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Preliminary communication

Studies on the synthesis and antibacterial activity of 3,6-disubstituted 1,2,4-triazolo[3,4-*b*]1,3,4-thiadiazoles

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ABSTRACT

Treating infections caused by drug-resistant bacterial strains constitutes one of the most essential challenges for medicine nowadays. A range of new derivatives of 1,2,4-triazolo[3,4-*b*]1,3,4-thiadiazole have been synthesized and evaluated for their *in vitro* antimicrobial activity. Compounds **1**–**8** indicated high activity towards Gram-positive bacteria, which was up to 16 times more than currently used antibiotics. To the best of our knowledge, the derivatives obtained by us are the most active among the 3-aryl-6-arylamino-1,2,4-triazolo[3,4-*b*]1,3,4-thiadiazoles known until now.

1. Introduction

Since 1942, when the Commercial Solvents Corporation launched penicillin, invented by Alexander Fleming, a considerable decrease in the number of deaths caused by bacterial infections has been noticed. Optimists have even announced an end to the era of bacterial diseases. However, too frequent, and frequently improper, applications of antibiotics have resulted in the formation of drug-resistant bacteria strains, and the world has begun to face another problem; that of treating nosocomial infections [1]. In the USA, 2 million patients a year come down with hospital-acquired infections, which are among the top five causes of death (90 000 deaths per year) and whose treatment costs amount to $4.5–5.7 billion a year [2,3]. Treating infections caused by drug-resistant bacterial strains constitutes one of the most essential challenges for medicine nowadays. Methicillin- and vancomycin-resistant strains of *Staphylococcus aureus* are responsible for most infections of this type [4,5].

Condensed heterocyclic systems containing an s-triazole ring are characterised by significant pharmacological activity. These types of compounds exhibit antitumour, antioxidant, analgesic, anti-inflammatory, antibacterial, antifungal activity, *etc.* [6–11]. In the eighties, it was confirmed that 3,6-disubstituted 1,2,4-triazolo [3,4-*b*]1,3,4-thiadiazole derivatives may be active against strains of *S. aureus* [12]. The attempt at exchanging a fragment of thiadiazole for a fragment of thiadiazine led to a considerable weakening of antimicrobial activity [13]. Further studies were to demonstrate the impact of substituents located at positions 3 and 6 on antimicrobial activity. The results obtained by Almajan *et al.* [14] suggest that the large volume of substituents at position 3 may reduce antimicrobial activity. At the same time, Badr *et al.* [15] studies indicate that the role of substituents at position 6 is secondary. There has been no material change of antimicrobial activity for compounds with different (both aliphatic and aromatic) substituents at position 6 of 1,2,4-triazolo[3,4-*b*]1,3,4-thiadiazole core. Taking into account the above observations, we decided to synthesize the group of 3,6-disubstituted 1,2,4-triazolo[3,4-*b*]1,3,4-thiadiazole derivatives and see if the substituents (at position 3) that are smaller in volume can beneficially affect antimicrobial activity of the compounds obtained. Substituents at position 6, as less important for activity (compared to the substituents at position 3), showed no significant structural diversity in our studies.

2. Results and discussion

2.1. Chemistry

A synthesis pathway of compounds **1**–**8** has been presented in Scheme 1. Aminotriazoles (**A**–**D**) – precursors of 3,6-disubstituted 1,2,4-triazolo[3,4-*b*]1,3,4-thiadiazoles – were obtained in

