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ORIGINAL PAPER

Antistaphylococcal Activity of Selected Thiourea Derivatives

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Abstract

Five of thiourea derivatives were prepared using as a starting compound 3-(trifluoromethyl)aniline, 4-chloro-3-nitroaniline, 1,3-thiazol-2-amine, 2*H*-1,2,3-triazol-4-amine and commercial isothiocyanates. All compounds were evaluated *in vitro* for antimicrobial activity. Derivatives 2 and 3 showed the highest inhibition against Gram-positive cocci (standard and hospital strains). The observed MIC values were in the range of 0.5–8 μg/ml. The products effectively inhibited the formation of biofilms of methicillin-resistant and standard strains of *Staphylococcus epidermidis*. Inhibitory activity of thioureas 2 and 3 against *Staphylococcus aureus* topoisomerase IV was studied. The examined compounds were nongenotoxic.

Key words: antistaphylococcal activity, anti-biofilm activity, genotoxicity, thiourea derivatives

Introduction

Staphylococci belong to the generally present, most important biofilm-formed pathogens and are responsible for a large number of serious nosocomial infections acquired after surgery or hospital (Leclercq, 2009; Agarwal *et al*., 2010). Methicillin resistant strains (both, coagulase-positive and coagulase-negative) often show resistance to many other antibiotics and chemotherapeutics, such as fluoroquinolones, macrolides, tetracyclines or glicopeptides. *Staphylococcus aureus* produces numerous virulence factors, *e.g.* hemolysin, exotoxin, enzymes (hyaluronidase, lipase, nuclease), surface proteins (that promote attachment to host proteins such as laminin and fibronectin) or enterotoxins (responsible for food poisoning) (Heczko *et al*., 2014). This bacterium can generate various types of infections, such as severe skin, subcutaneous tissue and bone infections, scalded skin syndrome, pneumonia, endocarditis, as well as can even cause severe sepsis (Chambers and DeLeo, 2009). Another, commonly present *Staphylococcus* species – *Staphylococcus epidermidis* – has the ability to produce extracellular mucous and is able to adhere to a variety of surfaces, *e.g.* joint implants, vascular lines or artificial heart valves (Arciola *et al*., 2005; Mack *et al*., 2006). Colonization of such biomaterials or medical devices can generate difficult-to-combat local and systemic infections (Otto, 2008; 2009).

Bacterial biofilm constitutes a complex multidimensional structure formed by cells conglomerated with one another and with the base. It is composed of extracellular mucous produced by bacteria, proteins, polysaccharides, nucleic acids and water (Costerton *et al*., 1999). Biofilm can be formed by microorganisms belonging to one or different species and can develop on abiotic surfaces or on living tissues (Costerton *et al*., 1999; Donlan and Costerton, 2002).

The cells of micro-organisms living in the biofilm are, in comparison to planktonic forms, more resistant to antibiotics and chemotherapeutics used in pharmacotherapy (Høiby *et al*., 2010) as well as antiseptics or disinfectant (Bridier *et al*., 2011). This causes numerous therapeutic problems, especially in combating chronic infections. The formation of the biofilm by bacteria constitutes also an essential clinical problem linked to infections associated with medical devices, *e.g.* bone implants, catheters or vascular lines (Donlan, 2001; Maki *et al*., 2006).

Effective methods of fight down bacterial biofilm are still missing. It is extremely important to seek new

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compounds having antibacterial activity which would be effective in combating biofilm. They could be used, for instance, to cover surfaces of biomaterials or protective clothing, to prevent micro-organisms from settling on them and thus making it impossible for the difficult-to-eradicate biofilm to form (Meng *et al*., 2013).

The thiourea fragment represents an important synthon which is responsible for numerous biological activities, such as antimicrobial (Struga *et al*., 2010; Vega-Pérez *et al*., 2012), antiviral (Ranise *et al*., 2003), anticancer (Saeed *et al*., 2010), cytotoxic (Vega-Pérez *et al*., 2012) and anti-inflammatory (Keche *et al*., 2012) properties. In many cases, antistaphylococcal potency of this class of compounds is the result of the type II topoisomerase inhibition, that includes topoisomerase IV and DNA gyrase (Basarab *et al*., 2013; Bielenica *et al*., 2015).

As a part of our research program of rapidly assemble novel bioactive compound we have synthesized five disubstituted thioureas. The compounds with various substituents at the thiourea moiety (aryl, heteroaryl and benzyl) were evaluated for their antimicrobial, as well as investigated as potential inhibitors on biofilm formation of Gram-positive pathogens. The mechanism of their action through topoisomerase IV inhibition was proved.

