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EVALUATION OF THE ANTIMICROBIAL AND ANTI-BIOFILM ACTIVITY OF SOME 4,2 AND 5,2 BISTHIAZOLES DERIVATIVES

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Due to the emergence of antimicrobial resistance, new antibiotics or anti-pathogenic drugs are strongly needed. In this context, 15 new 4,2 and 5,2 bisthiazole derivatives were investigated in order to determine their antimicrobial activity and influence on microbial biofilm development and subsequent inert substratum colonization. The obtained compounds were tested on five bacterial strains and one fungal strain. The antimicrobial activity was assessed by using both qualitative (an adapted disk diffusion technique) and quantitative assays (broth micro-dilution and biofilm microtiter methods). The compounds showed a low to good antimicrobial activity especially against *Escherichia coli* ATCC 25922. A moderate anti-biofilm effect was exhibited against *Pseudomonas aeruginosa* ATCC 27857 and *E. coli* ATCC 25922 strains. Five of the tested compounds exhibited good antibacterial and anti-biofilm activities against *E. coli* ATCC 25922.

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1. Introduction

Infectious diseases remain one of the major world health problems, partly due to the rapid development of resistance to the existing antimicrobial drugs. Bacterial resistance to antibiotics is in the top of the major public health problems identified by ECDC. In order to survive and adapt to different environmental conditions (pH, oxygen, nutrients, redox status etc.) microbial cells adhere between them and to a surface, forming biofilms. The adherence process is dynamic; if the environmental conditions became unfavourable the microbial cells are released from the surface, while in favourable conditions a rapid multiplication process is initiated [1-3]. The growing problem of antibiotic resistance is aggravated by microbial biofilms development on natural tissues or artificial devices [4-6], which according to NIH (National Institute of Health) are implicated in the etiology of 80% of human infections [7,8], characterized by chronic evolution and phenotypic, behavioural resistance to antibiotic treatment [3,9-12]. Although in the recent years more and more money and efforts were channelled for discovering new antibiotics, the number of new approved molecules is still small [1].

A relatively new approach is to design and synthesize molecules that act dually, against planktonic microbial cells (microbicidal activity) as well as on adherent microbial cells grown in biofilms (anti-biofilm activity) [2].

It is already known that the thiazole ring could provide a rich spectrum of biological activities [13,14]), being also present in some well known antibacterial molecules, such as ceftriaxone, ceftazidime, cefixime, aztreonam. Our previous research showed that this moiety can

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be further exploited in different molecular scaffolds [15-17]. Our research group has a long experience with the synthesis of potentially antimicrobial molecules containing the thiazole nucleus or its derivatives [18,19].

In this context our aim was to test new derivatives with bisthiazole scaffold for their antimicrobial activity, as well as for their influence on the microbial biofilm development on the inert substratum.

2. Experimental

Chemistry

The tested molecules were synthesized by our team as presented in our previous paper [20]. All 15 compounds were characterized physico-chemically and analyzed to confirm the proposed structures. The compounds belong to two different structural classes: seven of them being 4,2 bisthiazoles derivatives, while eight are 5,2 bisthiazoles.

The synthesis of the 5,2 bisthiazoles was performed in four different steps in order to yield the key intermediate **5,** called 4-methyl-2-phenyl-thiazole-5-carbothioamide**.** The synthesis of the 4,2 bisthiazoles is a more complex process that requires six different steps, and is dependent of the key intermediate **4,** called 2-phenyl-thiazole-4-carbothioamide (see **figure 1**).

The last reaction, in both synthesis processes, is identical. It involves a Hantzsch condensation between the key thioamide intermediates **4** and **5** and a series of α-bromo-ketones, in order to yield the corresponding bisthiazole derivatives **4BT 1-9** and **5BT1-9** (see **Table I**) [20].