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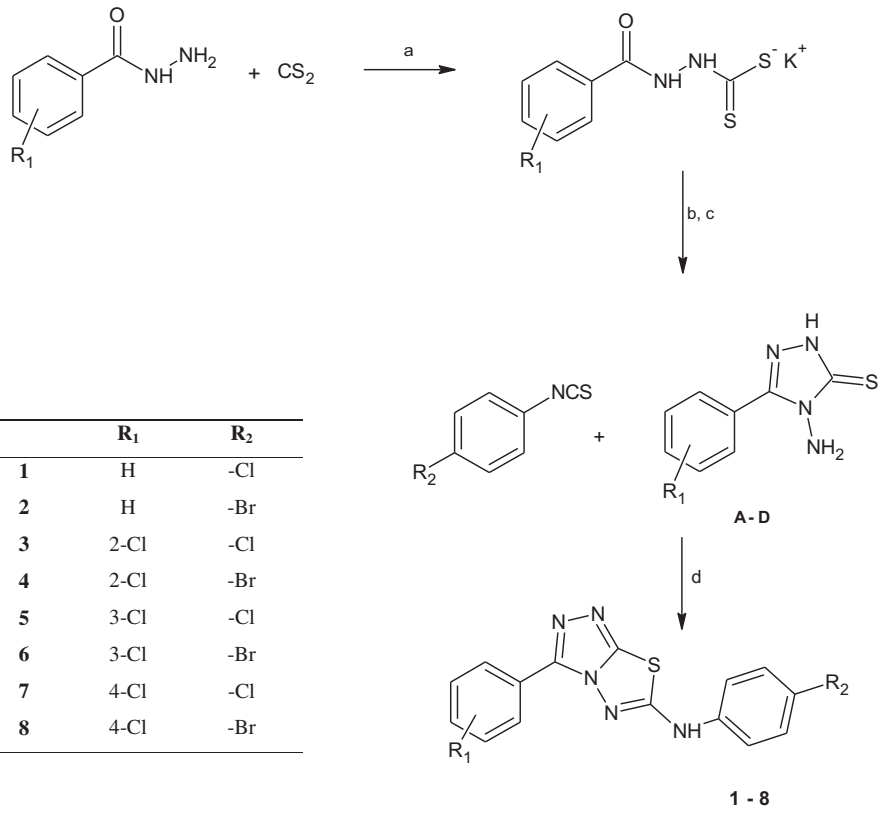

	R_1	R_2
1	H	-Cl
2	H	-Br
3	2-Cl	-Cl
4	2-Cl	-Br
5	3-Cl	-Cl
6	3-Cl	-Br
7	4-Cl	-Cl
8	4-Cl	-Br

Scheme 1. Reagents and conditions: (a) EtOK, rt., 2 min; (b) N_2H_4, reflux, 4 h; (c) HCl; (d) DMF, 80 °C, stirring, 24 h.

accordance with widely used procedures [16–22]. Next, by heating appropriate aminotriazoles (**A**–**D**) with 4-chlorophenyl isothiocyanate or 4-bromophenyl isothiocyanate in the environment of anhydrous dimethylformamide (DMF), 3-aryl-6-arylamino-1,2,4-triazolo[3,4-*b*]1,3,4-thiadiazoles (**1**–**8**) were obtained. In order to avoid creation of adventitious products of reaction, the reaction mixture was heated for 24 h at the temperature of 80 °C (instead of refluxing). It was found that although the same reaction carried out at the boiling temperature was quicker, there were also more adventitious products. Structures of the newly synthesized compounds were defined on the basis of the following spectra: ^{1}H NMR, IR and MS. The spectral data fully confirm the structures suggested for compounds **1**–**8** (presented in Scheme 1). In the MS spectra, molecular ion peaks (intensities: 10–88%) confirm the molecular weight of the synthesized compounds. In turn, the appearance of isotope peaks confirms the presence of halogen atom(s) in compounds **1**–**8**. In the infrared spectra, characteristic bands are visible: for the NH group – in the range of 3204–3320 cm^{-1}; for aromatic rings – in the range of 3011–3068 cm^{-1}; and for C=N fragments – in the range of 1577–1621 cm^{-1}. The ^{1}H NMR spectra of the compounds revealed the signals of aromatic protons at 6.97–7.85 ppm (the presence of the characteristic pattern of *para* substituted aromatic rings was detected), while the signals of the protons of the NH group appeared in the 9.21–10.84 region as a broad singlet.