Experimental

Materials and Methods

Chemistry. 3-(Trifluoromethyl)aniline, 1,3-thiazol-2-amine, 2*H*-1,2,3-triazol-4-amine, 4-chloro-3-nitroaniline were supplied from Alfa Aesar. Isothiocyanates were purchased from Alfa Aesar or Sigma Aldrich. Acetonitrile, chloroform and methanol were supplied from POCh (Polskie Odczynniki Chemiczne SA). All chemicals were of analytical grade and were used without any further purification. Before using, dry acetonitrile was kept in crown cap bottles over anhydrous phosphorus pentoxide (Carl Roth). The IR spectra were obtained on Perkin Elmer Spectrum 1000 spectrometer in KBr pellets. The NMR spectra were recorded on Varian VNMRS 300 Oxford NMR spectrometer, operating at 300 MHz (^{1}H NMR, relax. delay 1.000 sec, pulse 30.0 degrees) and 75.4 MHz (^{13}C NMR, relax. delay 3.700 sec, pulse 45.0 degrees, Waltz-16 modulated). Chemical shifts (δ) were expressed in parts per million relative to tetramethylsilane used as the internal reference. Mass spectral ESI measurements were carried out on Waters ZQ Micro-mass instruments with quadruple mass analyzer. The spectra were performed in the negative ion mode at a declustering potential of 40–60 V. The sample was previously separated on a UPLC column (C18) using UPLC ACQUITYTM system by Waters connected with DPA detector. Flash chromatography was performed on Merck silica gel 60 (200–400 mesh) using chloroform eluent. Analytical TLC was carried out on silica gel F254 (Merck) plates (0.25 mm thickness).

Synthesis of thiourea derivatives (1–5). A solution of amine derivative (3-(Trifluoromethyl)aniline, 4-chloro-3-nitroaniline, 1,3-thiazol-2-amine, 2*H*-1,2,3-triazol-4-amine) (0.01 mol) in acetonitrile (25 ml) was treated with appropriate isothiocyanate (0.011 mol) and the mixture was refluxed for 8 h. Then solvent was removed on rotary evaporator. The residue was purified by column chromatography (chloroform: methanol; 9.5:0.5 vol.). The final product was crystallized from acetonitrile.

1-(1-phenylethyl)-3-[3-(trifluoromethyl)phenyl] thiourea (1). Yield 75%, white powder, m.p. 113–115°C. ^{1}H NMR (300 MHz, DMSO) *δ*: 9.66 (s, 1H, NH), 8.42 (d, J = 7.8 Hz, 1H, H_{arom}), 8.03 (s, 1H, NH), 7.66 (d, J = 8.4 Hz, 1H, H_{arom}), 7.51 (t, 1H, J = 7.8 Hz, H_{arom}), 7.41–7.33 (m, 5H, H_{arom}), 7.30–7.23 (m, 1H, H_{arom}), 5.55–5.51 (m, 1H, CH), 1.48 (d, J = 6.9 Hz, 3H, CH_3). ^{13}C NMR (75.4 MHz, DMSO) *δ*: 179.80, 143.60, 140.58, 129.50, 128.94 (q), 128.34 (2C), 126.88, 126.24 (2C), 126.05, 124.08 (q), 119.91, 118.59, 52.60, 21.83. HRMS (ESI) calc. for $C_{16}H_{14}F_3N_2S$ [M – H]$^-$: 323.0830, found: 323.0834.

1-(3,4-dichlorophenyl)-3-[3-(trifluoromethyl) phenyl]thiourea (2) has been synthesized as described previously (Bielenica *et al*., 2015).

1,3-bis(4-chloro-3-nitrophenyl)thiourea (3). Yield 40%, yellow powder, m.p. 176–178°C. ^{1}H NMR (300 MHz, DMSO) δ: 10.47 (s, 2H, NH), 8.31 (d, 2H, J = 2.4 Hz, H_{arom}), 7.79 (dd, 2H, $J_1 = J_2$ = 2.4 Hz, H_{arom}), 7.74 (d, 2H, J = 8.4 Hz, H_{arom}).^{13}C NMR (75.4 MHz, DMSO) δ: 180.03, 147.02 (2C), 139.16 (2C), 131.50 (2C), 128.73 (2C), 120.16 (2C), 120.09 (2C). HRMS (ESI) calc. for $C_{13}H_8Cl_2N_4S$ [M – H]$^-$: 385.6756 found: 385.6759.

1-(2,3-dichlorophenyl)-3-(1,3-thiazol-2-yl)thiourea (4). Yield 68 %, white powder, m.p. 167–169°C. ^{1}H NMR (300 MHz, DMSO) δ: 12.5 (s, 1H, NH), 10.21 (s, 1H, NH), 7.75 (dd, 1H, $J_1 = J_2$ = 1.5 Hz, H_{arom}), 7.52 (dd, 1H, $J_1 = J_2$ = 1.5 Hz, H_{arom}), 7.44 (d, 1H, J = 4.2 Hz, H_{arom}), 7.37(d, 1H, J = 3.6 Hz, H_{arom}), 7.06 (t, 1H, J = 8.1 Hz, H_{arom}). ^{13}C NMR (75.4 MHz, DMSO) δ: 180.03, 159.17, 138.31, 137.57, 137.15, 131.77, 128.32, 124.46, 119.82, 112.90. HRMS (ESI) calc. for $C_{10}H_7Cl_2N_3S_2$ [M – H]$^-$: calc. 303.9390 found: 303.9399.

1-(4-chloro-3-nitrophenyl)-3-(1*H*-1,2,4-triazol-3-yl)thiourea (5). Yield 68 %, white powder, m.p. 234–236°C. ^{1}H NMR (300 MHz, DMSO) δ: 14.05 (s, 1H, NH), 11.96 (s, 1H, NH), 11.50 (s, 1H, NH), 8.57 (s, 1H, CH=); 8.11(d, 1H, J = 2.4 Hz, H_{arom}), 7.92 (dd, 1H, $J_1 = J_2$ = 2.7 Hz, H_{arom}), 7.75 (d, 1H, J = 8.7 Hz, H_{arom}). ^{13}C NMR (75.4 MHz, DMSO) δ: 177.58, 156.81, 148.92,

146.91, 142.93, 138.87, 131.41, 129.30, 120.66. HRMS (ESI) calc. for $C_9H_7ClN_6O_2S$ $[M-H]^-$: calc. 296.9969 found: 296.9967.

