Full Paper

Synthesis and Biological Evaluation of 2-Mercapto-1,3-benzothiazole Derivatives with Potential Antimicrobial Activity

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The enhancement of bacterial resistance of pathogens to currently available antibiotics constitutes a serious public health threat. So, intensive efforts are underway worldwide to develop new antimicrobial agents. To identify compounds with a potent antimicrobial profile, we designed and synthesized low molecular weight 2-mercaptobenzothiazole derivatives **2a – 2l** and **3a – 3l**. Both series were screened for *in-vitro* antibacterial activity against the representative panel of Gram-positive and Gram-negative bacteria strains. The biological screening identified compounds **2e** and **2l** as the most active ones showing an interesting antibacterial activity with MIC values of 3.12 μg/mL against *Staphylococcus aureus* and 25 μg/mL against *Escherichia coli*, respectively. The replacement of the S-H by the S-Bn moiety resulted in considerable loss of the antibacterial action of the **3a – 3l** series. The antibiotic action of compounds **2e** and **2l** was also investigated by testing their activity against some clinical isolates with different antimicrobial resistance profile. Moreover, the involvement of the NorA efflux pump in the antibacterial activity of our molecules was evaluated. Finally, in this paper, we also describe the cytotoxic activity of the most interesting compounds by MTS assay against HeLa and MRC-5 cell lines.

Keywords: Antibacterial activity / Benzothiazole / Efflux pump / MTS assay

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Introduction

The current interest in the development of new antimicrobial agents can be partially ascribed both to the increasing emergence of bacterial resistance to antibiotic therapy and to newly emerging pathogens [1, 2]. Despite advances in antibacterial therapy, many problems remain to be solved for most available antimicrobial drugs. For example, in the hospital setting, the re-emergence of Gram-negative pathogens is of major concern. In fact, the most important cases of sepsis were caused by virulent Gram-negative bacteria such as *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*, and *Enterobacter* spp. [3, 4]. Furthermore, emerging resistance among new pathogens such as *Acinetobacter baumannii*, and also the appearance of multidrug resistant Gram-positive bacteria, in particular, methicillin-resistant *Staphylococcus aureus (MRSA)* and vancomycin-resistant *Enterococci*, are causing a serious menace to public health. Therefore, the development of new and different antimicrobial drugs is a very important goal, and most of the research program efforts in this field are directed towards the design of new agents. A review of the recent literature revealed that many effective antimicrobial agents show a heterocyclic moiety within their structure [5] and, in particular, that substituted benzimidazole, benzoxazole,

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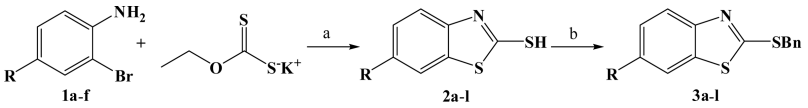
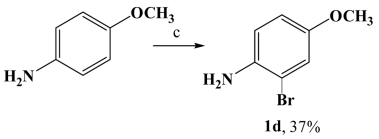
Abbreviations: National Committee on Clinical Laboratory Standards (NCCLS); norfloxacin (NRF); oxacillin (OXA); vancomycin (VAN)



Entry (aniline)	R	Yield	Yield
1a	$CH(CH_3)_2$	**2a**, 88%	**3a**, 98%
1b	Cl	**2b**, 83%	**3b**, 92%
1c	CH_3	**2c**, 77%	**3c**, 75%
1d	OCH_3	**2d**, 90%	**3d**, 76%
1e	CF_3	**2e**, 99%	**3e**, 98%
1f	F	**2f**, 98%	**3f**, 71%
	H	**2g**, comm. av.[§]	**3g**, 91%
	NH_2	**2h**, comm. av.[§]	**3h**, 69%
	OCH_2CH_3	**2i**, comm. av.[§]	**3i**, 96%
	NO_2	**2l**, comm. av.[§]	**3l**, 77%



Reagents and conditions: a) DMF, 160°C, 1 h; b) K_2CO_3, H_2O/dioxane, 75°C, 15 min; c) Ar-CH_2-Br, Bu_4NBr_3, *p*-anisidine, CH_2Cl_2/CH_3OH, r.t., 2 h.

Scheme 1. Synthetic routes of compounds **1d**, **2a–2f**, and **3a–3l**.

and benzothiazole derivatives bring different biological properties such as chemotherapeutical, antibacterial, antifungal, and antiviral activities, with a low toxicity for the antimicrobial therapeutic use in man [6–8]. Structure-activity relationship (SAR) studies carried out on these types of heterocycles have shown that positions 2 and 6 are crucial for antibacterial activity against Gram-positive and Gram-negative bacteria strains [9].

All these observations prompted us to start a research program for the synthesis of small molecules potentially useful as antimicrobial agents. After a careful screening of various heteronuclei, we have chosen to focus our attention on benzothiazole derivatives. In particular, we synthesized 2-mercaptobenzothiazole derivatives **2a–2f** and **3a–3l** in order to explore the effects of substituents at positions 2 and 6 on the antibacterial activity. Especially, the main objective of our program was to investigate how the potency and selectivity against different Gram-positive (*Staphylococcus aureus, Bacillus cereus, Bacillus subtilis, Enterococcus faecalis*) and Gram-negative (*Escherichia coli, Acinetobacter baumannii, Klebsiella pneumoniae, Pseudomonas aeruginosa*) bacteria, can be modulated by the replacement of the hydrogen at the 6-position of the heterocyclic nucleus with groups that can generate electronic and electrostatic effects as well as different steric properties. In addition, we also investigated the role of lipophilicity on the antibacterial activity through the synthesis of the benzylated series **3a–3l**. Although compounds of the **2a–2l** series are commercially available, they have been included in our research program to screen their antimicrobial profile in order to have more data for SAR (structure-activity relationship) proposal. Because of the prohibitive price, we synthesized compounds **2a–2f** *ex novo* in short reaction time, with very good yields and in a cheap synthetic procedure.