2.2. Antimicrobial evaluation

The antimicrobial activity of compounds **1**–**8** was tested against drug-sensitive bacteria (Gram-positive as well as Gram-negative ones) and towards methicillin-resistant *S. aureus* (MRSA). None of the compounds analysed showed activity against Gram-negative bacteria. However, their activity related to Gram-positive bacteria (both drug-sensitive and drug-resistant) was very promising. The reference drugs were commonly applied antibiotics such as ampicillin, cefuroxime and vancomycin (considered as "the last-resort drug" in treatment of infections caused by methicillin-resistant strains of *S. aureus*). Compounds **5** and **6** turned out to be the most active in relation to the methicillin-sensitive strains of *S. aureus* ATCC 25923 and *S. aureus* ATCC 6538 (Table 1). Their activity against *S. aureus* ATCC 6538 was twice as high as the activity of cefuroxime and similar to the activity of vancomycin. In turn, *S. aureus* ATCC 25923 was the most sensitive to compounds **2** and **6** (the same activity as of vancomycin). The synthesized compounds were also highly active against *Staphylococcus epidermidis* ATCC 12228. In relation to this strain, five out of eight compounds were characterised by activity similar to or higher (in the case of compound **2**) than vancomycin. All the substances described were characterised by very high activity (higher than the activity of ampicillin and cefuroxime) against *Bacillus subtilis* ATCC 6633 and *Bacillus cereus* ATCC 10876. In the case of *B. subtilis* ATCC 6633, the activity of compounds **1**–**8** was 4–16 times higher than the activity of ampicillin. The activity in relation to *B. cereus* ATCC 10876 was up to 16 times higher than the activity of ampicillin and up to 8 times higher than the activity of cefuroxime. The activity of the derivatives **1**–**8** against the methicillin-resistant *S. aureus* ATCC 14001 varied, and the minimum inhibitory concentration (MIC) values ranged between 0.98 and 7.81 μg/mL (Table 2). 3-(3-Chlorophenyl) derivatives (**5**, **6**) were found to have the highest anti-MRSA activity, comparable to the activity of vancomycin (MIC = 0.98 μg/mL). On the basis of MBC/MIC ratio, it was found that compounds **1**–**8** have bacteriostatic effect (MBC/MIC > 4) towards the MRSA strain.

2.3. Structure–activity relationships (SAR)

All the obtained compounds were active (high or average activity) irrespective of the type of substituent at position C-3. This

Table 1
In vitro antibacterial screening of the compounds **1**–**8** against drug-susceptible strains.

Microorganisms												
Compounds	*S. aureus* ATCC 25923		*S. aureus* ATCC 6538		*S. epidermidis* ATCC 12228		*B. subtilis* ATCC 6633		*B. cereus* ATCC 10876		*M. luteus* ATCC 10240	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
1	3.91	31.25	15.63	31.25	0.98	3.91	1.95	7.81	15.63	15.63	7.81	15.63
2	0.98	31.25	15.63	125	0.49	0.98	0.98	1.91	15.63	15.63	3.91	7.81
3	15.63	31.25	15.63	15.63	3.91	15.63	3.91	15.63	3.91	3.91	7.81	15.63
4	1.95	15.63	7.81	62.5	0.98	3.91	1.95	15.63	7.81	15.63	7.81	7.81
5	1.95	31.25	0.49	31.25	0.98	3.91	1.95	1.95	7.81	15.63	3.91	3.91
6	0.98	31.25	0.49	31.25	0.98	3.91	1.95	1.95	15.63	31.25	3.91	3.91
7	3.91	62.5	0.98	62.5	3.91	15.63	3.91	7.81	7.81	31.25	15.63	31.25
8	1.95	31.25	0.98	31.25	1.95	1.95	1.95	1.95	15.63	15.63	15.63	15.63
Ampicillin	–	–	–	–	–	–	–	–	62.5	–	–	–
Cefuroxime	0.49	–	0.98	–	0.24	–	15.63	–	31.25	–	0.98	–
Vancomycin	0.98	7.81	0.49	1.95	0.98	1.95	0.25	0.49	0.98	31.25	0.25	0.49