Biological evaluation

Antimicrobial studies. Antimicrobial activities of the thiourea derivatives were tested *in vitro* against six reference Gram-positive strains: *S. aureus*: NCTC 4163, ATCC 25923, ATCC 6538, ATCC 29213, *S. epidermidis*: ATCC 12228, ATCC 35984, and against a series 16 of clinical methicillin-resistant *S. aureus* (MRSA) and *S. epidermidis* (MRSE) strains. Microorganisms were obtained from the collection of Department of Pharmaceutical Microbiology, Medical University of Warsaw, Poland.

Minimal Inhibitory Concentration (MIC) of tested compounds were examined by the twofold serial broth (Mueller-Hinton Broth medium, Becton Dickinson) dilution methods using 96-well microtitre plates (Medlab Products) according to CLSI guidelines (CLSI, 2012). Concentrations of tested compounds in liquid medium ranged from 0.0625 to 256 μg/ml. The final inoculum of all strains studied was about 10^5 cfu/ml. MIC values were read after 18 h incubation at 35°C. Minimal bactericidal concentration of the compounds (MBC – 99.9% killing of the final inoculums) were determined by subculturing 10 μl of suspension from each negative well (no visible bacterial growth) from the MIC test, onto TSA plates and incubated at 37°C for 24 h (CLSI, 1999). Ciprofloxacin was used as the reference antibacterial drug.

Biofilm inhibitory assay. Three thiourea derivatives (2, 3, 5) and Ciprofloxacin (as reference antibacterial drug) were studied for their ability to inhibit the formation of staphylococcal biofilm. Selected methicillin-resistant clinical strains of *Staphylococcus* (four *S. aureus* and four *S. epidermidis*) and two reference *S. epidermidis* strains were used in this assay. The clinical strains were isolated from blood of hospitalized patients. *S. epidermidis* ATCC 35984 was used as high biofilm-producer (positive control), *S. epidermidis* ATCC 12228 was used as low-biofilm producer (negative control). Ciprofloxacin was the reference antibacterial agent.

Inhibition of bacterial biofilm formation was screened using the method, described previously (Stefańska *et al.*, 2015). All strains were cultured overnight in Tryptic Soy Broth medium supplemented with 0.5% glucose (BTL, Poland). The solution of tested compounds in above medium was mixed (1:1) with the bacterial inoculums (10^7 cfu/ml) in sterile 96-well polystyrene microtiter plates (Karell-Medlab, Italy) and incubated at 37°C for 24 h. The final concentrations of tested compounds ranged from 1 to 16 μg/ml.

The positive control (biofilm formation) was bacterial culture in TSB-glucose; a negative control was the medium devoid of the bacteria. After incubation, medium was removed from wells and washed twice with sterile PBS buffer (phosphate-buffered saline) to take out the non-adherent bacteria. Adherent bacterial cells, which usually formed biofilm on wells surface, were uniformly stained with 3-(4,5-dimethyl-2-thiazolyl)-2,5 diphenyl-2H-tetrazolium bromide (MTT; 0.5% in PBS) and incubated for 2 hours at 37°C. After incubation, the solution was removed and bacterial biofilm was solubilized by DMSO (Merck) with glycine buffer (pH 10.2) and mixed 15 minutes at room temperature. The solution absorbance (A_{554}) was measured at 554 nm using a spectrophotometer PowerWave XS (BioTek).

The biofilm-inhibition results were interpreted from dose (concentrations) – response graphs.

Due to the transparency image on the graphs shows the results for selected four clinical and two reference strains. Results in the graphs are averages of four repetitions ± the standard error of the mean. The standard deviation values were very low, is they are sometimes invisible in the figures.

Genotoxicity. DNA-damaging activity of salinomycin and its derivatives was tested by *rec*-assay using two genetically modified *Bacillus subtilis* strains: M45 (rec^-) and H17 (rec^+) (Saide and Kada, 1976; Kada *et al.*, 1980). *B. subtilis* M45 is devoid of the recombinant – based DNA repair mechanism and is much more susceptible to genotoxic substances compared to *B. subtilis* H17 strain. Tested compounds were dissolved in DMSO and 10 μl of each solution were dripped onto sterile filter paper discs (Whatman No 3MM) to load 256 μg of a given compound per 9 mm disc. Discs were placed on the surface of Nutrient agar plates (BTL, Poland) inoculated with 100 μl of bacterial culture overnight and incubated for 24 h at 35°C. After incubation the growth inhibition zones were measured. NOQ (4-nitroquinoline N-oxide) was used as the reference genotoxin in concentration 2 μg per disc.

Results of the genotoxicity test were read after 18 h of incubation at 35°C by comparing the diameter of the inhibition zone for the *B. subtilis* M45 (rec^-) strain with that observed for the *B. subtilis* H17 (rec^+) strain.

Inhibition of bacterial topoisomerase IV

Decatenation assay. The assay was performed using *S. aureus* topoisomerase IV decantation kit (Inspiralis). Kinetoplast DNA (kDNA) was the substrate for topoisomerase IV. 1 U of topoisomerase IV 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 and in the presence of different concentrations of the test compounds. The reactions were terminated by adding 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 1.5 h at 80 V. Gels were stained with ethidium bromide and visualized under UV light in a transilluminator (ChemiDoc MP, Bio Rad).