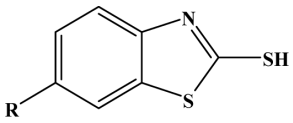
In the present work, we report the synthesis of two series of 2-mercaptobenzothiazoles **2a–2f** and **3a–3l** and their preliminary antibacterial profile against different Gram-positive and Gram-negative bacterial strains belonging to American Type Culture Collection (ATCC). The most active compounds of the series were also studied by using clinical isolates *S. aureus* with different antibiotic resistance profile. Finally, the most interesting molecules were also characterized with regard to their cytotoxic effects by testing them against a human cervical cancer cell line (HeLa) and a normal human lung fibroblasts cell line (MRC-5).

Results and discussion

Chemistry

The synthetic routes of compounds **1d**, **2a–2f**, and **3a–3l** are reported in Scheme 1. The 6-substituted-2-mercapto-

Table 1. Antimicrobial activity results[a] of 2-mercaptobenzothiazole derivatives **2a**–**2l** (MIC in μg/mL).



		Microorganism											
		Gram-positive						Gram-negative					
Compd	R	*S. a.*[b] 6538P	*S. a.*[b] 25923	*S. a.*[b] 29213	*E. f.*[c] 19433	*E. f.*[c] 29212	*B. s.*[d] 6633	*B. c.*[e] 11778	*E. c.*[f] 8739	*E. c.*[f] 35218	*A. b.*[g] 19606	*K. p.*[h] 13883	*P. a.*[i] 27853
2a	$CH(CH_3)_2$	12.5	50	25	50	50	25	25	R	R	R	R	R
2b	Cl	25	25	12.5	50	100	50	50	R	R	R	R	R
2c	CH_3	25	50	50	100	100	50	25	100	50	100	100	R
2d	OCH_3	12.5	50	50	50	50	25	25	50	100	R	R	R
2e	CF_3	6.25	6.25	3.12	100	100	50	50	R	R	100	R	R
2f	F	50	100	25	R	R	100	100	R	R	R	R	R
2g	H	50	100	50	100	100	50	50	50	50	100	100	R
2h	NH_2	12.5	25	25	50	50	25	25	R	R	R	R	R
2i	OCH_2CH_3	25	100	50	R	R	50	50	R	R	R	R	R
2l	NO_2	12.5	25	12.5	100	100	50	50	25	50	100	50	R
NRF[+]		0.25	0.5	0.5	4	2	0.125	0.25	0.06	0.06	4	0.25	2

[a] Antimicrobial activity was estimated by using NCCLS assay [12].
Abbreviations: [b] *S. a., S. Aureus*, [c] *E. f., E. Faecalis*, [d] *B. s., B. Subtilis*; [e] *B. c., B. Cereus*, [f] *E. c., E. Coli,* [g] *A. b., A. Baumannii*, [h] *K. p., K. Pneumoniae*, [i] *P. a., P. aeruginosa.*
+ NRF, norfloxacin; R, resistant.
Acceptable quality-control range of MICs for reference strains [NCCLS M7-A4]: *S. aureus* ATCC 29213: 0.5-2; *E. faecalis* ATCC 29212: 2-8; *P. aeruginosa* ATCC 27853: 1 – 4.

benzothiazole derivatives **2a**–**2f** were prepared according to the literature procedure [10]. Reaction of the appropriate anilines **1a**–**1c**, **1e**, **1f**, commercially available, with potassium ethyl xanthate in DMF gave the corresponding 6-substituted-1,3-benzothiazole-2-thiols **2a**–**2c**, **2e**, **2f**, respectively. Compounds **2g**–**2l** are commercially available.

The **3a**–**3l** series was prepared by the treatment of 6-substituted-1,3-benzothiazole-2-thiols **2a**–**2l** with benzyl bromide, K_2CO_3, dissolved in a mixture of dioxane/water, providing the corresponding 2-benzylthio-6-substituted-1,3-benzothiazoles derivatives.

The starting 2-bromo-4-methoxyaniline **1d** was synthesized according to a reported methodology via a bromination strategy by using tetrabuthylammonium tribromide (Bu_4NBr_3) following a synthetic procedure described in the literature [11].

As it is outlined in Scheme 1, compounds **2a**–**2f** and **3a**–**3l** were synthesized in high yields. The structures of the obtained compounds were elucidated by spectral data.

Biological evaluation

The antibacterial activity of compounds **2a**–**2l** was evaluated *in vitro* against an assortment of Gram-positive and Gram-negative bacteria belonging to the ATCC collection. All MIC determinations were carried out using NCCLS guidelines [12]. MIC values are given in μg/mL and were compared to MIC values for the standard antibacterial drug norfloxacin. Screening results are summarized in Table 1.

The combined data showed that compounds **2a**–**2l** exerted inhibitory activity against the tested bacterial strains with MIC values between 3.12 and 100 μg/mL. The obtained results generally indicate that most of the tested molecules are more active against Gram-positive than Gram-negative bacterial strains. Among the mentioned derivatives **2a**–**2l**, the most promising results were obtained with compounds **2e** and **2l**. In particular **2e** was the most active derivative giving the best antibacterial activity against *S. aureus* with a MIC value of 3.12 μg/mL. On the other hand, compound **2l** showed a wide antimicrobial activity toward Gram-positive such as *S. aureus* (MIC: 12.5 μg/mL) and Gram-negative such as *E. coli* (MIC: 25 μg/mL). Furthermore, it is noteworthy that the data registered in Table 1 reveal that **2a**–**2l** derivatives generally have a significant influence on the antibacterial profile of *S. aureus*. In this series, compounds **2a**, **2b**, **2d**, **2h**, and **2l** were found to inhibit *S. aureus* at a MIC

value of 12.5 μg/mL. In addition, compounds **2c, 2d**, and **2g** revealed moderate antimicrobial activity against Gram-negative bacteria such as *E. coli* strains.