MIC and MBC values are given in μg/mL. "–": not determined.

may suggest that the volume of the substituent is more important, and its type is only secondary. It can be assumed that the 1,2,4-triazolo[3,4-*b*]1,3,4-thiadiazole core, together with a substituent at position C-3, mainly participates in the process of binding of such molecules to the drug-target site. Taking into account the influence of an aryl fragment at position C-3 of the 1,2,4-triazolo[3,4-*b*]1,3,4-thiadiazole system, what seems the most beneficial for the anti-MSSA and anti-MRSA activity is the presence of chlorine atom at the *meta* position of the phenyl ring. Although the impact of substituents at position C-6 on antimicrobial activity is smaller, it cannot be ignored. In the case of *S. aureus* ATCC 25923, all 6-(4-bromophenyl) derivatives were more active than respective 6-(4-chlorophenyl) analogues. A similar dependency can be noticed in terms of *S. epidermidis* ATCC 12228 and *B. subtilis* ATCC 6633 strains (apart from compounds **5** and **6**, whose activity is equal). Thus, by proper selection of the type of substituent the activity of newly obtained derivatives can be also modified. In the present study, these SAR conclusions are presented only tentatively due to the relatively small number of the synthesized compounds. It is necessary to widen the base of compounds, which we plan to do in the near future.

It is also worth of mentioning that described substances are absolutely inactive in relation to Gram-negative bacteria. One needs to guess that it results from differences in structure of cell wall of Gram-positive and Gram-negative bacteria. In case of Gram-negative bacteria there exists an additional outer membrane rich of lipopolysaccharides (LPS). LPS confers a negative charge and also repels hydrophobic compounds [23–25]. This specific structure of the cell wall makes that very large or intensively hydrophobic molecules are not able to penetrate an interior of the bacterial cell.

Table 2
In vitro antibacterial screening of the compounds **1**–**8** against methicillin-resistant *Staphylococcus aureus* (MRSA).

Compounds	*Staphylococcus aureus* ATCC 14001 (MRSA)		MBC/MIC
	MIC	MBC	
1	1.95	62.5	32
2	7.81	62.5	8
3	1.95	62.5	32
4	1.95	15.63	8
5	0.98	62.5	64
6	0.98	125	128
7	7.81	62.5	8
8	7.81	125	16
Vancomycin	0.98	3.91	4

C log *P* values (calculated acc. to the atom/fragment contribution method [26]) for compounds **1**–**8** were close to 5 which may explain lack of activity in relation to the Gram-negative bacteria. On the other hand, the cell wall of the Gram-positive bacteria, generally made of peptidoglycan, does not constitute a barrier for penetration of the interior of a cell by hydrophobic substances.

Furthermore, compounds **1**–**8** exhibit bacteriostatic (MBC/MIC > 4) or bactericidal (MBC/MIC ≤ 4) effects, which lead to the assumption that there may be two completely different mechanisms of action of the described derivatives. Therefore, not only one molecular target for this type of compounds should be searched for.

3. Conclusions

Eight derivatives of 1,2,4-triazolo[3,4-*b*]1,3,4-thiadiazole substituted at position 3 with an aryl group, and at position 6 with arylamino group have been obtained. Their structures were confirmed on the basis of spectral data and elemental analyses. Compounds **1**–**8** described in the present paper indicated high activity towards Gram-positive bacteria, which was up to 16 times more than currently used antibiotics. Two new derivatives (**5** and **6**) were as effective towards the MRSA strain as vancomycin. Such promising activity against the methicillin-resistant strain bids to intensify studies on the use of 1,2,4-triazolo[3,4-*b*]1,3,4-thiadiazoles in the fight against infections caused by drug-resistant bacteria.