Results

The preparation of 5 thiourea derivatives was accomplished according the reactions described in Scheme 1.

The first step was to determine the antibacterial activity of tested thiourea derivatives against standard and clinical methicillin-resistant *S. aureus* (MRSA) and *S. epidermidis* strains (MRSE). Moreover derivatives were tested for their genotoxicity, ability to inhibition biofilm formation by various clinical *Staphylococcus* strains and activity of selected compounds against bacterial topoisomerase.

Antibacterial activities of tested compounds were expressed by minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC). The results were listed in Tables I–II. Two derivatives, 2 and 3 showed very potent anti-staphylococcal activity. For the compound 2 MIC values were from 0.5 to 1.0 µg/ml and MBC values ranged from 4 to 8 µg/ml. For compound 3 MIC values ranged from 2 µg/ml to 8 µg/ml and MBC values was 256 µg/ml and above. For the reference antibacterial agent – Ciprofloxacin MIC values ranged from 0.125 µg/ml to 0.5 µg/ml and MBC values ranged from 0.5 µg/ml to 2 µg/ml for standard *Staphylococcus* strains. For clinical methicillin-resistant isolates Ciprofloxacin MIC values ranged from 0.5 µg/ml to 64 µg/ml and MBC values ranged from 4 µg/ml to above 256 µg/ml.

Only for the compound 2 the bactericidal activity was observed at a low concentration (4–8 µg/ml). Compounds 1 and 3 showed only bacteriostatic effect (MBC values much higher as compared to the MIC values). Compound 4 and 5 showed weak activity against tested staphylococcal strains.

Sometimes antimicrobial activity of various compounds is directly connected with their genotoxicity. This effect is not profitable to microbiologically active compounds. Genotoxicity of thiourea derivatives was tested by *rec*-assay using two genetically modified *Bacillus subtilis* strains: H17 (rec^+) and M45 (rec^-). *B. subtilis* M45 is more susceptible to genotoxic substances (for example NOQ) compared to *B. subtilis* H17 strain. As shown in Table III there was no significant difference in the diameter of the inhibition zones for both bacterial strains. Examined compounds are nongenotoxic *in vitro* for the tested bacterial cells.

Three of obtained compounds were tested for their ability to inhibit the staphylococcal biofilm formation. Four selected clinical isolates (four *S. aureus* and four *S. epidermidis* isolated from blood of hospitalized patients) and two standard *S. epidermidis* strains (positive and negative control) were used in this study. The biofilm inhibitory activity of derivatives 2 and 3

Table I
In vitro activity of tested compounds against planktonic cells of standard and clinical methicillin-resistant *S. aureus* strains.

No. *S. aureus*	**1** MIC (MBC)	**2** MIC (MBC)	**3** MIC (MBC)	**4** MIC (MBC)	**5** MIC (MBC)	Ref* MIC (MBC)
NCTC 4163	16 (>256)	0.5 (4)	4 (256)	64 (>256)	64 (>256)	0.25 (0.5)
ATCC 25923	16 (>256)	0.5 (4)	8 (>256)	64 (>256)	64 (>256)	0.5 (2)
ATCC 6538	16 (>256)	0.5 (4)	8 (>256)	32 (>256)	64 (>256)	0.25 (1)
ATCC 29212	16 (>256)	0.5 (4)	4 (256)	64 (>256)	128 (>256)	0.5 (2)
452/11	32 (>256)	0.5 (4)	2 (256)	64 (>256)	128 (>256)	32 (>256)
462/11	16 (>256)	1 (8)	2 (256)	64 (>256)	256 (>256)	64 (>256)
514/12	16 (>256)	1 (8)	8 (>256)	64 (>256)	64 (>256)	32 (256)
522/12	16 (>256)	1 (8)	4 (>256)	32 (>256)	64 (>256)	64 (>256)
572/12	32 (>256)	0.5 (4)	8 (>256)	32 (>256)	256 (>256)	64 (>256)
573/12	16 (>256)	1 (8)	2 (256)	64 (>256)	128 (>256)	32 (256)
585/12	16 (>256)	0.5 (4)	4 (>256)	32 (>256)	128 (>256)	64 (>256)
586/12	16 (>256)	0.5 (4)	2 (256)	64 (>256)	256 (>256)	64 (>256)

Ref* – Ciprofloxacin (reference antimicrobial drug), MIC – minimal inhibitory concentration (µg/ml), MBC – minimal bactericidal concentration (µg/ml).

Table II
In vitro activity of tested compounds against planktonic cells of standard and clinical methicillin-resistant *S. epidermidis* strains.

