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Preliminary communication

Synthesis and antibacterial activity of 5-ylidenethiazolidin-4-ones and 5-benzylidene-4,6-pyrimidinediones

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ABSTRACT

5-Benzylidenethiazolidin-4-ones and 5-benzylidenepyrimidine-4,6-diones (compounds **1**–**9**), carrying 2,3,4-trifluoro or 3,4,5-trimethoxy groups on the benzylidene moiety, and rhodanine derivatives **10** and **11** were synthesized and assayed in vitro for their antimicrobial activity against four standard bacterial strains (*Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853). Compounds **1**–**3** and **9** that were active against *S. aureus*, were also tested against methicillin-resistant *S. aureus* (MRSA) ATCC 43300, *Streptococcus pneumoniae* ATCC 49619 and *Streptococcus pyogenes* ATCC 19615. (*Z*)-5-(2,3,4-Trifluorobenzylidene)rhodanine (**1**) inhibited the growth of *S. aureus* at 0.5 μg/mL and MRSA at 32 μg/mL. Stronger antimicrobial activity against *S. aureus* was observed for compounds bearing the rhodanine ring than those containing other heterocyclic moieties. Neither of the compounds **1**–**11** inhibited the growth of Gram-negative bacteria *E. coli* or *P. aeruginosa*.

1. Introduction

The treatment of bacterial infections still remains an important and challenging therapeutic problem because of factors that include emerging infectious diseases and the increasing number of multi-drug resistant microbial pathogens. In spite of the large number of antibiotics and chemotherapeutics available for medical use, the emergence of old and new antibiotic resistant bacterial strains in the last decades constitutes a substantial need for new classes of antibacterial agents [1].

The diverse biological activities of rhodanines (2-thioxothiazolidin-4-ones) and their analogues have been known from the beginning of the 20th century. Rhodanines and thiazolidine-2,4-diones have become a pharmacologically important class of heterocyclic compounds since the introduction of various glitazones and epalrestat into clinical use for the treatment of type II diabetes and diabetic complications, respectively [2,3]. Chemical modifications of these heterocycles have consistently resulted in compounds with a broad spectrum of other pharmacological activities. Since the antimicrobial activity of rhodanines has been known for more than 50 years, there have been several attempts to design antibacterial agents based on this heterocycle [4].

5-Arylmethylidenerhodanines have a number of structural features responsible for their antimicrobial activity, such as an arylmethylidene group at position 5 of the rhodanine, and the non-substituted rhodanine ring nitrogen at position 3 [5–7]. The incorporation of a halide-substituted benzylidene group at position 5 of 4-thioxothiazolidin-2-one, in which, in comparison to the rhodanine ring, only 4-thioxo and 2-oxo groups are exchanged, resulted in antimicrobial activity against Gram-positive bacteria, including multi-drug-resistant isolates [8]. Although the structure–activity relationships of thiazolidin-4-one derivatives have been studied extensively, very little is known about the contributions to antibacterial activity of the trifluoro- and trimethoxybenzylidene moieties attached to position 5 of rhodanine, 2-(4-oxo-2-thioxothiazolidin-3-yl)acetic acid (rhodanine-*N*-acetic acid), thiazolidine-2,4-dione, pyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione (barbituric acid) and 2-thioxodihydropyrimidine-4,6(1*H*,5*H*)-dione (thiobarbituric acid).

In view of these facts, and as part of our ongoing studies in developing new antimicrobial agents [9–12], we synthesized 5-benzylidenerhodanines **1**, **2**, **5** and **6**, 5-benzylidenethiazolidine-2,4-diones **3** and **4**, 5-benzylidenethiobarbituric acid **7**, 5-benzylidenebarbituric acids **8** and **9**, and rhodanine derivatives **10** and **11** (Fig. 1). They were evaluated as antimicrobial agents on four standard bacterial strains (*Staphylococcus. aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922 and

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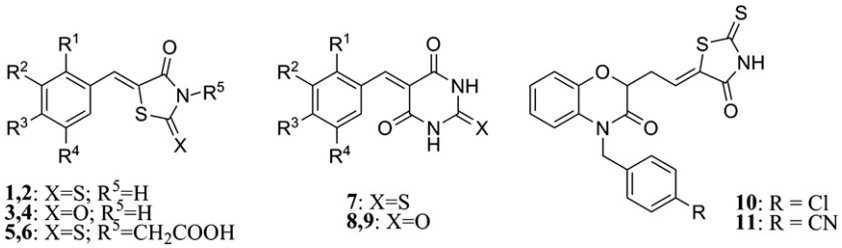


Fig. 1. Thiazolidin-4-ones and pyrimidine-4,6-diones **1**–**11**.

Pseudomonas aeruginosa ATCC 27853). The most potent compounds were also tested on MRSA ATCC 43300, *Streptococcus pneumoniae* ATCC 49619 and *Streptococcus pyogenes* ATCC 19615.

