The final concentrations of tested compounds ranged from 1/2 to 8 × MIC (0.5–512 μg/mL).

The negative control was TSB-glucose medium, and the positive control (biofilm formation) was bacterial culture in TSB-glucose. After incubation, medium was removed from wells and washed twice with sterile phosphate-buffered saline (PBS) to remove the non-adherent bacteria. Alive bacterial cells in each well of the microtitre plate were stained with 3-(4,5-dimethyl-2-thiazolyl)-2,5 diphenyl-2H-tetrazolium bromide (MTT; 0.5% in PBS) for 2 h at 37 °C (protected from light). Adherent bacterial cells, which usually formed biofilm on wells, were uniformly stained with MTT. After incubation, the solution was removed and bacterial biofilm was solubilized by DMSO with glycine buffer and mixed 15 min at room temperature. The absorbance (A554) was measured at 554 nm using spectrophotometer (PowerWave XS, BioTek, Winooski, VT, USA). The IC_{50} values were calculated from the concentration–response curves by nonlinear regression analysis. All the experiments were carried out in quadruplicates. The biofilm survival (%) was determined using the formula: (%) = {(At–Ab)/(Ac–Ab)} × 100%, where At = Absorbance value of biofilm growth in the presence of a tested compound, Ab = Absorbance value of blank, Ac = Absorbance value of biofilm growth without any compound.

Staphylococcus epidermidis strain ATCC 12228 and *S. epidermidis* ATCC 35984 were used in eradication assays as a negative (low biofilm-producing) and positive (high biofilm-producing) control, respectively. Ciprofloxacin was used as the reference antimicrobial compound; its final concentration ranged from ½ to 8 × MIC (0.125–512 μg/mL). The initial steps for the preparation of bacterial strains are the same as for biofilm inhibitory assay. Tested strains were cultured on microplates and then incubated at 37 °C, 24 h for biofilm development (without shaking). After incubation, the supernatant containing non-adherent cells was removed, and 200 μL of a solution of a tested compound was re-filed into the wells in the microtitre plate, and the whole was incubated for 24 h at 37 °C. Further procedure corresponded to that described for biofilm inhibitory assay.

Results and Discussion

Chemistry

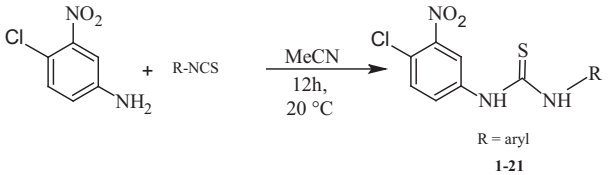
In the present study, new asymmetric 1,3-disubstituted thioureas **1-21** were synthesized from commercially available 4-chloro-3-nitroaniline and variety of isothiocyanates (Scheme 1, Table 1). Reactions were carried out in anhydrous acetonitrile at room temperature. The structures of compounds were determined using different spectroscopic methods (^{1}H NMR, ^{13}C NMR, MS and IR).

Biological studies

In vitro antimicrobial activity

All the newly synthesized compounds **1-21** were evaluated for their *in vitro* antimicrobial activity against a number of bacteria and fungi. The preliminary screening by the disc-diffusion method (26,27) was aimed to select derivatives with significant antimicrobial properties. As all of them exhibited this potency against Gram-positive strains (GIZ ≥ 13 mm), they were further tested for their minimal inhibitory concentration (MIC) by the twofold serial microdilution method (28,29).

The results revealed that 18 of 21 investigated compounds exhibited high and broad antibacterial activity, especially against standard staphylococcal and *M. luteus* strains (Table 1). Within this group, the observed MIC values were in the range 0.5–4 μg/mL. The remaining three derivatives were able to display moderate activity against tested Gram-positive cocci. Derivative **11** was equipotent to Ciprofloxacin against *S. aureus* ATCC 25923 (MIC 0.5 μg/



Scheme 1: Synthetic scheme of thiourea derivatives **1-21**.