With regards to the antimicrobial activity, the behaviour of the **3a–3l** series was very different compared with the **2a–2l** series: under the same experimental conditions it was found that derivatives having a thiobenzyl group at the 2-position of the heterocyclic nucleus did not inhibit the bacterial growth in spite of biological results previously observed on benzothiazole derivatives [9].

Due both to the small numbers of evaluated compounds and the low diversity of the involved chemical features of the series reported herein, an attempt to analyse the structure-activity relationships does not seem reasonable. Several comparisons on the results could be made. Initially, it should be noted that small structural changes at the 6-position do not significantly alter the antibacterial activity, except for compounds **2e** and **2l** carrying a trifluoromethyl group and a nitro group at the 6-position of the 2-mercapto 1,3-benzothiazole, respectively. In detail, the biological results identified compound **2e** as a potent and selective inhibitor of *S. aureus* strains (MIC: 3.12 μg/mL) and whereas compound **2l** was less active than **2e** against Gram-positive bacteria, it was able to inhibit the growth of Gram-negative microorganism such as *E. coli* (MIC: 25 μg/mL).

By evaluating the biological results, it is possible to observe that the presence of a hydrogen atom (**2g**) or a methyl group (**2c**) at the 6-position on the heterocyclic nucleus, preserves a wide spectrum of action. In addition, it is interesting to consider that an increase of steric hindrance, though an increment of lipophilicity, probably could lead to a loss of activity against Gram-negative strains, as observed for compound **2a**.

Moreover, the displacement of the hydrogen atom (**2g**) at the 6-position with chlorine (**2b**) and fluorine (**2f**) atoms as well as with methoxy (**2d**) and ethoxy (**2i**) groups generally produced an increment in the activity against *S. aureus* with MIC values in the range of 12.5 to 100 μg/mL. Furthermore, the introduction of a polar group such as the aminic one (**2h**) led to an interesting antibacterial activity against Gram-positive strains.

Because among the mentioned derivatives a remarkable activity was registered against *S. aureus* strains, we focused our attention on our best molecules, **2e** and **2l**, to investigate their antimicrobial profile against seven clinical isolates of *S. aureus* belonging both to NARSA and private collections with a different antimicrobial resistance profile as detailed in the experimental section. In Table 2 the microbiological data obtained according to NCCLS protocol [12] are reported and compared with norfloxacin (NRF), vancomycin (VAN), and oxacillin (OXA) used as standard drugs. From the results reported in Table 2, it appears that compound **2e** generally revealed better growth inhibitory effects against the clinical isolates than compound **2l**. In particular, compound **2e** was surprisingly efficacious against two vancomycin-resistant clinical isolate strains (VRS2 and NRS52) which were close to VAN in the control experiments. In detail, compound **2e** displayed an interesting antibacterial activity against VRS2, a vancomycin fully resistant strain, showing a MIC value three-fold smaller than the MIC value registered for the commercially available drug VAN (MIC values: 8 μg/mL *vs.* 64 μg/mL, respectively). Additionally, compound **2e** was active against NRS52, a vancomycin intermediate resistant strain (VISA), having a MIC value of 8 μg/mL comparable to the reference drug VAN.

Table 2. Antimicrobial activity results (MIC in μg/mL) of compounds **2e** and **2l** against clinical isolates of *S. aureus*.

	2e	**2l**	**NRF**[a]	**VAN**[b]	**OXA**[c]
S. a. 25923	4	16	1	n. e.	n. e.
VRS2	8	32	128	64	128
NRS52	8	16	256	4	0.5
N4120032	16	32	256	1	16
N4112910	8	32	256	1	128
NRS100	16	32	1	1	256
STA268	8	32	1	1	n. e.

[a] NRF, Norfloxacin.
[b] VAN, Vancomycin.
[c] OXA, Oxacillin.

To further investigate the antibacterial effects in several clinical isolates, molecules **2e** and **2l** were tested against two different methicillin-resistant bacteria strains (MRSA) such as N4120032 and N4112910. As shown in Table 2, both compound **2e** and **2l** possessed interesting antimicrobial effects against N4112910 bacteria strain in comparison to the control drugs NRF and VAN. The biological data reported in Table 2 indicate that compound **2e** was more active against N4112910 rather than N4120032 when compared to OXA, showing MIC values of 8 μg/mL and 16 μg/mL, respectively. Despite this relevant data, it is worthy to note that compound **2e** inhibited the growth of the clinical isolate N4120032 with a MIC value similar to the reference drug OXA. Taking into account that the last-mentioned bacteria strain belongs to the HA-MRSA family characterized by high morbidity and mortality degrees, data registered for compound **2e** appear to be very significant.

Moreover, compound **2e** showed a remarkable antimicrobial profile against the multi-drug resistant strain NRS100, an oxacillin/tetracycline-resistant strain, with a MIC value of 16 μg/mL in comparison to 256 μg/mL of OXA.

Table 3. Antimicrobial activity results (MIC in μg/mL) on NorA over-expressing strain with and without reserpine.

	ATCC 25923		SA-1 (NorA over-expr.)	
Compound	Res -	Res +	Res -	Res +
NRF	1	1	16	4
2e	4	4	64	64
2l	16	16	128	128

Finally, the antibacterial activity exhibited by both compound **2e** and **2l** against STA268, a potentially lethal strain producing the Panton–Valentine Leukocidin (PVL+) [13], seems to be negligible in comparison to NRF and VAN.