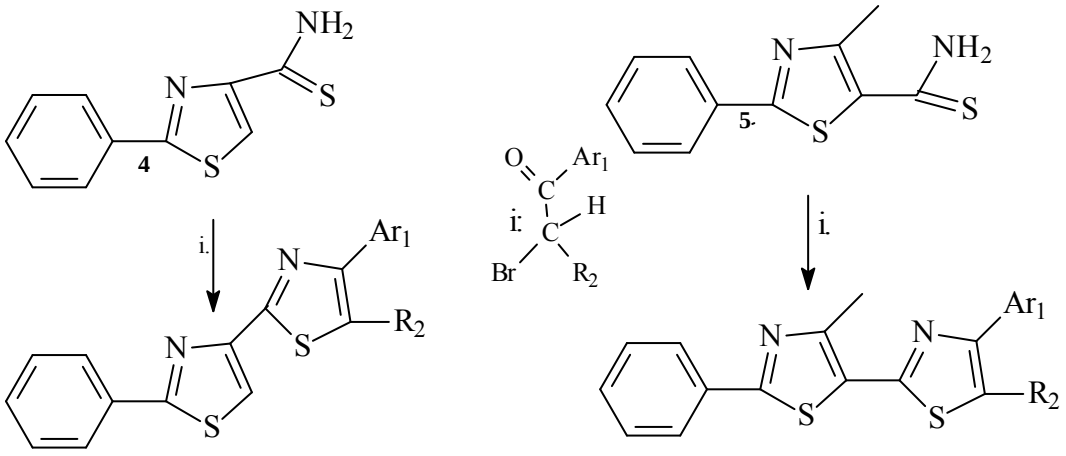
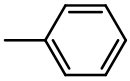
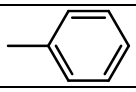
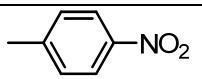
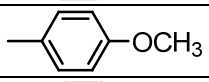
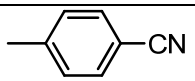
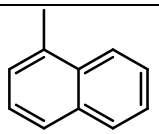
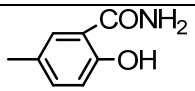
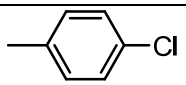


Fig. 1. The final chemical reactions for the synthesis of the 2 series of related compounds, i: the corresponding alpha-bromo-ketones and acetone, stirred at room temperature for 24 h [20].

Table I. The chemical structures of the analyzed molecules [20]

4,2-Bisthiazoles	Ar $_1$	R_2	5,2-Bisthiazoles
4BT1		- CH_3	**5BT1**
-		H	**5BT2**
4BT3		H	**5BT3**
4BT4		H	**5BT4**
4BT6		H	**5BT6**
4BT7		H	**5BT7**
4BT8		H	**5BT8**
4BT9		H	**5BT9**

Antimicrobial activity evaluation

The evaluation of the potential antimicrobial activity was performed by using the following microbial strains: *Enterococcus faecium E5, Staphylococcus aureus* ATCC 6538, *Bacillus subtillis* ATCC 6683, *Pseudomonas aeruginosa* ATCC 27857, *Klebsiella pneumoniae* IC 13420, *Escherichia coli* ATCC 25922 and *Candida albicans* 393. These strains are either reference strains or recently isolated strains form clinical specimens. The VITEK 1 automatic system was used to identify the microbial strains. All determinations were performed in accordance with the manufacturer's recommendations. The results were interpreted using the AMS RO9.1 software.

1. The qualitative screening of the antimicrobial activity

The qualitative screening aimed at assessing the susceptibility of different microbial strains to the potentially antimicrobial molecules being tested.

This screening was performed by an adapted disk diffusion technique [21-23]. An amount of 5 µl of the chemical compound solution prepared in dimethyl sulfoxide (DMSO) (10 mg/ml) was spotted on the Muller-Hinton medium, previously seeded with the microbial standardized inocula of a 0.5 McFarland density, prepared in sterile saline suspension with 24 h microbial cultures developed on a solid medium.