Table 1: Activity of compounds against bacteria – minimal inhibitory concentrations (MIC, μg/mL)

Comp.	R	*S. aureus NCTC 4163*	*S. aureus ATCC 25923*	*S. aureus ATCC 6538*	*S. aureus ATCC 29213*	*S. epidermidis ATCC 35984*	*S. epidermidis ATCC 12228*	*B. subtilis ATCC 6633*	*B. cereus ATCC 11778*	*E. faecalis ATCC 29212*	*M. luteus ATCC 9341*	*M. luteus ATCC 10240*
1	2-Cl-Ph	8	8	8	8	16	16	8	8	32	16	16
2	3- Cl-Ph	2	2	2	2	4	4	2	1	8	4	4
3	4- Cl-Ph	2	4	2	2	4	8	4	2	32	4	4
4	2-Br-Ph	2	1	1	1	2	1	2	4	16	8	8
5	3-Br-Ph	1	1	1	2	4	4	2	1	4	2	2
6	4-Br-Ph	1	1	1	1	2	2	16	16	32	16	16
7	2-F-Ph	2	1	1	1	1	1	1	2	64	2	1
8	3-F-Ph	4	4	8	8	16	32	16	8	16	16	16
9	4-F-Ph	4	4	4	4	2	4	4	4	16	8	4
10	3-Cl-4-F-Ph	1	1	2	2	1	1	1	2	4	1	1
11	3,4-diCl-Ph	0.5	0.5	0.5	1	1	1	1	2	8	1	1
12	2,4- diCl-Ph	1	1	1	2	2	2	2	1	4	2	2
13	3-Cl-4-CH_3-Ph	0.5	1	0.5	1	1	1	1	0.5	4	1	0.5
14	5-Cl-2-CH_3-Ph	2	4	2	2	4	4	2	2	–	2	2
15	3-(CF_3)Ph	1	1	1	1	2	4	2	1	2	2	1
16	4-(CF_3)Ph	4	4	4	4	8	8	8	4	32	4	4
17	4-I-Ph	1	1	1	2	2	2	4	1	128	1	1
18	4-NO_2-Ph	4	4	8	4	16	16	16	8	16	8	4
19	4-CN-Ph	2	1	1	1	2	1	2	4	4	8	8
20	CH_2-Ph	8	8	8	8	8	8	8	8	–	32	8
21	CH(CH_3)-Ph	8	4	8	8	8	8	8	4	16	8	8
	Ciprofloxacin	0.25	0.5	0.25	0.5	0.25	0.25	<0.12	0.25	1	1	2

–, Lack of activity.

Table 2: Activity of compounds against hospital methicillin-resistant strains of *Staphylococcus epidermidis* (MRSE) – minimal inhibitory concentrations (MIC, μg/mL)

Comp.	*S. epidermidis* MRSE *403/11*	*S. epidermidis* MRSE *404/11*	*S. epidermidis* MRSE *405/11*	*S. epidermidis* MRSE *406/11*	*S. epidermidis* MRSE *407/11*	*S. epidermidis* MRSE *409/11*	*S. epidermidis* MRSE *411/11*	*S. epidermidis* MRSE *412/11*	*S. epidermidis* MRSE *413/11*	*S. epidermidis* MRSE *422/11*
1	16	16	16	16	16	16	16	16	16	16
2	8	8	8	8	8	8	8	8	8	8
3	4	4	4	8	8	4	4	4	4	4
4	16	16	16	16	16	16	8	16	8	16
5	4	4	4	4	4	4	4	4	4	4
6	2	2	2	2	2	2	2	2	2	2
7	16	16	16	32	16	16	16	16	16	16
8	8	8	8	8	8	8	8	16	8	8
9	8	16	8	8	8	8	8	16	8	8
10	4	4	4	4	4	4	4	4	4	4
11	1	1	1	1	1	1	1	2	1	1
12	4	2	2	2	2	2	2	2	2	4
13	1	1	1	1	1	1	1	1	1	1
14	4	4	4	4	4	4	4	4	4	4
15	2	2	2	4	2	2	2	4	2	2
16	4	4	4	4	4	4	4	4	4	4
17	1	1	1	2	1	1	1	1	1	1
18	16	16	16	32	16	16	16	32	16	16
19	16	16	16	32	16	16	16	16	16	16
20	8	8	8	8	8	8	8	8	8	8
21	8	8	8	16	8	8	8	8	8	8
***Ref.**	16	64	4	0.5	64	2	4	64	32	4

*Ref. – Ciprofloxacin.