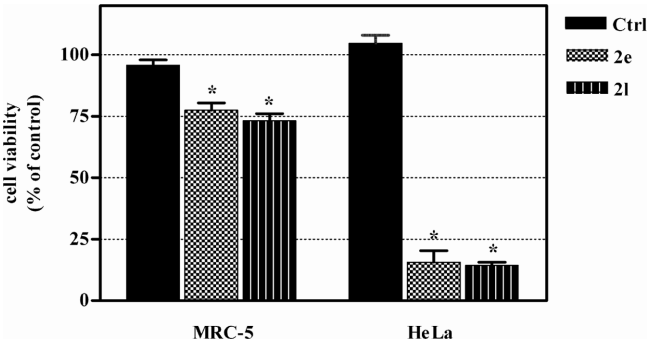
Results obtained during our preliminary investigation caused us to carry out additional tests using multidrug-resistant bacteria SA-1, a modified *S. aureus* strain that overexpresses the NorA, the most studied efflux pump at the present time [14].

Generally, the overexpression of multidrug-resistance (MDR) efflux pumps confer clinically relevant resistance to antibiotics (*e.g.* fluoroquinolones), dyes, detergents, and disinfectants. In particular, the typical substrate profile of NorA includes quinolones, chloramphenicol, and several unrelated substances [15].

The literature clearly describes a detailed biological assay used to evaluate the NorA involvement in the mechanism of antibacterial multidrug resistance, by using reserpine as NorA blocker [16, 17]. In fact, this alkaloid seems to be able to inhibit multidrug transporters like NorA, increasing the intracellular concentration of fluorochinolones, thus potentially lowering MICs.

In light of this evidence, we investigated the involvement of the NorA efflux pump in the mechanism of action of our best molecules **2e** and **2l** by using the biological assay reported before and detailed in the experimental section, herein. The antimicrobial results exhibited by compounds **2e** and **2l** against both wild-type and SA-1 bacteria strains were compared with the control drug NRF as reported in Table 3. By reading Table 3, it is clear that the standard drug NRF produced a lower SA-1 bacteria growth in the presence, rather than in absence, of reserpine (MIC values: 4 μg/mL *vs.* 16 μg/mL, respectively) suggesting that the latter is a good substrate for the NorA efflux pump. Herein, we also noticed that molecules **2e** and **2l** exhibited comparable inhibition of bacteria growth in absence so as in presence of reserpine indicating that these compounds are not involved in this active efflux system.

This interesting result suggests that both compounds **2e** and **2l** could be considered as candidates for new tools for the synthesis of antibacterial compounds acting on resistant bacterial strains overexpressing NorA efflux pump.



Vehicle (DMSO 0.1%) was used as control. Each experiment was performed in quadruplicate; data are expressed as mean ± S.E.M of three to five experiments. * $p < 0.0001$ *vs.* respective control.

Figure 1. Cytotoxicity of compounds **2e** and **2l** (100 μM) in MRC-5 and HeLa cells.

Finally, taking into account the well-known antimicrobial and anticancer activities of benzothiazole derivatives, structurally related to our 2-mercaptobenzothiazole compounds but, of course, with different chemical features, we also investigated the cytotoxic activity of the molecules with most meaningful antibacterial activity, **2e** and **2l**. This study was realized by using the MTS assay against HeLa and MRC-5 cell lines, following a 3-days exposure [18].

In general, the cancer cells were more sensitive to the tested agents. As shown in Fig. 1, the derivatives **2e** and **2l** tested at the single dose of 100 μM, did not produce a relevant change in cell viability in MRC-5 cells (maximal inhibition of cell growth never exceeded 25%), whereas their cytotoxic effect in HeLa cells was remarkable (about 80% of inhibition).

Data obtained confirm that our tested molecules can be considered moderately toxic for HeLa cells while they lack of any toxicity for normal cells like MRC-5.

Conclusion

In summary, in the present study, we report the synthesis and the antimicrobial studies of 2-mercapto 1,3-benzothiazole derivatives. It was observed that the synthesized compounds substituted with a S-H moiety at the 2-position of the heterocyclic nucleus (**2a**–**2l**) favored the antibacterial activity especially against the Gram-positive strains. On the contrary, compounds bearing the S-Bn moiety **3a**–**3l** at the 2-position of the benzothiazole nucleus did not show any antimicrobial profile.

Among the series **2a–2l**, the most prominent and consistent antimicrobial activity was obtained with compound **2e** (MIC: 3.12 μg/mL) carrying a trifluoromethyl moiety at the 6-position of the heterocycle. Compound **2l** showed an appreciable broad spectrum of action against both Gram-positive and Gram-negative bacteria. Its MIC value (25 μg/mL) toward *E. coli* is very significant.

Interesting, cytotoxicity against the MRC-5 cells was not observed for compounds **2e** and **2l**. In light of the results presented in this work and taking into account that this preliminary study does not produce conclusive evidence regarding a structure-antibacterial relationship, we stopped our attention on the most promising compounds **2e** and **2l** as an interesting starting point for the development of a new class of antimicrobial agents. Therefore, the synthesis of novel 2-mercaptobenzothiazole derivatives aimed to optimize the chemical features involved in the antibacterial activity such as to investigate the mechanism of action is currently going on our laboratory.

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The authors have declared no conflict of interest.