The seeded plates were left at room temperature for 30 minutes in order to allow the adsorption of the liquid onto the solid medium. Then the plates were incubated, face down, at 37° C for 24 h. Results were assessed by measuring the diameters of the growth inhibition zone with the help of a scale, the obtained results being expressed in mm.

The DMSO solvent was also tested comparatively in order to assess its intrinsic antimicrobial activity.

The microbicidal effect was quantified through the presence of a clear growth inhibition zone, around the applied spot.

2. Quantitative assay of the antimicrobial activity

The quantitative assay of the antimicrobial activity allowed the establishment of the Minimum Inhibitory Concentration (MIC) value, defined as the minimal amount of the chemical compound capable to inhibit the growth of microbial cells.

This assay was performed by using a serial micro-dilution method in liquid medium (Mueller-Hinton), distributed in 96 multi-well plates. To a volume of 100 μl of medium, serial binary dilutions of the 10 mg/ml stock solutions were added. In the first well 180 μl liquid culture medium and 20 μl stock solution were pipetted. A volume of 100 μl from the first well was then added to the second well, and 100 μl from the second well was then added to the third, and so on, up until the last well from which 100 μl where eliminated [21-26].

The wells were then inoculated with 20 μl of microbial suspension of 0.5 MacFarland density. The microbial inocula were made out of a sterile saline suspension obtained from 24 h cultures on a solid medium.

For each measurement a set of sterile blank wells (containing only the culture medium) and a set of positive control wells (containing the culture medium and the microbial inocula) were used.

Then the plates were incubated for 24 h at 37° C and then subjected to macroscopic evaluation. In the positive control wells the culture medium became turbid because of microbial growth. The sterile blank wells were clear and transparent due to the lack of microbial growth. The concentration of the tested chemical compound from the last well that did not show the signs of culture growth was considered the MIC for that molecule.

3. The study of the influence of the tested molecules on microbial biofilm development on the inert substratum

The anti-biofilm properties were measured by the microtiter method [12-24]. This involves a series of steps, i.e.: i) the microbial cells were grown in plates of 96 multi-well plates containing nutritive broth and in the presence of the tested compounds (following the same steps as for the determination of MIC) were incubated for 24 h at 37° C for bacterial strains and for 48 h at 28° C for fungal strains; ii) the plates were subsequently emptied and washed twice with phosphate buffered saline; iii) the adherent cells were fixed with 100 μl methanol 80%.; iv) the cells were stained with an alkaline 1% violet crystal solution (100μl/well) for 15 minutes; v) after the removal of the staining solution, the plates were washed with water; vi) the microbial biofilms formed on the plastic plates were resuspended in a 33% acetic acid solution. The intensity of the tinted suspension was macroscopically assessed in order to establish the minimal biofilm eradication concentration (MBEC).

3. Results

1. Qualitative screening of the antimicrobial activity

The initial qualitative screening was performed for identifying the antimicrobial spectrum of which of the tested molecules. An example of the obtained results is presented in the picture below (**figure 2**).

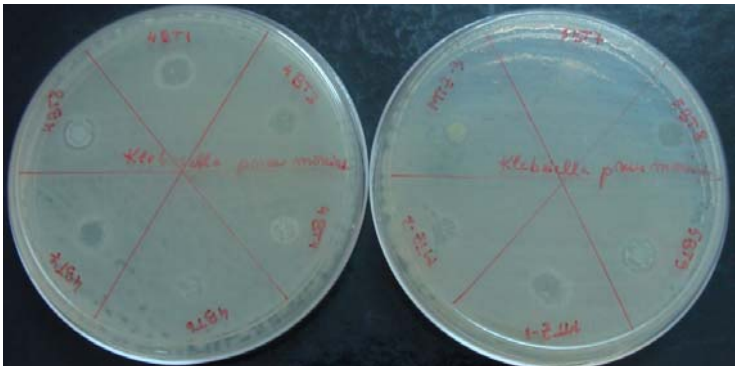


Fig. 2. The qualitative assessment of the antimicrobial activity- adapted disk diffusion technique

The diameters of the growth inhibition zones are presented in table II.