mL), while compounds **4-7, 10, 12, 13, 15, 17, 19** showed 50–25% activity of the standard antibiotic against various *S. aureus* isolates (MIC 1 μg/mL). Further, derivatives **4, 7, 10, 11, 13, 19** showed 25% of the potency of Ciprofloxacin (MIC 2 μg/mL) against *S. epidermidis* representatives. Noticeable inhibitory effect towards tested Staphylococci was also observed for analogues **2, 3, 20, 21**. However, **5, 7, 10-15, 17, 19** were equally or more potent against *B. subtilis* than the standard compound (MIC 0.5–2 μg/mL). Derivative **13** acted four times stronger for the selected *M. luteus* isolate than Ciprofloxacin. Compounds were not active against Gram-negative rods and fungi. On the other hand, the starting 4-chloro-3-nitroaniline exerted no relevant activity against both types of staphylococcal strains, being moderately active only against *B. cereus*, *M. luteus*, *P. vulgaris* and *C. albicans* (GIZ 12–14 mm). It confirms that high biological activity of the synthesized group is the result of the appropriate substitution at thiourea nitrogen.

Next, the activity of thioureas against hospital methicillin-resistant strains of *S. epidermidis* (MRSE) was assigned. While chosen bacterial strains were not susceptible to the presence of control Ciprofloxacin, almost all new derivatives had strong influence on the bacterial growth (Table 2). For the most active compounds (**6, 11-13, 15, 17**), MIC values ranged from 1 to 2 μg/mL and were 16–64 times lower than these achieved for the reference. Only one hospital strain (*S. epidermidis* MRSE *406/11*) was more resistant to the presence of tested compounds.

The highest potency against standard Gram-positive strains was achieved with derivatives possessing 3,4-dichlorophenyl (**11**) and 3-chloro-4-methylphenyl ring (**13**) (MIC 0.5–1 μg/mL), as well as with 3-chloro-4-fluorophenyl (**10**) and 2-fluorophenyl (**7**) analogues (MIC 1 μg/mL for most of the tested strains). Next, the linear decrease in activity was observed in the direction of the following substituents at the benzene ring: 3-(trifluoro)methyl = 4-iodo > 2-bromo = 4-bromo > 2,4-dichloro = 4-cyano > 3-bromo >3-chloro = 4-chloro > 5-chloro-2-methyl > 4-fluoro. Moderate activity (MIC 4–8 μg/mL) was denoted for the group of *N*-alkylthiourea derivatives (**20, 21).** The results of compounds screening against hospital methicillin-resistant strains of *S. epidermidis* proved that disubstituted thioureas (**11-13**) with at least one chlorine atom at the C3 position at the ring, as well as 4-iodophenyl derivative (**17**), were the most effective antibacterial agents (MIC 1 μg/mL). Introducing 4-bromophenyl (**6**) or 3-(trifluoro)methylphenyl (**15**) terminal fragment at the thiourea chain also resulted in high inhibitory potency against hospital isolates (MIC 1–2 μg/mL).

The encouraging results from our antibacterial studies prompted us to investigate the title compounds for their antitubercular potency. For derivatives, **1-19** moderate to weak activity was observed (MIC 25–100 μg/mL, data not shown).

Table 3: Activity of compounds **20** and **21** against *Mycobacterium tuberculosis* strains – minimal inhibitory concentrations (MIC, μg/mL)

	MIC (μg/mL)		
Strain	20	21	*Ref.
M. tuberculosis $H_{37}R_v$ ATCC 25618	6.25	12.5	6.25
M. tuberculosis Spec. 192	6.25	12.5	6.25
M. tuberculosis Spec. 210	6.25	12.5	12.5
M. bovis	25	12.5	25
M.scrofulaceum	50	50	50
M. fortuitum	100	25	100
M. avium	>100	100	50
M. kansasii	50	100	50
M. xenopi	25	12.5	50
M. intracellulare	50	50	100

*Ref. – Isonicotinic acid hydrazide (INH).