Experimental

Chemistry

Melting points were recorded on Gallenkamp melting point apparatus (Weiss-Gallenkamp, London, UK) in open glass capillary tubes. The IR spectra were recorded on a Perkin-Elmer Spectrum One FT spectrophotometer (Perkin-Elmer, Norwalk, CT, USA) and band positions are given in reciprocal centimeters (cm^{-1}). ^{1}H-NMR spectra were recorded on a FT Bruker Aspect 3000 spectrometer (Bruker Bioscience, USA) using $CDCl_3$ as the solvent, unless otherwise indicated. Chemical shifts are reported in part per million (ppm) relative to the solvent resonance: $CDCl_3$, δ = 7.26 (^{1}H-NMR). Amino-proton assignments were confirmed by D_2O exchange. *J* values are given in Hz. EIMS spectra were recorded with a Hewlett-Packard 6890-5973 MSD gas chromatograph / mass spectrometer at low resolution (Hewlett-Packard, Palo Alto, CA, USA). Elemental analyses were performed on a Eurovector Euro EA 3000 elemental analyzer (EuroVector, Milan, Italy). The data for C, H, and N were within ± 0.4 of the theoretical values for all final compounds. Silica gel chromatographic separations were performed by chromatography with silica gel (Kieselgel 60, 40–63 μm; Merck, Germany) packed in glass columns, using the technique described in the literature [19]. The weight of the silica gel was approximately 100-times that of the substance. The eluting solvent indicated in parentheses for each purification was determined by TLC performed on precoated silica gel on aluminum sheets (Kieselgel 60, F_{254}, Merck). TLC plates were visualized with UV light and/or in an iodine chamber. All chemicals, compounds **2g–2l** included, were purchased from Aldrich Chemical Co. (Sigma-Aldrich, Germany) in the highest quality commercially available. The structures of the compounds were confirmed by routine spectrometric and spectroscopic analyses. Only spectra for compounds not previously described are given.

*Synthesis of 2-bromo-4-methoxyaniline **1d***

Compound **1d** was prepared according to the literature procedure [11] starting from 4-methoxyaniline (commercially available). Column chromatography (silica gel, eluent: Et_2O/petroleum ether, 4:6) of the reaction crude provided 2-bromo-4-methoxyaniline **1d** as purple oil (37% yield). ^{1}H-NMR and MS spectra were in agreement with those reported in the literature.

*General procedure for the synthesis of 6-substituted-1,3-benzothiazole-2-thiols **2a–2f***

6-Substituted-1,3-benzothiazole-2-thiols **2a–2c**, **2e**, **2f** were prepared from the reaction of the corresponding aniline derivatives **1a–1c**, **1e**, **1f**, commercially available, with potassium ethyl xanthate by using a literature procedure [10], while **2d** was prepared using aniline derivative **1d** (see above).

*6-(1-Methylethyl)-1,3-benzothiazole-2-thiol **2a***

Yield: 88%; m.p.: 133–135°C (EtOAc); IR ($CHCl_3$): 3092, 2911, 1496, 1257, 895 cm^{-1}; ^{1}H-NMR was in agreement with the published data [10]. MS (70 eV) *m/z* (%): 211 [M^+ + 2] (8), 210 [M^+ + 1] (10), 209 [M^+] (72), 194 (100), 161 (15). Anal. Calcd. for $C_{10}H_{11}NS_2$: C, 57.38; H, 5.30; N, 6.69; found: C, 57.17; H, 5.14; N, 6.71.

*6-Chloro-1,3-benzothiazole-2-thiol **2b***

Yield: 83%; m.p.: >245°C (EtOAc/petroleum ether); IR (KBr): 3081, 2913, 1486, 1240, 874 cm^{-1}; ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 13.9 (br s, 1H), 7.81 (s, 1H), 7.40 (d, *J* = 8.5 Hz, 1H), 7.24 (d, *J* = 8.8 Hz, 1H); MS (70 eV) *m/z* (%): 203 [M^+ + 2] (47), 202 [M^+ + 1] (12), 201 [M^+] (100), 166 (35). Anal. calcd. for $C_7H_4ClNOS_2$: C, 41.69; H, 2.00; N, 6.94; found: C, 41.38; H, 2.15; N, 6.92.

*6-Methyl-1,3-benzothiazole-2-thiol **2c***

Yield: 77%; m.p.: 180–181°C (EtOAc/petroleum ether); IR ($CHCl_3$): 3094, 2914, 1494, 1253, 893 cm^{-1}; ^{1}H-NMR and MS were in agreement with the published data [10]. Anal. calcd. for $C_8H_7NS_2 \cdot 0.25\ H_2O$: C, 51.72; H, 4.07; N, 7.54; found: C, 51.76; H, 3.73; N, 7.48.

*6-Methoxy-1,3-benzothiazole-2-thiol **2d***

Yield: 90%; m.p.: 206–207°C (EtOAc/petroleum ether); IR (KBr): 3036, 2936, 1495, 1296, 893 cm^{-1}; ^{1}H-NMR (300 MHz) δ 13.2–13.9 (br s, 1H), 7.26 (s, 1H), 7.02–6.97 (m, 2H), 3.82 (s, 3H); MS (70 eV) *m/z* (%): 199 [M^+ + 2] (12), 198 [M^+ + 1] (13), 197 [M^+] (100), 182 (78), 154 (17). Anal. calcd. for $C_8H_7NOS_2 \cdot 0.12\ H_2O$: C, 48.16; H, 3.66; N, 7.02; found: C, 48.02; H, 3.35; N, 6.85.

*6-Trifluoromethyl-1,3-benzothiazole-2-thiol **2e***

Yield: 99%; m.p.: >240°C (EtOAc/petroleum ether); IR (KBr): 3106, 2931, 1487, 1262, 882 cm^{-1}; ^{1}H-NMR was in agreement with the published data [20]. MS (70 eV) *m/z* (%): 237 [M^+ + 2] (12), 236 [M^+ + 1] (13), 235 [M^+] (100), 157 (10). Anal. calcd. for $C_8H_4F_3NS_2$: C, 40.84; H, 1.71; N, 5.95; found: C, 41.08; H, 1.71; N, 5.86.