A relatively large spectrum of antimicrobial activity was exhibited by the compound **4BT1,** which was active against all tested strains, excepting *E. faecium* E5, followed by **4BT4**, active on all microbial strains, excepting the gram positive cocci. None of the tested molecules exhibited activity against *E. faecium* E5.

All tested compound exhibited antifungal activity, and the great majority were also active against *B. subtilis* ATCC 6683. Considering the high diameter value (10 mm), it seems that the most active compound against *B. subtilis* ATCC 6683 was **5BT1**.

The tested compounds were equally ineffective against *S. aureus* ATCC 6538, **4BT1** being the only active one.

A diverse spectrum of antimicrobial activity was also exhibited by compounds **4BT3**, **4BT7, 5BT1**, **5BT2, 5BT3 and 5 BT8,** which were active against four of the seven microbial tested strains.

It is to be noticed that the compounds **5BT1**, **5BT2** and **5BT3** exhibited a similar antimicrobial spectrum.

Table II. The results of the qualitative evaluation of the antibacterial activity (diameters of the growth inhibition zone are expressed in mm)

Microbial strain / **Compound**	*Enterococcus faecium E5*	*Staphylococcus aureus* ATCC 6538	*Bacillus subtillis* ATCC 6683	*Pseudomonas aeruginosa* ATCC 27857	*Klebsiella pneumoniae* IC 13420	*Escherichia coli* ATCC 25922	*Candida albicans* 393
4BT1	0	8	6	8	6	6	7
4BT3	0	0	5	8	5	0	5
4BT4	0	0	7	6	5	7	5
4BT6	0	0	0	5	0	0	5
4BT7	0	0	0	8	6	7	8
4BT8	0	0	6	5	0	0	6
4BT9	0	0	0	5	0	0	6
5BT1	0	0	10	7	0	6	6
5BT2	0	0	6	8	0	6	5
5BT3	0	0	5	5	0	5	7
5BT4	0	0	8	8	0	0	5
5BT6	0	0	4	5	0	0	5
5BT7	0	0	5	0	0	7	6
5BT8	0	0	6	0	7	6	5
5BT9	0	0	5	5	0	6	5
DMSO	0	0	0	0	0	0	0

On the other hand, compounds **4BT6**, **5BT6** and **4BT9** seem to be the least active compounds, in the studied series.

2. Quantitative assay of the antimicrobial activity

The MIC values obtained from this assay are shown in table III. Most of the results show a good correlation with the growth inhibition values obtained in the qualitative screening. The tested compounds showed a moderate antimicrobial action with MIC values ranging in the interval of 0.5 -1 mg/ml.

The most promising compound was **4BT4** that showed a good antibacterial action against *B. subtilis* ATCC 6683 and *E. coli* ATCC 259222, with MIC values of 0.5 mg/ml.

The tested molecules seem to have a particularly good antibacterial effect against *E. coli* ATCC 259222, as shown by the MIC values of 0.5 mg/ml obtained for 6 compounds: **4BT1**, **4BT4**, **4BT7**, **5BT7**, **5BT8**.

Also, a good antibacterial activity was shown by **4BT8** against *P. aeruginosa* ATCC 27857 (MIC=0.5 mg/ml). The compound **5BT3** showed a had a good anti-fungal activity against *C. albicans* 393.

Table III. The quantitative assay of the antimicrobial activity for the bisthiazoles derivatives studied. Results are expressed as MIC mg/ml.