Table 4: Affinity of selected compounds towards bacterial type II topoisomerases, expressed as IC_{50} ± SEM (μg/mL)

	*IC_{50}	
Comp.	*S. aureus* DNA Gyrase	*S. aureus* Topoisomerase IV
7	46.25 ± 2.65	19.55 ± 1.22
10	283.07 ± 8.94	18.38 ± 0.89
11	3.35 ± 0.41	50.35 ± 0.96
13	36.40 ± 1.22	59.40 ± 1.55
20	4.48 ± 0.43	24.45 ± 0.96
21	283.68 ± 6.93	39.75 ± 1.40
Ciprofloxacin	22.20 ± 1.30	3.43 ± 0.28

*Concentration (μg/mL) of tested compound required to inhibit 50% of enzyme.

The optimal activity towards *M. tuberculosis* was achieved for derivatives **20** and **21**, possessing alkylphenyl substituents at the thiourea branch (Table 3). Compound **20** was found to be equipotent or even stronger inhibitor than INH against *M. tuberculosis* $H_{37}Rv$, *M. tuberculosis* 210 and *M. tuberculosis* 192 (MIC 6.25 μg/mL). Derivative **21** possessed the same as the reference drug or 50% of its potency, depending on the type of *M. tuberculosis* isolate. It has been shown to be the most effective inhibitor against atypical *Mycobacterium* strains, possessing a significant twofold to fourfold increase in the potency against *M. bovis, M. fortuitum, M. xenopi* and *M. intracellulare*, as compared to INH. The analogue **20** was able to produce noticeable growth inhibitory effect against *M. xenopi* and *M. intracellulare*, which represents twice of the INH activity.

To exclude potential carcinogens, the most active compounds (**2-7, 10-15, 17, 19**) were tested for DNA-damaging activity by *rec*-assay. For this purpose, two genetically modified *B. subtilis* strains were used, and no significant differences were observed between diameters of the inhibition zones between them (Table S2). It led to

Table 5: Biofilm inhibitory (as IC_{50} ± SD values (μg/mL)) and biofilm eradication activity (as EC_{50} ± SD values (μg/mL)) of compounds **11** and **13** against standard and hospital methicillin-resistant strains of *S. epidermidis*

Strain	11	13	***Ref	Strain	11	13	***Ref
	*IC_{50} [μg/mL]				**EC_{50} [μg/mL]		
#*S. e.* ATCC 35984	19.31 ± 0.31	5.14 ± 0.55	1.31 ± 0.33	*S. e.* ATCC 35984	6.44 ± 0.50	7.09 ± 1.51	0.55 ± 0.48
S. e. ATCC 12228	7.53 ± 0.18	4.25 ± 0.15	1.08 ± 0.50	*S. e.* ATCC 12228	2.87 ± 0.77	5.97 ± 0.58	1.10 ± 0.68
S. e. 404/11	2.16 ± 1.16	2.07 ± 1.44	355.84 ± 1.03	*S. e.* 404/11	4.65 ± 0.62	4.97 ± 1.04	173.44 ± 0.17
S. e. 405/11	5.31 ± 0.30	1.93 ± 0.37	29.28 ± 1.29	*S. e.* 405/11	3.25 ± 1.39	4.29 ± 0.14	17.08 ± 1.46
S. e. 411/11	9.78 ± 1.30	6.15 ± 0.89	34.52 ± 1.61	*S. e.* 411/11	3.81 ± 0.46	3.80 ± 0.28	30.48 ± 0.40
S. e. 412/11	8.72 ± 0.86	6.11 ± 0.60	355.84 ± 0.51	*S. e.* 412/11	9.00 ± 0.96	4.92 ± 0.96	240.00 ± 0.73

*Concentration (μg/mL) of tested compound required to inhibit 50% of biofilm formation under the assay conditions. **Concentration (μg/mL) of tested compound required to eradicate 50% of established biofilm under the assay conditions. ***Ref. – Ciprofloxacin. #*S. e.* – *Staphylococcus epidermidis*.

the conclusion that examined thiourea derivatives are non-genotoxic and their antimicrobial potency is not an effect of their genotoxic activity.

Type II topoisomerase inhibition assay

Enzyme inhibition profiles were assessed on a panel of bacterial topoisomerases comprising DNA gyrase and topoIV isolated from *S. aureus* strains (Figures S2–S6). The aim of these studies was to verify the hypothesis that antimicrobial activity of thioureas depended on the level of their inhibitory effect on bacterial type II topoisomerases. According to the results of the *in vitro* antibacterial assay, chosen *N*-aryl derivatives **7, 10**, **11** and **13** gave the most favourable effect; on the other hand, *N*-alkylphenylthioureas **20** and **21** were the strongest inhibitors of growth of *M. tuberculosis* pathogens, being also quite potent towards Gram-positive pathogens.