6-Fluoro-1,3-benzothiazole-2-thiol ***2f***

Yield: 98%; m.p.: 227 – 228°C (EtOAc/petroleum ether); IR (KBr): 3442, 2938, 1946, 1287, 911 cm^{-1}; ^{1}H-NMR (300 MHz) *d*: 10.5 (br s, 1H), 7.21 – 7.03 (m, 3H).; MS (70 eV) *m/z* (%): 187 [M^+ + 2] (1), 186 [M^+ + 1] (4), 185 [M^+] (100), 153 (27), 126 (16). Anal. calcd. for $C_7H_4FNS_2 \cdot 0.75\ H_2O$: C, 42.30; H, 2.79; N, 7.05; found: C, 42.19; H, 2.38; N, 6.70.

General procedure for the synthesis of 2-(benzylthio)-6-substituted-1,3-benzothiazoles ***3a – 3l***

The preparation of 2-(benzylthio)-6-isopropyl-1,3-benzothiazole **3a** can be taken as the reference for the synthesis of 2-(benzylthio)-6-substituted-1,3-benzothiazoles **3a – 3l**. 6-Isopropyl-1,3-benzothiazole-2-thiol **2a** (1.91 mmol) in dioxane (16 mL), was added to a solution of K_2CO_3 (2.87 mmol) in water (5 mL). The reaction mixture was stirred at 75°C. Then, a solution of benzyl bromide (2.1 mmol) in dioxane (16 mL) was added dropwise over 15 min. When the addition was completed, the resulting mixture was stirred for another 15 min. The dioxane was evaporated under reduced pressure and the residue was extracted with EtOAc and washed with 2 N NaOH (3 × 20 mL) up to pH 11. The organic phase was dried over Na_2SO_4, filtered, and evaporated to dryness. Column chromatography (silica gel, eluent: EtOAc/petroleum ether, 3:7) of the reaction crude afforded the desired compound **3a**.

2-(Benzylsulphanyl)-6-(1-methylethyl)-1,3-benzothiazole ***3a***

Yield: 98%; m.p.: 80 – 81°C (EtOAc); IR (KBr): 3058, 2950, 1438, 1237, 830 cm^{-1}; ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 7.85 (s, 1H), 7.76 (d, *J* = 8.5 Hz, 1H), 7.46 (d, *J* = 8.2 Hz, 2H), 7.38 – 7.25 (m, 4H), 4.61 (s, 2H), 2.92 (m, *J* = 7.2 Hz, 1H), 1.21 (d, *J* = 7.2 Hz, 6H); MS (70 eV) *m/z* (%): 301 [M^+ + 2] (11), 300 [M^+ + 1] (21), 299 [M^+] (100), 267 (17), 266 (85), 91 (63.4). Anal. calcd. for $C_{17}H_{17}NS_2$: C, 68.19; H, 5.72; N, 4.68; found: C, 68.47; H, 5.84; N, 4.62.

2-(Benzylsulphanyl)-6-chloro-1,3-benzothiazole ***3b***

Purified by chromatography (EtOAc/petroleum ether, 3:7). Yield: 92%; m.p.: 80 – 82°C (EtOAc/petroleum ether); IR (KBr): 3068, 2922, 1431, 1297, 861 cm^{-1}; ^{1}H-NMR (300 MHz) δ: 7.78 (d, *J* = 8.5 Hz, 1H), 7.72 (s, 1H), (m, 6H), 4.59 (s, 2H); MS (70 eV) *m/z* (%): 293 [M^+ + 2] (26), 292 [M^+ + 1] (11), 291 [M^+] (62), 258 (38), 91 (100). Anal. calcd. for $C_{14}H_{10}ClNS_2$: C, 57.62; H, 3.45; N, 4.80; found: C, 57.86; H, 3.51; N, 4.83.

2-(Benzylsulphanyl)-6-methyl-1,3-benzothiazole ***3c***

Yield: 75%; m.p.: 54 – 55°C (EtOAc); IR (KBr): 3053, 2915, 1436, 1240, 873 cm^{-1}; ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 7.79 – 7.71 (m, 2H), 7.47 (d, *J* = 5.9 Hz, 2H), 7.36 – 7.23 (m, 4H), 4.61 (s, 2H), 2.39 (s, 3H); MS (70 eV) *m/z* (%): 273 [M^+ + 2] (11), 272 [M^+ + 1] (19), 271 [M^+] (100), 238 (82), 91 (85.1). Anal. calcd. for$C_{15}H_{10}NS_2$: C, 66.38; H, 4.83; N, 5.16; found: C, 66.56; H, 4.87; N, 5.16.

2-(Benzylsulphanyl)-6-methoxy-1,3-benzothiazole ***3d***

Yield: 76%; m.p.: 93 – 95°C (EtOAc); IR (KBr): 3444, 2922, 2851, 1702, 1641, 1600, 1435, 1059, 999 cm^{-1}; ^{1}H-NMR (300 MHz) δ: 7.80 (d, *J* = 9.07 Hz, 1H), 7.46 – 7.42 (m, 1H), 7.36-7.21 (m, 5H), 7.03 (dd, $J_{1\text{-}2}$ = 2.74 Hz, J_{1-3} = 8.35 Hz, 1H), 4.59 (s, 2H), 3.89 (s, 3H); MS (70 eV) *m/z* (%): 289 [M^+ + 2] (11), 288 [M^+ + 1] (19), 287 [M^+] (90), 254 (100), 91 (73.3). Anal. calcd. for $C_{15}H_{13}NOS_2$: C, 62.69; H, 4.56; N, 4.87; found: C, 63.01; H, 4.72; N, 4.74.