4. Experimental section

4.1. Chemistry

4.1.1. General comments

All reagents were purchased from Lancaster (Ward Hill, USA) and Merck Co. (Darmstadt, Germany). Melting points were determined by using Fischer-Johns apparatus (Sanyo, Japan) and are uncorrected. The ^{1}H NMR spectra were recorded on a Bruker Avance 250 MHz instrument using $CDCl_3$ as a solvent and TMS as an internal standard. The IR spectra were recorded in KBr discs using a Perkin–Elmer 1725X FTIR spectrometer. The mass spectra were obtained on a Finnigan Trace DSQ spectrometer operating at 70 eV. Elemental analyses were performed on an AMZ 851 CHX analyser (PG, Gdańsk, Poland) and the results were within ±0.2% of the theoretical value. All the compounds were purified by flash chromatography (PuriFlash 430*evo*, Interchim, USA).

*4.1.2. General procedure for the synthesis of 4-amino-5-substituted-2,4-dihydro-3H-1,2,4-triazole-3-thiones (**A**–**D**)*

Solid potassium hydroxide (0.015 mol) and appropriate aryl hydrazide (benzhydrazide – for **A**, 2-chlorobenzhydrazide – for **B**, 3-chlorobenzhydrazide – for **C**, 4-chlorobenzhydrazide – for **D**) (0.01 mol) were dissolved in anhydrous ethanol (25 mL) and the resulted solution was cooled to 0–5 °C. Then carbon disulfide (CS_2, 1 mL) was added dropwise and the reaction mixture was stirred for 2 min. The precipitated solid of potassium dithiocarbazinate was filtered off, washed with diethyl ether and dried. Thus obtained (in quantitative yield) potassium salt was used in the next step without further purification. A solution of potassium dithiocarbazinate and 80% hydrazine hydrate (10 mL) was refluxed for 4 h, cooled to room temperature, diluted with water (50 mL) and acidified with 3 M HCl. The resulting solids of compounds **A**–**D** were filtered, dried and crystallized from anhydrous ethanol. Physicochemical/spectral properties of compounds **A**–**D** were described earlier [16–22].

*4.1.3. General procedure for the synthesis of 3-aryl-6-arylamino-1,2,4-triazolo[3,4-b]1,3,4-thiadiazoles (**1**–**8**)*

An equimolar mixture of appropriate aminotriazole (**A**–**D**) (0.01 mol) and 4-chlorophenyl- or 4-bromophenyl isothiocyanate (0.01 mol) in anhydrous dimethylformamide (DMF, 15 mL) was stirred at 80 °C for 24 h. The reaction mixture was cooled to room temperature, poured onto crushed ice (with stirring) and kept overnight. The resulting solid was filtered off, washed several times with distilled water, dried and crystallized from EtOH.

*4.1.3.1. 6-(4-Chlorophenyl)amino-3-phenyl-1,2,4-triazolo[3,4-b]1,3,4-thiadiazole (**1**).* CAS 882151-34-0. Yield: 81%, m.p. 260–261 °C. ^{1}H NMR (250 MHz) ($CDCl_3$) δ (ppm): 7.09–7.12 (m, 1H, Ar–H), 7.20 (d, 2H, Ar–H, $J = 8.4$ Hz), 7.23–7.62 (m, 4H, Ar–H), 7.71 (d, 2H, Ar–H, $J = 8.3$ Hz), 9.76 (br s, 1H, NH). IR (KBr, ν, cm^{-1}): 3248 (NH), 3026 ($CH_{arom.}$), 1597 (C=N). Mass *m/z*: 327 (M^+, 88%), 329 (M + 2, 32%). Anal. Calcd for $C_{15}H_{10}ClN_5S$ (%): C, 54.96; H, 3.07; N, 21.37. Found: C, 55.02; H, 3.00; N, 21.48.