The obtained results (Table 4) demonstrated that antistaphylococcal activity of studied compounds is not strictly dependent on the degree of their activity to DNA gyrase and topoIV. Even if thioureas **11** and **20** inhibited DNA gyrase stronger than the reference drug, derivative **20** has lower antibacterial activity in terms of MIC, as compared to **11** and the control compound. However, the strongest antitubercular potency of the alkylthiourea **20** could be explained just by the high level of gyrase inhibition. Its profile could be similar to Moxifloxacin, the third generation of quinolones, inhibitor of *S. aureus* and *S. pneumonia* growth that is also active against *M. tuberculosis*, which lacks topoIV (34). Derivatives **10** and **21** acted by visible topoIV inhibition, whereas thioureas **7** and **13** are dual class inhibitors.

Biofilm inhibitory and biofilm eradication studies

Preliminary antibacterial studies revealed that derivatives **11** and **13** are the most potent against free-floating planktonic cells of standard and clinical *S. epidermidis* strains; therefore, they were further studied for their ability to inhibit the formation of bacterial biofilm of the same pathogens. They were tested at doses ranging from ½ × MIC to 8 × MIC (according to Tables 1 and 2). Both compounds considerably inhibited biofilm formation of hospital strains at most of used concentrations (1–8 × MIC), in contrast to Ciprofloxacin (Fig. S3-S5, Supporting Information). Derivatives **11** and **13**, applied at 1 μg/mL (1 × MIC), in above 50% prevented from biofilm formation of *S. epidermidis* 404/11 isolate. It is also noteworthy that for this particular strain the doses of both compounds needed for 40% biofilm formation inhibition (1/2 × MIC) were lower than for planktonic cells inhibition. The concentration of compound **13** required to inhibit biofilm growth of *S. epidermidis* 405/11 strain was also definitely lower for biofilm than for free-floating cultures. Low-biofilm producers (*S. epidermidis* 411/11 and 412/11) were susceptible only to the highest concentrations of thiourea compounds (4–8 × MIC). The inhibitory potency of derivative **13** against the formation of biofilm of standard pathogens was equal to Ciprofloxacin. The ATCC 35984 isolate appeared to be the least susceptible to the presence of tested compounds, probably due to its highest biofilm-producing level.

Calculated IC_{50} values indicate that derivative **13** was the most potent among evaluated thioureas (Table 5). As compared to the standard drug, these values were from 5.6 (*S. epidermidis* 411/11) to 172 (*S. epidermidis* 404/11) times lower. Similarly, compound **11** inhibited by 50% the formation of biofilm of *S. epidermidis* 404/11 at concentration 165 times lower than standard, being also multiple times more potent for other clinical staphylococcal strains.

Next, compounds **11** and **13** were examined for their influence on mature biofilm (Figures S6–S8). The strongest activity was observed only for compound **11**, which at higher concentrations (2–8 × MIC) has eradicated biofilm in 40–80%. Its potency was similar to the reference Ciprofloxacin; however, the activity described as EC_{50} for *S. epidermidis* clinical strains was even stronger (Table 5). Although Ciprofloxacin was the strongest inhibitor of standard staphylococci growth (EC_{50} 0.55–1.10 μg/mL), compounds **11** and **13** showed higher activity against hospital isolates – up to 37–49 times higher than the reference drug.

Acknowledgments

Research subject carried out with the use of CePT infrastructure financed by the European Union – the European Regional Development Fund within the Operational Programme 'Innovative economy for 2007–2013'.

Conflict of Interest

The authors confirm that this article content has no conflict of interests.