2-(Benzylsulphanyl)-6-trifluoromethyl-1,3-benzothiazole ***3e***

Yield: 98%; m.p.: 102-104°C (EtOAc); IR (KBr): 2925, 1566, 1437, 1308, 1235, 993 cm^{-1}; ^{1}H-NMR (300 MHz) δ: 8.15 – 7.92 (m, 2H), 7.65 (d, *J* = 7.9 Hz, 1H), 7.45 (d, *J* = 7.65 Hz, 2H), 7.38 – 7.22 (m, 3H), 4.62 (s, 2H); MS (70 eV) *m/z* (%): 327 [M^+ + 2] (6), 326 [M^+ + 1] (10), 325 [M^+] (52), 292 (19), 91 (100). Anal. calcd. for $C_{15}H_{10}F_3NS_2$: C, 55.37; H, 3.10; N, 4.30; found: C, 55.40; H, 3.09; N, 4.32.

2-(Benzylsulphanyl)-6-fluoromethyl-1,3-benzothiazole ***3f***

Yield: 71%; m.p.: 69 – 70°C (EtOAc); IR (KBr): 2923, 1565, 1437, 1307, 1238, 991 cm^{-1}; ^{1}H-NMR (300 MHz) δ: 7.86 – 7.79 (m, 1H), 7.47 – 7.41 (m, 3H), 7.38 – 7.27 (m, 3H), 7.18 – 7.11 (m, 1H), 4.59 (s, 2H); MS (70 eV) *m/z* (%): 277 [M^+ + 2] (7), 276 [M^+ + 1] (10), 275 [M^+] (61), 242 (33), 91 (100). Anal. calcd. for $C_{14}H_{10}FNS_2$: C, 61.37; H, 3.66; N, 5.09; found: C, 61.45; H, 3.87; N, 4.91.

2-(Benzylsulphanyl)-benzothiazole ***3g***

Purified by chromatography (acetone/petroleum ether, 3:7). Yield: 91%; m.p.: 46 – 47°C (EtOAc/petroleum ether) (lit. 38 – 40°C, [21]) IR, ^{1}H-NMR, and MS were in agreement with the published data [21]. Anal. calcd. for $C_{14}H_{11}NS_2$: C, 65.34; H, 4.31; N, 5.44; found: C, 65.24; H, 4.40; N, 5.44.

2-(Benzylsulphanyl)-6-amino-1,3-benzothiazole ***3h***

Yield: 69%; MS (70 eV) *m/z* (%): 274 [M^+ + 2] (7), 273 [M^+ + 1] (13), 272 [M^+] (70), 239 (100), 181 (47), 91 (83). The biological data were carried out on **3h** · HCl. Yield: 74%; m.p.: 219 – 221°C; IR (KBr): 2850, 1494, 1275 cm^{-1}; ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 10.6 (br s, 1H), 8.21 – 7.90 (m, 2H), 7.55 – 7.21 (m, 6H), 5.60 (br s, 2H, exchangeable with D_2O), 4.61 (s, 2H). Anal. calcd. for $C_{14}H_{12}NO_2S_2 \cdot 0.16$ HCl: C, 53.40; H, 4.21; N, 8.90; found: C, 53.74; H, 3.81; N, 8.51.

2-(Benzylsulphanyl)-6-ethoxy-1,3-benzothiazole ***3i***

Yield: 96%; m.p.: 63 – 64°C (EtOAc); IR (KBr): 2929, 1443, 998 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 7.78 (d, *J* = 9.05 Hz, 1H), 7.41 – 7.40 (m, 2H), 7.36 – 7.25 (m, 3H), 7.20 (d, *J* = 2.47 Hz, 1H), 7.02 (dd, $J_{1\text{-}2}$ = 3.22 Hz, J_{1-3} = 9.07 Hz, 1H), 4.56 (s, 2H), 4.06 (q, *J* = 6.8 Hz, 2H), 1.44 (t, *J* = 6.8 Hz, 3H); MS (70 eV) *m/z* (%): 303 [M^+ + 2] (13), 302 [M^+ + 1] (22), 301 [M^+] (100), 268 (97), 240 (18), 210 (69), 182 (22), 91 (80). Anal. calcd. for $C_{16}H_{15}NOS_2$: C, 63.76; H, 5.02; N, 4.65; found: C, 63.94; H, 4.83; N, 4.93.

2-(Benzylsulphanyl)-6-nitro-1,3-benzothiazole ***3l***

Yield: 77%; m.p.: 116.2 – 117.4°C (EtOAc); IR (KBr): 3072, 1494, 1265, 892 cm^{-1}; ^{1}H-NMR (300 MHz) δ: 8.67 (d, *J* = 2.20 Hz, 1H), 8.31 (dd, $J_{1\text{-}2}$ = 2.20 Hz, J_{1-3} = 9.07 Hz, 1H), 7.93 (d, *J* = 8.8 Hz, 1H), 7.59 – 7.43 (m, 2H), 7.39 – 7.30 (m, 3H), 4.65 (s, 2H); MS (70 eV) *m/z* (%): 304 [M^+ + 2] (9), 303 [M^+ + 1] (14), 302 [M^+] (73), 91 (100), 65 (13). Anal. calcd. for $C_{14}H_{10}NO_2S_2$: C, 55.61; H, 3.33; N, 9.26; found: C, 56.00; H, 3.34; N, 9.21.

Biology

Test organisms

Twelve bacteria strains belonging to the ATCC collection were used: Gram-positive such as *Staphylococcus aureus* ATCC 6538P, ATCC 25923, and ATCC 29213, *Enterococcus faecalis* ATCC 19433 and ATCC 29212, *Bacillus subtilis* ATCC 6633, *Bacillus cereus*

ATCC 11778, and Gram-negative *Escherichia coli* ATCC 8739 and ATCC 35218, *Acinetobacter baumannii* ATCC 19606, *Klebsiella pneumoniae* ATCC 13883 and *Pseudomonas aeruginosa* ATCC 27853.