No. *S. epidermidis*	**1** MIC (MBC)	**1** MIC (MBC)	**1** MIC (MBC)	**1** MIC (MBC)	**1** MIC (MBC)	Ref.* MIC (MBC)
ATCC 12228	32 (>256)	1 (8)	4 (256)	64 (>256)	128 (>256)	0.25 (1)
ATCC 35984	32 (>256)	1 (8)	4 (256)	32 (256)	64 (>256)	0.125 (0.5)
409/11	16 (>256)	1 (8)	2 (>256)	32 (>256)	128 (>256)	2 (32)
455/11	16 (>256)	0.5 (4)	2 (>256)	64 (>256)	128 (>256)	32 (256)
459/11	16 (>256)	0.5 (4)	4 (>256)	32 (>256)	64 (>256)	32 (256)
469/11	16 (>256)	0.5 (4)	4 (>256)	64 (>256)	128 (>256)	8 (64)
517/12	16 (>256)	1 (8)	2 (>256)	32 (>256)	128 (>256)	32 (256)
519/12	16 (>256)	1 (8)	2 (>256)	32 (>256)	64 (>256)	0.5 (4)
526/12	32 (>256)	0.5 (4)	4 (>256)	64 (>256)	128 (>256)	4 (32)
528/12	16 (>256)	1 (8)	4 (>256)	64 (>256)	128 (>256)	32 (256)

Ref* – Ciprofloxacin (reference antimicrobial drug), MIC – minimal inhibitory concentration (μg/ml), MBC – minimal bactericidal concentration (μg/ml).

Table III
Genotoxicity of tested compounds in *rec-assay.*
Concentration of tested compounds – 256 μg per 9 mm disc.
*NOQ (4-nitroquinoline N-oxide) – reference genotoxic agent, 2 μg per 9 mm disc

Tested compound	Diameter of growth inhibition zones (mm)	
	Bacillus subtilis H17 (rec^+)	*Bacillus subtilis* M45 (rec^-)
1	12	12
2	na	na
3	14	13
4	na	na
5	na	na
NOQ*	12	24

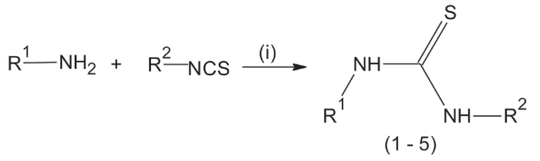
na – no growth inhibition zone

were higher than the reference agent – Ciprofloxacin (Fig. 1–4). The more active compound 2 inhibited biofilm formation in the range from 40% to above 90% in concentration 1 μg/ml (MIC for planktonic cells) (Fig. 1). The same concentration of compound 3 caused inhibition of bacterial biofilm formation in 30% to 50% by 5 of 10 tested strains (Fig. 2).

Biofilm inhibitory activity of tested thiourea derivatives 2 and 3 is the result of inhibiting of multiplication of bacterial cells, in consequence they prevent from biofilm formation and reduce its amount. In the presence of the compound 2 the lower amount of staphylococcal biofilm was produced and it was more susceptible to the mechanical damage and removal of the staining process, as shown in the Figure 5.

The last step was the investigation of mechanism of the antimicrobial activity. Considering the results in the *in vitro* antibacterial assay, we have investigated the inhibitory effect on topoisomerase IV of compounds 2 and 3, showed highest therapeutic potential against strains of *S. aureus* and *S. epidermidis*, including clinically relevant resistant isolates. Compounds 2 and 3 were used in wide range of concentrations (32 μg/ml, 8 μg/ml, 2 μg/ml, 0.5 μg/ml, 0.25 μg/ml).

The affinity of the selected compounds towards bacterial type II topoisomerases, such as topoisomerase IV from *S. aureus* was analysed. The results obtained



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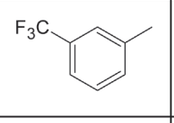
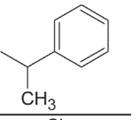
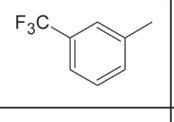
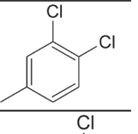
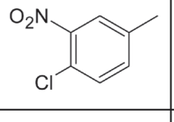
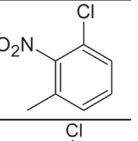
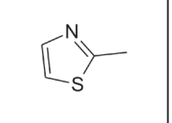
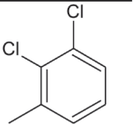
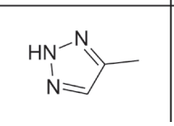
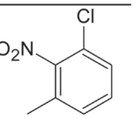
1		
2		
3		
4		
5		

Scheme 1. Synthesis and structure of tested compounds. Reagents and conditions: (i) MeCN, reflux 8 h.