References

1. Trovatti E., Cotrim C.A., Garrido S.S., Barros R.S., Marchetto R. (2008) Peptides based on CcdB protein as novel inhibitors of bacterial topoisomerases. Bioorg Med Chem Lett;18:6161–6164.
2. Ehmann D.E., Lahiri S.D. (2014) Novel compounds targeting bacterial DNA topoisomerase/DNA gyrase. Curr Opin Pharmacol;18:76–83.
3. Alt S., Mitchenall L.A., Maxwell A., Heide L. (2011) Inhibition of DNA gyrase and DNA topoisomerase IV of *Staphylococcus aureus* and *Escherichia coli* by aminocoumarin antibiotics. J Antimicrob Chemother; 66:2061–2069.
4. Basarab G.S., Manchester J.I., Bist S., Boriack-Sjodin P.A., Dangel B., Illingworth R., Sherer B.A., Sriram S., Uria-Nickelsen M., Eakin A.E. (2013) Fragment-to-hit-to-lead discovery of a novel pyridylurea scaffold of ATP competitive dual targeting type II topoisomerase inhibiting antibacterial agents. J Med Chem;56:8712–8735.
5. Finn J. (2013) Evaluation of WO2012177707 and WO2012097269: vertex's phosphate prodrugs of gyrase and topoisomerase antibacterial agents. Expert Opin Ther Pat;23:1233–1237.
6. Chopra S., Matsuyama K., Tran T., Malerich J.P., Wan B., Franzblau S.G., Lun S., Guo H., Maiga M.C., Bishai W.R., Madrid P.B. (2012) Evaluation of gyrase B as a drug target in *Mycobacterium tuberculosis*. J Antimicrob Chemother;67:415–421.
7. Bjarnsholt T. (2013) The role of bacterial biofilms in chronic infections. APMIS Suppl;136:1–51.
8. Blackledge M.S., Worthington R.J., Melander C. (2013) Biologically inspired strategies for combating bacterial biofilms. Curr Opin Pharmacol;13:699–706.
9. Gutiérrez D., Ruas-Madiedo P., Martínez B., Rodríguez A., García P. (2014) Effective removal of staphylococcal biofilms by the endolysin LysH5. PLoS ONE;9:e107307.
10. Rogers S.A., Huigens R.W. 3rd, Melander C. (2009) A 2-aminobenzimidazole that inhibits and disperses gram-positive biofilms through a zinc-dependent mechanism. J Am Chem Soc;131:9868–9869.
11. Anghel I., Limban C., Grumezescu A.M., Anghel A.G., Bleotu C., Chifiriuc M.C. (2012) In vitro evaluation of anti-pathogenic surface coating nanofluid, obtained by combining Fe3O4/C12 nanostructures and 2-((4-ethylphenoxy)methyl)-N-(substituted-phenylcarbamo-thioyl)-benzamides. Nanoscale Res Lett;7:513.
12. Minvielle M.J., Bunders C.A., Melander C. (2013) Indole/triazole conjugates are selective inhibitors and inducers of bacterial biofilms. Medchemcomm;4:916–919.
13. Böttcher T., Kolodkin-Gal I., Kolter R., Losick R., Clardy J. (2013) Synthesis and activity of biomimetic biofilm disruptors. J Am Chem Soc;135:2927–2930.
14. Bordi C., de Bentzmann S. (2011) Hacking into bacterial biofilms: a new therapeutic challenge. Ann Intensive Care;1:19.
15. Chan W.C., Coyle B.J., Williams P. (2004) Virulence regulation and quorum sensing in staphylococcal infections: competitive AgrC antagonists as quorum sensing inhibitors. J Med Chem;47:4633–4641.
16. Izano E.A., Amarante M.A., Kher W.B., Kaplan J.B. (2008) Differential roles of poly-N-acetylglucosamine surface polysaccharide and extracellular DNA in *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms. Appl Environ Microbiol;74:470–476.
17. Suresha G.P., Suhas R., Kapfo W., Gowda D.C. (2011) Urea/thiourea derivatives of quinazolinone-lysine conjugates: synthesis and structure-activity relationships of a new series of antimicrobials. Eur J Med Chem;46:2530–2540.
18. Chikhalia K.H., Patel M.J. (2009) Design, synthesis and evaluation of some 1,3,5-triazinyl urea and thiourea derivatives as antimicrobial agents. J Enzyme Inhib Med Chem;24:960–966.
19. Sriram D., Yogeeswari P., Dinakaran M., Thirumurugan R. (2007) Antimycobacterial activity of novel 1-(5-cyclobutyl-1,3-oxazol-2-yl)-3-(sub)phenyl/pyridylthiourea compounds endowed with high activity toward multidrug-resistant *Mycobacterium tuberculosis*. J Antimicrob Chemother;59:1194–1196.
20. Bhowruth V., Brown A.K., Reynolds R.C., Coxon G.D., Mackay S.P., Minnikin D.E., Besra G.S. (2006) Symmetrical and unsymmetrical analogues of isoxyl; active agents against *Mycobacterium tuberculosis*. Bioorg Med Chem Lett;16:4743–4747.
21. Karakuş S., Güniz Küçükgüzel S., Küçükgüzel I., De Clercq E., Pannecouque C., Andrei G., Snoeck R., Sahin F., Bayrak O.F. (2009) Synthesis, antiviral and anticancer activity of some novel thioureas derived from N-(4-nitro-2-phenoxyphenyl)-methanesulfonamide. Eur J Med Chem;44:3591–3595.
22. Verlinden B.K., de Beer M., Pachaiyappan B., Besaans E., Andayi W.A., Reader J., Niemand J., van Biljon R., Guy K., Egan T., Woster P.M., Birkholtz L.M. (2015) Interrogating alkyl and arylalkylpolyamino (bis)urea and (bis)thiourea isosteres as potent antimalarial chemotypes against multiple lifecycle forms of *Plasmodium falciparum* parasites. Bioorg Med Chem;23:5131–5143.
23. Zhao F., Xiao J.H., Wang Y., Li S. (2009) Synthesis of thiourea derivatives as CCR4 antagonists. Chin Chem Lett;20:296–299.