Microbial strain / **Compound**	*Staphylococcus aureus* ATCC 6538	*Bacillus subtillis* ATCC 6683	*Pseudomonas aeruginosa* ATCC 27857	*Klebsiella pneumoniae* IC 13420	*Escherichia coli* ATCC 25922	*Candida albicans* 393
4BT1	>1	>1	>1	>1	0.5	1
4BT3	-	-	>1	-	-	-
4BT4	-	0.5	>1	-	0.5	-
4BT6	-	-	-	-	-	-
4BT7	-	-	>1	>1	0.5	>1
4BT8	-	>1	0.5	-	-	-
4BT9	-	-	-	-	-	-
5BT1	-	>1	>1	-	>1	-
5BT2	-	>1	>1	-	>1	-
5BT3	-	-	-	-	-	0.5
5BT4	-	>1	>1	-	-	-
5BT6	-	-	-	-	-	-
5BT7	-	-	-	-	0.5	-
5BT8	-	>1	-	1	0.5	-
5BT9	-	-	-	-	>1	-

*No quantitative tests were performed on *E. faecium* E5, considering the lack of activity seen in the preliminary screening assay.

3. Influence of the tested molecules on microbial biofilm development on inert substrata

The results of the anti-biofilm effect evaluation are shown in table IV. MBEC values of 0.25-0.5 mg/ml were considered as indicators for a good anti-biofilm activity. The most active

compound was **5BT8** with MBEC=0.25 mg/ml against against *E. coli* ATCC 259222 biofilm development. Moreover, 8 of the 15 tested compounds, including **5BT8**, exhibited a good anti-biofilm activity against *E. coli* ATCC 259222 strain.

Also, the compounds **4BT1**, **4BT3**, **4BT7**, **4BT8** and **5BT2** exhibited a good anti-biofilm activity against the gram negative strain *P. aeruginosa* ATCC 27857 with a MBEC of 0.5 mg/ml.

The compound **5BT3** is the only molecule that exhibits good anti-biofilm activity against the fungal strain *C. albicans* 393, which is consistent with the findings of the quantitative antimicrobial assay.

Table IV. Results of the anti-biofilm activity assay. Minimal biofilm eradication concentrations (MBEC) values are expressed in mg/ml

Microbial strain / **Compound**	*Staphylococcus aureus* ATCC 6538	*Pseudomonas aeruginosa* ATCC 27857	*Bacillus subtillis* ATCC 6683	*Klebsiella pneumoniae* IC 13420	*Escherichia coli* ATCC 25922	*Candida albicans* 393
4BT1	>1	0.5	>1	>1	0.5	1
4BT3	-	0.5	-	-	-	-
4BT4	-	>1	>1	-	0.5	-
4BT6	-	-	-	-	-	-
4BT7	-	0.5	-	1	0.5	>1
4BT8	-	0.5	>1	-	-	-
4BT9	-	-	-	-	-	-
5BT1	-	1	>1	-	0.5	-
5BT2	-	0.5	1	-	0.5	-
5BT3	-	-	-	-	-	0.5
5BT4	-	>1	>1	-	-	-
5BT6	-	-	-	-	-	-
5BT7	-	-	-	-	0.5	-
5BT8	-	-	1	>1	0.25	-
5BT9	-	-	-	-	0.5	-

*No quantitative tests were performed on *E. faecium* E5, considering the lack of activity seen in the preliminary assays.

4. Discussions

The increasing problem antimicrobial resistance amplified by the phenotypic tolerance of microbial biofilms to antibiotics and biocides is difficult to be solved, when considering the very limited number of new antimicrobial agents found in development on the market [26-29].

The development of new analogues starting from the current classes of antimicrobial substances could represent an easier alternative than the synthesis of novel compounds.

Current research is focusing on obtaining novel anti-pathogenic agents that interfere with the biofilm formation or with the expression of other virulence factors. Instead of microbicidal substances, these agents do not interfere with microbial growth, but with the coordinated expression of virulence potential, rendering pathogenic microbial agents harmless and also, decreasing the probability of selecting resistance.

The purpose of this work was to assess the microbicidal and anti-biofilm activity of novel derivatives bearing the thiazole ring, previously reported in the scientific literature for different biological activities, including the antimicrobial one.

The results of the qualitative antimicrobial assay showed a rather diverse antibacterial spectrum of the tested compounds. Moderate to good activity was shown against the majority of bacterial tested strains, with the exception of *E. faecium* E5 and *S. aureus* ATCC 6538.