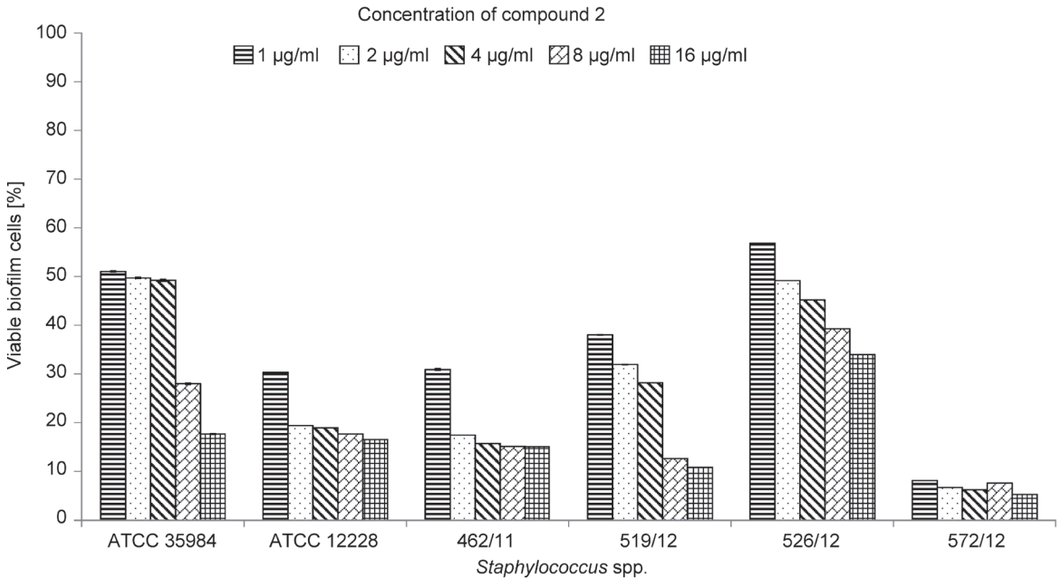


Fig. 1. Inhibitory effect of compound 2 for biofilm formation by standard and selected hospital methicillin-resistant *Staphylococcus* spp. strains.
All presented results are mean from experiments performed in quadruplicate ± S.D.

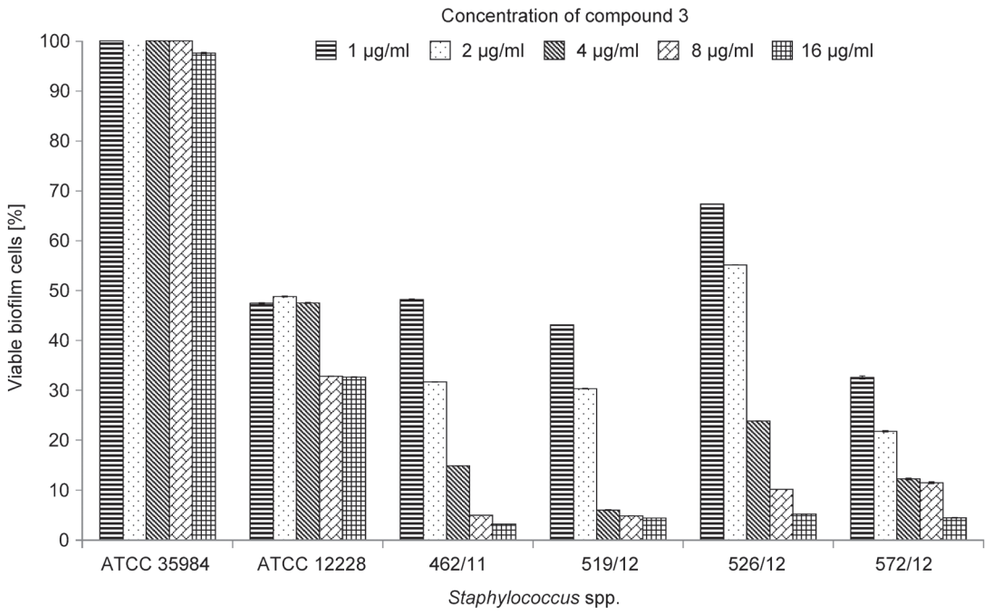


Fig. 2. Inhibitory effect of compound 3 for biofilm formation by standard and selected hospital methicillin-resistant *Staphylococcus* spp. strains.
All presented results are mean from experiments performed in quadruplicate ± S.D.

demonstrated that synthesized compound 2 applied at concentration 32 µg/ml was equally active as Ciprofloxacin, however the compound 3 did not show inhibitory potency towards *S. aureus* topoisomerase IV (Fig. 6). For 3 the inhibition of bacterial type II topoisomerases in not the sole factor responsible for the antibacterial activity.

Discussion

When the effect of the substituent at thiourea nitrogen (N1) was evaluated, it was found that the functionalities could be valued in order of their decreasing influence as follows: 3-trifluoromethylphenyl > 4-chloro-3-nitrophenyl > 1,3-thiazol > 2*H*-1,2,3-tria-