We also used clinical isolates of *S. aureus* belonging both to NARSA (Network on Antimicrobial Resistance in Staphylococcus aureus) collection (NRS52 and VRS2 with glycopeptide intermediate and fully resistant profile, respectively) and Prof. Glupczynski's private collection with different antimicrobial resistance profiles such as N4112910 a methicillin-resistant *S. aureus* (MRSA) and N4120032 a healthcare-associated methicillin-resistant *S. aureus* (HA-MRSA). Moreover, we used *S. aureus* STA268 such as Panton–Valentine Leukocidin (PVL+) [13] isolated by Y. C. Huang in the Chang Gung Children's Hospital of Taiwan and *S. aureus* SA-1, a NorA overexpressing strain, selected from *S. aureus* ATCC 25923 [14].

Antimicrobial assay

The *in-vitro* Minimal Inhibitory Concentrations (MICs, μg/mL) of the prepared compounds were determined by the broth microdilution method according to the National Committee for Clinical Laboratory Standards (NCCLS, 2003).

Drug dilutions

For antibacterial assays the microdilution method, encoded by CLSI, formerly NCCLS, using 96-wells plates (Microtiter1), was used [12]. Stock solutions of the tested compounds **2a–2l**, **3a–3l**, were obtained in absolute ethanol at a concentration of 800 μg/mL. Stock solutions of lower concentrations were prepared for those substances which did not dissolve well. Further two-fold serial dilutions in the test medium between 100 and 0.78 μg/mL were plated. In each well, 200 μL of these solutions was added. To be sure that the solvent had no an adverse effect on bacterial growth, a control test was carried out by using ethanol at its maximum concentration along with the medium. Norfloxacin (NRF) was used as the standard drug.

To evaluate the antimicrobial profile towards the clinical isolates, the microdilution method [12] was used. Stock solutions of the tested compounds **2e** and **2l**, were obtained in absolute ethanol at a concentration of 1024 μg/mL. NRF, VAN, and OXA were used as standard drugs. The antibacterial activity against SA-1 strain was registered in the presence as well as in absence of reserpine added at 10 μg/mL following the experimental procedure suggested by Brenwald *et al*. [16].

Inoculum preparation

Cultures were grown on Petri dishes with Müller–Hinton agar (Merck, Darmstadt, Germany) for 24 h at 37 ± 1°C. A number of colonies were drawn using a sterile metal loop. They were then dissolved in MHB (Müller–Hinton broth) and were incubated for approximately 3 to 4 h. The absorbance of these cellular suspensions calibrated at a wavelength of 625 nm using spectrophotometric method (Thermo Spectronic, Genesis 20), should be 0.08 to 0.10 for the 0.5 McFarland standard, corresponding approximately to 10^8 CFU (Colony Forming Unit)/mL. The inocula were diluted to a ratio of 1:40 (100 μL of inocula in 3.9 mL of MHB) and 20 μL of this dilution were pipetted into each well. The final inoculum value was 2.5×10^6 CFU/mL. A number of wells containing only inoculated broth as control growth were prepared. After incubation for 24 h at 37 ± 1°C, the last well containing no microbial growth was recorded to show the MIC, in values of μg/mL. The MIC were determined by using an antibacterial assay repeated twice in triplicates.

Cytotoxicity assay

The tested compounds were assayed against cell lines HeLa (human epithelial cervical cancer cells) and MRC-5 (normal human lung fibroblasts) for their cytotoxic effect, by using the MTS cytotoxicity assay a variant of the widely used MTT assay [18].

Cell culture

HeLa and MRC-5 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), 50 units/mL of penicillin G and 0.05 mg/mL streptomycin (Sigma–Aldrich), 2 mM glutamine (Sigma–Aldrich), 0.25 μg/mL amphotericin B (Sigma–Aldrich) in a humidified atmosphere consisting of 5% CO_2 and 95% air. HeLa cells were subcultured three times weekly to maintain continuous logarithmic growth. MRC-5 cells were grown to confluence without subculturing; the medium was renewed twice a week. Confluent cells were harvested after trypsinization using Trypsin-EDTA solution 1X (Sigma–Aldrich).

Agent treatment

The MTS cytotoxicity assay (Cell Titer 96® Aq One Solution Cell Proliferation Assay, Promega), was used to screen the viability of the cells incubated with the test compounds. This assay measures cell viability and is based on the bioreduction of MTS tetrazolium into formazan by NADH and NADPH produced by dehydrogenase enzymes only in metabolically active, viable cells.

Compounds were prepared as 100 mM top stock solutions, dissolved in DMSO, and stored at 4°C. For each cytotoxicity assay with HeLa cells, cells were seeded into 96-well microtiter plates at a density of 200 cells per well and allowed 24 h to adhere before drugs were introduced (final concentration 100 μM). For the cytotoxicity assay with MRC-5, 600 cells per well were seeded in 96-well microplates and allowed 24 h to adhere before drug addition (final concentration 100 μM). Compounds that exhibited activity in single-dose testing were further evaluated to generate dose-response curves, which were used to determine CC_{50} (cytotoxic concentration able to destroy 50% of the initial cell amount). To perform dose-response curves, serial drug dilutions (final concentration 1 to 100 μM) were prepared in medium immediately prior to each assay. Cells were incubated for 72 h at 37°C in a humidified atmosphere and 5% CO_2.

The reagent (16% total well volume) was added to each well. Plates were incubated at 37°C until sufficient colour development had occurred (usually 3–4 h). The purple formazan product was then measured spectrophotometrically at 490 nm. The optical density (O.D.) value of each culture is a function of the amount of formazan produced and is proportional to the number of viable cells [18].

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