A more accurate characterization of the antimicrobial potential was obtained through the two quantitative assays.

The MIC and MBEC assays indicated the most significant antibacterial activity of the tested compounds against *E. coli* ATCC 259222 strain. Five of the tested compounds, i.e. **4BT1**, **4BT4**, **4BT7**, **5BT7** and **5BT8** exhibited both a good antibacterial activity (MIC=0.5 mg/ml) and, in the same time, a good anti-biofilm development activity (MBEC=0.25-0.5 mg/ml). These correlations between the two properties suggest that these compounds could exhibit a more potent antibacterial activity, by coupling the bactericidal or bacteriostatic activity with the potential ability to prevent bacterial adhesion and colonization. The compounds **5BT1**, **5BT2**, and **5BT9,** also exhibited a good ability to prevent biofilm formation and colonization of inert substrata by the *E. coli* ATCC 259222 strain, but were not effective as antibacterial agents at concentrations <1 mg/ml.

Considering the activity against *P. aeruginosa* ATCC 27857, although five of the tested compounds have shown a potential to inhibit biofilm formation, only one of them (**4BT8**) has also shown a good antibacterial activity against this strain. The compounds that inhibit only biofilm formation and do not have a good antibacterial effect can be considered as adjuvants for usage together with other antibiotic therapy or for the design of anti-microbial surfaces.

The antifungal activity of the tested compounds was modest, only **5BT3** exhibiting both a good antimicrobial and anti-biofilm activity at MIC/MBEC=0.5 mg/ml, while the compound **4BT1** was active in both quantitative assays at MIC/MBEC= 1 mg/ml.

The promising activity shown by **4BT1** against *S. aureus* ATCC 6538 strain, in the preliminary qualitative assay (diameter of the growth inhibition zone= 8 mm) was not supported by any of the quantitative assays.

The 2 series of compounds 4,2 bisthiazoles and 5,2 bisthiazoles are very similar from a structural point of view, both of them having a 2-phenylthiazole core substituted in the 4 or 5 position of the thiazole ring with a 2-thiazolyl- 4-aryl moiety that is identical for the compounds with the same number (e.g. **4BT1** and **5BT1**).

The comparison of the antimicrobial activity of the tested compounds revealed how important the small structural changes are. For example compounds **4BT8** and **5BT8** share a common 2-hidroxy-5-benzamidyl moiety**.** While **4BT8** is the most active compound against *P. aeruginosa* ATCC 27857, its 5,2 bisthiazoles counterpart **5BT8** proved to be totally ineffective against this strain (d=0 mm). In the same time, **5BT8** is the most active tested compound against the *E. coli* ATCC 259222 strain (MIC=0.25 mg/ml) and is also moderately active against *K. pneumoniae* IC 13420, while **4BT8** is totally ineffective against these strains (d=0 mm, Table II). These differences could be probably explained by the different 3D shape of the molecules and thus their different ability to interact with the biological targets.

Compounds from the same series, with mild structural differences showed similar antimicrobial patterns, an example being supported by the compounds **5BT1**, **5BT2** and **5BT3** that only differ through the R_2 methyl substituent on the thiazole ring (see table I) and have similar antimicrobial properties (see tables II and III).

5. Conclusions

A number of 15 new molecules with a 4,2 or a 5,2 bisthiazoles structure were tested, through qualitative and quantitative assays, for their antimicrobial and anti-biofilm properties. The tested compounds showed a relatively diverse spectrum of antimicrobial activity, with moderate to good inhibitory effects. The most significant results were obtained for *P. aeruginosa* ATCC 27857 and *E. coli* strains, demonstrating a good interaction of the obtained compounds with the Gram-negative bacterial cellular structures; further studies are required to elucidate the specific targets of these compounds, in order to improve the design of future bisthiazoles derivatives active of Gram-negative bacteria, for which the need for new drugs is critical.

Acknowledgements

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