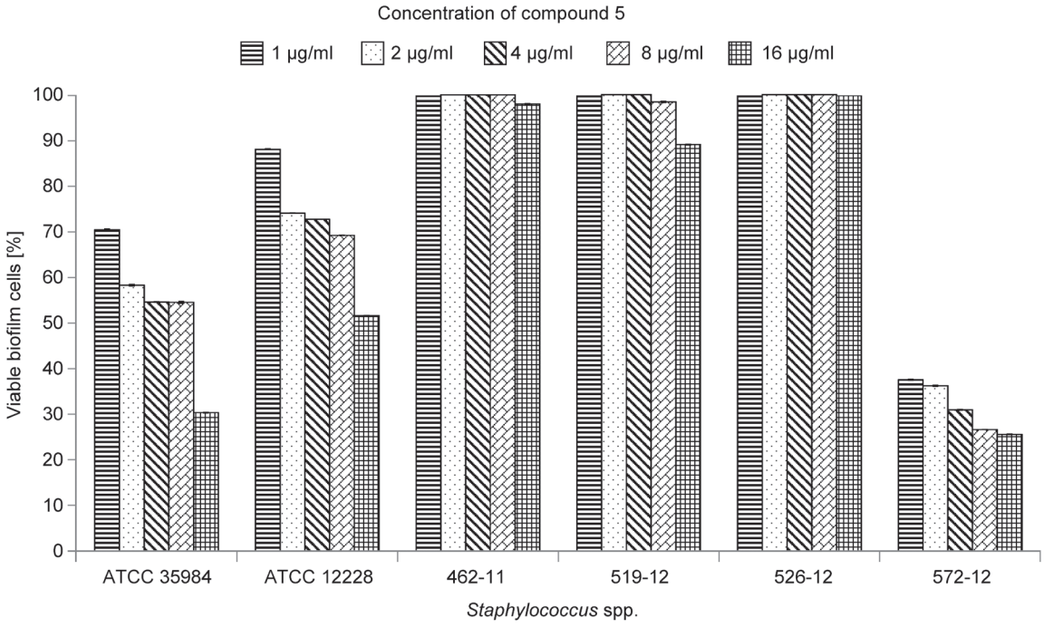


Fig. 3. Inhibitory effect of compound 5 for biofilm formation by standard and selected hospital methicillin-resistant *Staphylococcus* spp. strains.
All presented results are mean from experiments performed in quadruplicate ± S.D.

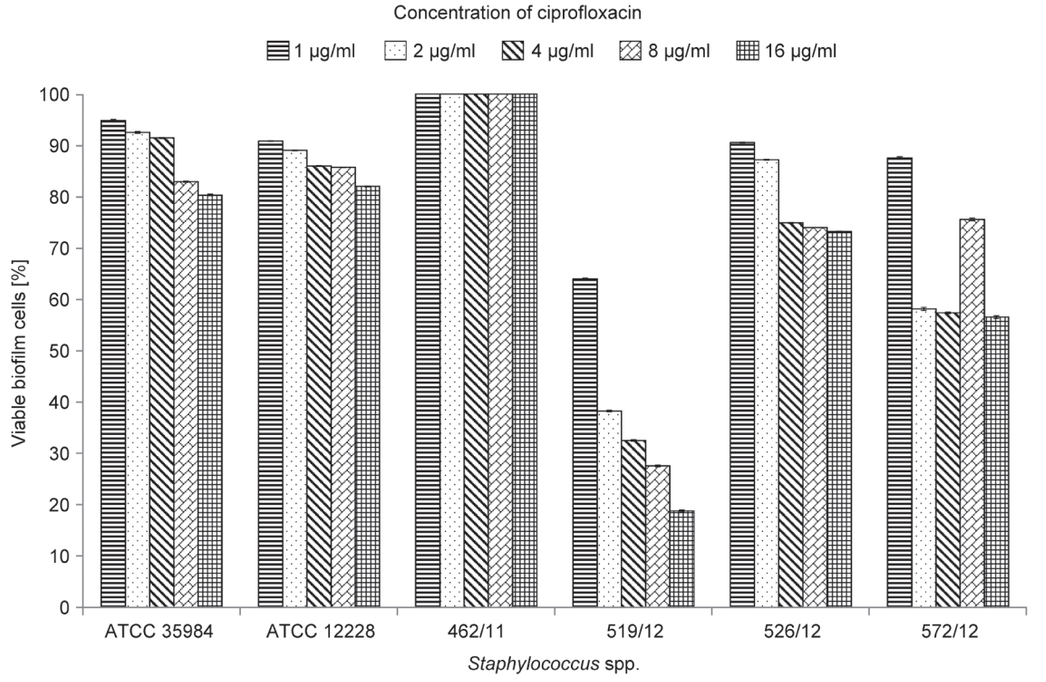


Fig. 4. Inhibitory effect of ciprofloxacin for biofilm formation by standard and selected hospital methicillin-resistant *Staphylococcus* spp. strains.
All presented results are mean from experiments performed in quadruplicate ± S.D.

zol. Interesting was activity comparison of activity of compound 1 and 2 because only substituent connected to N2 nitrogen atom was changed. Antimicrobial activity decreased when between thiourea and aryl substituent alkyl group was introduced. Only compound 2 with trifluoromethylphenyl and 3,4-dichlorophenyl connected to thiourea moiety possessed bactericidal activity (Tables I, II). The rest of tested compounds

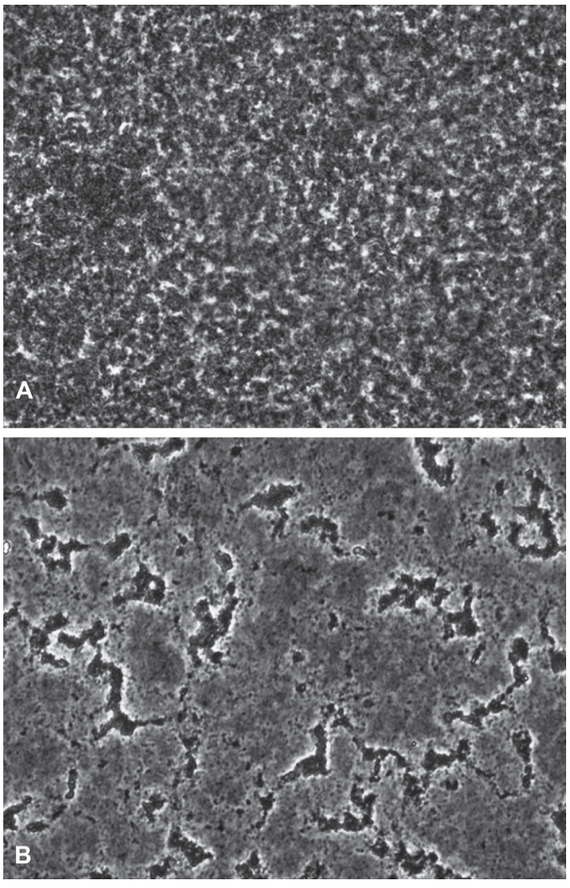


Fig. 5. Biofilms of *S. epidermidis* ATCC 35984 growth for 24 h on polystyrene microtiter plates: A – in TSB medium supplemented with 0.5% glucose; B – in TSB medium supplemented with 0.5% glucose in the presence of compound 2 in concentration 1 μg/ml. The digital images were visualized using the phase-contrast inverted microscope Eclipse TS 100F Inverted Routine Microscopes (Nikon, USA) equipped with the DeltaPix Invenio 5SCII camera using DeltaPixInSigh software.

showed bacteriostatic effect (MBC values much higher compared to the MIC values).