24. Bielenica A., Kedzierska E., Fidecka S., Maluszynska H., Miroslaw B., Koziol A.E., Stefanska J., Madeddu S., Giliberti G., Sanna G., Struga M. (2015) Synthesis, antimicrobial and pharmacological evaluation of thiourea derivatives of 4H-1,2,4-triazole. Lett Drug Des Discov;12:263–276.
25. Bielenica A., Stefańska J., Stępień K., Napiórkowska A., Augustynowicz-Kopeć E., Sanna G., Madeddu S., Boi S., Giliberti G., Wrzosek M., Struga M. (2015) Synthesis, cytotoxicity and antimicrobial activity of thiourea derivatives incorporating 3-(trifluoromethyl)phenyl moiety. Eur J Med Chem;101:111–125.
26. Clinical and Laboratory Standards Institute. (2012) Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically, Approved Standard M7-A9, CLSI, Pennsylvania, USA.
27. Clinical and Laboratory Standards Institute. (1999) Methods for determining bactericidal activity of antimicrobial agents, approved guideline M26-A. CLSI, Wayne, Pennsylvania, USA.
28. Clinical and Laboratory Standards Institute. (2006) Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically, Approved Standard M7-A7, CLSI, Pennsylvania, USA.
29. Clinical and Laboratory Standards Institute. (2008) Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts, Approved Standard. CLSI document M27-A3. CLSI, Pennsylvania, USA.
30. Youmans G.P. (1947) Test tube evaluation of tuberculostatic agents. Am Rev Tuberc;56:376.
31. Youmans G.P., Youmans A.S. (1949) A method for the determination of the rate of growth of tubercle bacilli by the use of small inocula. J Bacteriol;58:247–255.
32. Kada T., Hirano K., Shirasu Y. (1980) Bacillus subtilis rec-assay test. In: de Sevres F.E., Hollaender A., editors. Chemical Mutagens, vol 6. New York: Plenum Press; p. 149.
33. Sadaie Y., Kada T. (1976) Recombination-deficient mutants of *Bacillus subtilis*. J Bacteriol;125:489–500.
34. Mdluli K., Ma Z. (2007) Mycobacterium tuberculosis DNA gyrase as a target for drug discovery. Infect Disord Drug Targets;7:159–168.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Structures and atom numbering of thiourea derivatives **1-21.**

Table S2. Mutagenic activity of tested compounds in *Bacillus subtilis* rec-assay, expressed as diameter of growth inhibitory zone (GIZ, mm).