*4.1.3.2. 6-(4-Bromophenyl)amino-3-phenyl-1,2,4-triazolo[3,4-b]1,3,4-thiadiazole (**2**).* Yield: 76%, m.p. 276–278 °C. ^{1}H NMR (250 MHz) ($CDCl_3$) δ (ppm): 6.97–7.03 (m, 1H, Ar–H), 7.17 (d, 2H, Ar–H, $J = 8.4$ Hz), 7.19–7.24 (m, 2H, Ar–H), 7.41 (d, 2H, Ar–H, $J = 8.4$ Hz), 7.58–7.82 (m, 2H, Ar–H), 10.08 (br s, 1H, NH). IR (KBr, ν, cm^{-1}): 3271 (NH), 3052 ($CH_{arom.}$), 1607 (C=N). Mass *m/z*: 371 (M^+, 87%), 373 (M + 2, 86%). Anal. Calcd for $C_{15}H_{10}BrN_5S$ (%): C, 48.40; H, 2.71; N, 18.81. Found: C, 48.50; H, 2.68; N, 18.80.

*4.1.3.3. 3-(2-Chlorophenyl)-6-(4-chlorophenyl)amino-1,2,4-triazolo [3,4-b]1,3,4-thiadiazole (**3**).* Yield: 71%, m.p. 247–249 °C. ^{1}H NMR (250 MHz) ($CDCl_3$) δ (ppm): 7.20 (d, 2H, Ar–H, $J = 8.0$ Hz), 7.31 (d, 2H, Ar–H, $J = 8.2$ Hz), 7.40–7.82 (m, 4H, Ar–H), 9.72 (br s, 1H, NH). IR (KBr, ν, cm^{-1}): 3311 (NH), 3017 ($CH_{arom.}$), 1589 (C=N). Mass *m/z*: 361 (M^+, 19%), 363 (M + 2, 8%). Anal. Calcd for $C_{15}H_9Cl_2N_2S$ (%): C, 49.74; H, 2.50; N, 19.33. Found: C, 49.68; H, 2.61; N, 19.36.

*4.1.3.4. 6-(4-Bromophenyl)amino-3-(2-chlorophenyl)-1,2,4-triazolo [3,4-b]1,3,4-thiadiazole (**4**).* Yield: 75%, m.p. 281–283 °C. ^{1}H NMR (250 MHz) ($CDCl_3$) δ (ppm): 7.19 (d, 2H, Ar–H, $J = 8.5$ Hz), 7.32 (d, 2H, Ar–H, $J = 8.4$ Hz), 7.53–7.80 (m, 4H, Ar–H), 9.21 (br s, 1H, NH). IR (KBr, ν, cm^{-1}): 3204 (NH), 3045 ($CH_{arom.}$), 1621 (C=N). Mass *m/z*: 405 (M^+, 32%), 407 (M + 2, 35%). Anal. Calcd for $C_{15}H_9BrClN_5S$ (%): C, 44.30; H, 2.23; N, 17.22. Found: C, 44.33; H, 2.29; N, 17.15.

*4.1.3.5. 3-(3-Chlorophenyl)-6-(4-chlorophenyl)amino-1,2,4-triazolo [3,4-b]1,3,4-thiadiazole (**5**).* Yield: 76%, m.p. 250–252 °C. ^{1}H NMR (250 MHz) ($CDCl_3$) δ (ppm): 7.13 (d, 2H, Ar–H, $J = 8.6$ Hz), 7.27–7.33 (m, 1H, Ar–H), 7.40–7.49 (m, 2H, Ar–H), 7.76 (d, 2H, Ar–H, $J = 8.6$ Hz), 7.84 (s, 1H, Ar–H), 9.92 (br s, 1H, NH). IR (KBr, ν, cm^{-1}): 3219 (NH), 3066 ($CH_{arom.}$), 1577 (C=N). Mass *m/z*: 361 (M^+, 11%), 363 (M + 2, 4%). Anal. Calcd. for $C_{15}H_9Cl_2N_2S$ (%): C, 49.74; H, 2.50; N, 19.33. Found: C, 49.80; H, 2.64; N, 19.30.