3-(Trifluoromethyl)aniline, 4-chloro-3-nitroaniline, 1,3-thiazol-2-amine, 2*H*-1,2,3-triazol-4-amine were subjected to the reaction with isothiocyanates to obtain thiourea derivatives (Scheme 1). To assure structural variability, different aryl, heteroaryl and aryl connected by methylene group at thiourea moiety were introduced. Whereas unsubstituted 1,3-diphenylthiourea exerts no relevant antimicrobial activity (Cunha *et al*., 2007), howewer its various structural modifications improve the biological effectiveness of a compound (Mishra and Batra, 2013). Literature survey reveals that incorporation of halogen atom(s) within the molecule is one of the most effective strategies to enhance its biopotency, bioavailability and lipophilicity. The fluoro-containing arylthiourea compounds show better activity as compared to other analogues (Suresha *et al*., 2011), however fluoro-methyl substituent on the benzene ring also improve antimicrobial potency (Bielenica *et al*., 2015). According to other authors findings (Chikhalia and Patel, 2009; Saeed *et al*., 2009; 2010; Faidallah *et al*., 2013), the antibacterial and antifungal efficacy depends on the presence of such electron withdrawing (NO_2, Cl, CF_3) substituent at C-2 and C-4 position of the phenyl ring.

In this work in order to assure structural variability, different aryl substituents connected to N1 and N2 atom of thiourea moiety were introduced and the biological activity depended on the structure of new thiourea derivatives.

Potent Gram-positive antibacterial activity of several analogs of thiourea, urea (Ehmann and Lahiri, 2014) and thiosemicarbazide derivatives (Siwek *et al*., 2011) is explained by an inhibition of the catalytic site of bacterial type II topoisomerases, in particular DNA gyrase and topoisomerase IV. Topoisomerase IV is a bacterial type II topoisomerase that is essential for proper chromosome segregation and is a target for quinolone-based antimicrobial agents, such as Ciprofloxacin and Levofloxacin (Fournier *et al*., 2000).

Fluoroquinolones stimulate topoisomerase IV-mediated DNA cleavage both by increasing rate of DNA scission and by inhibiting relegation of cleaved DNA. As a result, quinolones inhibit the overall catalytic activity of topoisomerase IV by interfering with enzyme-ATP interactions (Anderson *et al*., 1998). Presented preliminary results showed that thiourea-derived compounds presented in this study were able to inhibit the activity of bacterial topoisomerases, such as *S. aureus* topoisomerase IV.

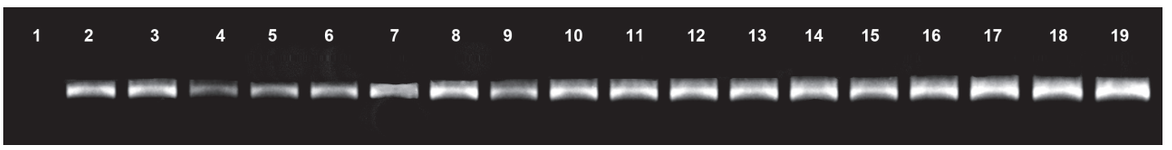


Fig. 6. The effect of studied compounds on topoisomerase IV in decatenation assays: 1 – control assay without enzyme; 2 – *S. aureus* topoisomerase IV control; 3 – *S. aureus* topoisomerase IV control with DMSO; 4 – Ciprofloxacin (CFX) (96 μg/ml), 5 – CFX (32 μg/ml), 6 – CFX (8 μg/ml), 7 – CFX (4 μg/ml), 8 – CFX (2 μg/ml); 9 – compound 2 (32 μg/ml), 10 – comp. 2 (8 μg/ml), 11– comp. 2 (4 μg/ml), 12 – comp. 2 (1 μg/ml), 13 – comp. 2 (0.5 μg/ml), 14 – comp. 2 (0.25 μg/ml); 15 – comp. 3 (32 μg/ml); 16 – comp. 3 (8 μg/ml), 17 – comp. 3 (4 μg/ml), 18 – comp. 3 (1 μg/ml), 18 – comp. 3 (0.5 μg/ml).

It is known, that 3-(trifluoromethyl)phenyl]thiourea derivatives, close analogs of 1 and 2, are not cytotoxic against normal (HaCaT) cells (Bielenica *et al*., 2015). They do not considerably decreased viability and have no visible influence on the mortality of tested cells. Similar tests for thiourea compounds presented in that paper will be conducted in the near future. To exploit their antibiofilm properties, the title compounds could be used to cover the surface of biomaterials or medical devices.

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Conflicts of interest

All authors declare, that there are not any potential conflicts of interest.

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