Figure S1. The influence of tested compounds on S. aureus gyrase DNA activity. Decreasing amounts of **11** or **20** were incubated with 200 ng kDNA and run on agarose gel. Lane 1: Control with 2% DMSO Lane 2: negative control without enzyme, containing diluted buffer. Lane 3-8: compound **11** (at concentration 32, 8, 4, 2, 1, 0.5 μg/mL, respectively). Lane 9-14: compound **20** (at concentration 32, 8, 4, 2, 1, 0.5 μg/mL, respectively).

Figure S2. The influence of tested compounds on *S. aureus* gyrase DNA activity. Decreasing amounts of derivatives **21** or **Ciprofloxacin** were incubated with 500 ng pBR322 plasmid DNA and run on agarose gel. Lane 1: negative control without enzyme, containing diluted buffer. Lane 2: Control with 2% DMSO. Lane 3-9: compound **21** (at concentration 32, 8, 4, 2, 1, 0.5, 0.25 μg/mL, respectively). Lane 10-16: Ciprofloxacin (at concentration 32, 8, 4, 2, 1, 0.5, 0.25 μg/mL, respectively).

Figure S3. The influence of tested compounds on *S. aureus* gyrase DNA activity. Decreasing amounts of derivatives **7, 10** and **13** were incubated with 500 ng pBR322 plasmid DNA and run on agarose gel. Lane 1: negative control without enzyme, containing diluted buffer. Lane 2: Control with 2% DMSO. Lane 3-7: compound **10** (at concentration 32, 8, 2, 1, 0.5 μg/mL, respectively). Lane 8-12: compound **7** (at concentration 32, 8, 2, 1, 0.5 μg/mL, respectively). Lane 13-17: compound **13** (at concentration 32, 8, 2, 1, 0.5 μg/mL, respectively).

Figure S4. The effect of tested compounds on relaxation of *S. aureus* topoisomerase IV. Decreasing amounts of **11** or **20** were incubated with 200 ng kDNA and run on agarose gel. Lane 1: Control with 2% DMSO Lane 2: negative control without enzyme, containing diluted buffer. Lane 3-8: compound **11** (at concentration 32, 8, 4, 2, 1, 0.5 μg/mL, respectively). Lane 9-14: compound **20** (at concentration 32, 8, 4, 2, 1, 0.5 μg/mL, respectively).

Figure S5. The effect of tested compounds on relaxation of *S. aureus* topoisomerase IV. Decreasing amounts of **21** or **Ciprofloxacin** were incubated with 200 ng kDNA and run on agarose gel. Lane 1: Control with 2% DMSO. Lane 2: negative control without enzyme, containing diluted buffer. Lane 3-9: compound **21** (at concentration 32, 8,4, 2, 1, 0.5, 0.25 μg/mL, respectively). Lane 9-16: Ciprofloxacin (at concentration 32, 8, 4, 2, 1, 0.5, 0.25 μg/mL, respectively).

Figure S6. The effect of tested compounds on relaxation of *S. aureus* topoisomerase IV. Decreasing amounts of **7, 10** and **13** were incubated with 200 ng kDNA and run on agarose gel. Lane 1: Control with 2% DMSO. Lane 2: negative control without enzyme, containing diluted buffer. Lane 3-8: compound **10** (at concentration 32, 8, 4, 2, 1, 0.5 μg/mL, respectively). Lane 9-14: compound **7** (at concentration 32, 8, 4, 2, 1, 0.5 μg/mL, respectively). Lane 15-20: compound **13** (at concentration 32, 8, 4, 2, 1, 0.5 μg/mL, respectively).

Figure S7. Biofilm inhibitory activity of compound **11** –

the percentage of *S. epidermidis* biofilm survival in the presence of the tested compound. (compared to drug-free control).

Figure S8. Biofilm inhibitory activity of compound **13** – the percentage of *S. epidermidis* biofilm survival in the presence of the tested compound. (compared to drug-free control).

Figure S9. Biofilm inhibitory activity of Ciprofloxacin – the percentage of *S. epidermidis* biofilm survival in its presence.

Figure S10. Percentage survival of *S. epidermidis* biofilm after 24 h growth and after 24 h treatment with compound

Figure S11. Percentage survival of *S. epidermidis* biofilm after 24 h growth and after 24 h treatment with compound **13.**

Figure S12. Percentage survival of *S. epidermidis* biofilm after 24 h growth and after 24 h treatment with Ciprofloxacin**.**