*4.1.3.6. 6-(4-Bromophenyl)amino-3-(3-chlorophenyl)-1,2,4-triazolo [3,4-b]1,3,4-thiadiazole (**6**).* Yield: 66%, m.p. 264–266 °C. ^{1}H NMR (250 MHz) ($CDCl_3$) δ (ppm): 7.24 (d, 2H, Ar–H, $J = 8.5$ Hz), 7.31–7.35 (m, 1H, Ar–H), 7.43–7.60 (m, 3H, Ar–H), 7.85 (d, 2H, Ar–H, $J = 8.6$ Hz), 10.39 (br s, 1H, NH). IR (KBr, ν, cm^{-1}): 3213 (NH), 3068 ($CH_{arom.}$), 1612 (C=N). Mass *m/z*: 405 (M^+, 10%), 407 (M + 2, 12%). Anal. Calcd for $C_{15}H_9BrClN_5S$ (%): C, 44.30; H, 2.23; N, 17.22. Found: C, 44.38; H, 2.18; N, 17.19.

*4.1.3.7. 3-(4-Chlorophenyl)-6-(4-chlorophenyl)amino-1,2,4-triazolo [3,4-b]1,3,4-thiadiazole (**7**).* Yield: 69%, m.p. 278–280 °C. ^{1}H NMR (250 MHz) ($CDCl_3$) δ (ppm): 7.19 (d, 2H, Ar–H, $J = 8.3$ Hz), 7.37 (d, 2H, Ar–H, $J = 8.4$ Hz), 7.56 (d, 2H, Ar–H, $J = 8.3$ Hz), 7.68 (d, 2H, Ar–H, $J = 8.4$ Hz), 10.70 (br s, 1H, NH). IR (KBr, ν, cm^{-1}): 3320 (NH), 3011 ($CH_{arom.}$), 1590 (C=N). Mass *m/z*: 361 (M^+, 53%), 363 (M + 2, 36%). Anal. Calcd for $C_{15}H_9Cl_2N_2S$ (%): C, 49.74; H, 2.50; N, 19.33. Found: C, 49.68; H, 2.51; N, 19.36.

*4.1.3.8. 6-(4-Bromophenyl)amino-3-(4-chlorophenyl)-1,2,4-triazolo [3,4-b]1,3,4-thiadiazole (**8**).* Yield: 74%, m.p. 268–269 °C. ^{1}H NMR (250 MHz) ($CDCl_3$) δ (ppm): 7.03 (d, 2H, Ar–H, $J = 8.5$ Hz), 7.21 (d, 2H, Ar–H, $J = 8.4$ Hz), 7.48 (d, 2H, Ar–H, $J = 8.5$ Hz), 7.65 (d, 2H, Ar–H, $J = 8.5$ Hz), 10.84 (br s, 1H, NH). IR (KBr, ν, cm^{-1}): 3287 (NH), 3015 ($CH_{arom.}$), 1614 (C=N). Mass *m/z*: 405 (M^+, 78%), 407(M + 2, 100%). Anal. Calcd for $C_{15}H_9BrClN_5S$ (%): C, 44.30; H, 2.23; N, 17.22. Found: C, 44.25; H, 2.27; N, 17.30.

4.2. Microbiological part

The antimicrobial activity of the compounds was tested on the Gram-positive bacteria (*S. aureus* ATCC 25923, *S. aureus* ATCC 6538, *S. aureus* ATCC 14001 [MRSA], *S. epidermidis* ATCC 12228, *B. subtilis* ATCC 6633, *B. cereus* ATCC 10876, *Micrococcus luteus* ATCC 10240) and on the Gram-negative strains (*Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Proteus mirabilis* ATCC 12453, *Pseudomonas aeruginosa* ATCC 9027). Ampicillin, cefuroxime and vancomycin were used as control antimicrobial agents. Determination of the MIC (minimal inhibitory concentration – defined as the lowest concentration of compound at which there was no visible growth of tested microorganisms) and MBC (minimal bactericidal concentration – defined as the lowest concentration of compound that resulted in >99.9% reduction in CFU of the initial inoculum) values was achieved by a broth microdilution method, according to CLSI recommendation [27]. The procedures used were described in details in an earlier article [28].

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