2. Chemistry

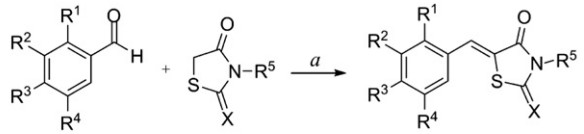
5-Benzylidene-substituted compounds **1**–**9** were prepared in good yields via a Knoevenagel condensation between the corresponding heterocyclic cores of rhodanine, rhodanine-*N*-acetic acid, thiazolidine-2,4-dione, barbituric acid and thiobarbituric acid and a range of substituted benzaldehydes (Schemes 1 and 2). Thiazolidin-4-one-based compounds were synthesized using microwave-assisted synthesis, with piperidine and glacial acetic acid as catalysts. The reaction conditions were optimized variously, depending on the heterocyclic nucleus. For example, rhodanine-*N*-acetic acid derivatives **5** and **6** were synthesized at lower temperature and prolonged reaction times than for their rhodanine and thiazolidine-2,4-dione counterparts **1**–**4**. Conventional heating of barbituric or thiobarbituric acid with the corresponding benzaldehyde derivative at reflux temperatures had to be employed for the synthesis of compounds **7**–**9**, since application of microwave irradiation proved to be unsuccessful. The synthesis of alkylidenerhodanine compounds **10** and **11** has been described elsewhere [13]. In theory, *E* and *Z* geometrical isomers around the exocyclic double bond (CH═C) are possible for 5-benzylidene derivatives **1**–**6** and 5-alkylidene compounds **10** and **11**. ^{1}H NMR spectra of compounds **1**–**6** show only one signal for the methyne proton in the range 7.46–7.82 ppm, at lower field values than those expected for the *E*-isomers, which strongly indicates that the compounds have the *Z*-configuration. The latter has been reported as thermodynamically more stable than the *E*-configuration [14,15]. The *Z*-configuration of compounds **10** and **11** was confirmed from the ^{1}H-coupled ^{13}C NMR spectrum of compound **11** followed by examination of the splitting pattern and coupling constant of the signal of the C═O group in the rhodanine system [13].

3. Results and discussion

3.1. Antimicrobial activity

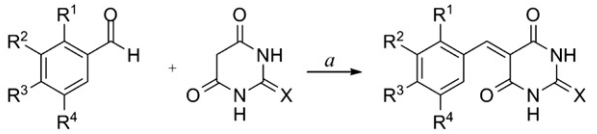
All the compounds **1**–**11** were tested for their in vitro antibacterial activity against *S. aureus* ATCC 29213, *E. faecalis* ATCC 29212, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853. The results are given in Table 1. The most active compounds **1**–**3** and **9** against *S. aureus* (minimal inhibitory concentration (MIC) < 32 μg/mL) were also tested against methicillin-resistant *S. aureus* (MRSA isolate ATCC 43300), *S. pneumoniae* ATCC 49619 and *S. pyogenes* ATCC 19615 (Table 2). Their antimicrobial activities were compared, using gentamicin as the reference antibacterial agent. Significant inhibitory activity against Gram-positive *S. aureus* is exhibited by compounds **1**–**3** and **9**–**11**, with MIC values in the 0.5–32 μg/mL range. Compounds **1**–**3** and **9** also demonstrated moderate inhibition of MRSA growth (MICs 32–128 μg/mL). None of the compounds **1**–**11** inhibited the growth of Gram-negative *E. coli* or *P. aeruginosa* (MIC > 128 μg/mL).

Thiazolidine-2,4-diones and 4-thioxo-thiazolidine-2-ones have been reported to be active against *S. aureus* but inactive against Gram-negative bacteria [7,8]. The significant activity of compounds **1**–**3** and the inactivity of compound **I** (Fig. 2), previously identified as a multitarget inhibitor of enzymes MurD-F [12a] that catalyze the intracellular steps of bacterial peptidoglycan biosynthesis [16], against *S. aureus* can be attributed to the hydrophilic character of the 2,3,4-trihydroxybenzylidene group that hinders the penetration of the molecule into the bacterial cell. Indeed, the calculated log *P* value, *c* Log *P*, for compound **I** is −0.13 [17]. Replacement of the 2,3,4-trihydroxybenzylidene group of **I** with the more lipophilic 2,3,4-trifluorobenzylidene group increased *c* Log *P* to 2.1, resulting in the most potent antibacterial compound in the series (**1**), which inhibited the growth of *S. aureus* with an MIC value of 0.5 μg/mL. Compound **1** was also evaluated for antibacterial activity against MRSA (MIC = 32 μg/mL), *S. pneumoniae* (minimal bactericidal concentration (MBC) = 64 μg/mL) and *S. pyogenes* (MBC > 128 μg/



Compound	X	R^1	R^2	R^3	R^4	R^5
1	S	F	F	F	H	H
2	S	H	OCH_3	OCH_3	OCH_3	H
3	O	F	F	F	H	H
4	O	H	OCH_3	OCH_3	OCH_3	H
5	S	F	F	F	H	CH_2COOH
6	S	H	OCH_3	OCH_3	OCH_3	CH_2COOH

Scheme 1. Reagents and conditions: (*a*) piperidine, AcOH, EtOH, 30 W, 18 bar, 140 °C, 30 min for **1**–**4** or 110 °C, 40 min for **5** and **6**.



Compound	X	R^1	R^2	R^3	R^4
7	S	H	OCH_3	OCH_3	OCH_3
8	O	H	OCH_3	OCH_3	OCH_3
9	O	F	F	F	H

Scheme 2. Reagents and conditions: (*a*) water, reflux, 10 h.

mL). Attachment of the 3,4,5-trimethoxybenzylidene moiety to the rhodanine heterocycle at position 5, instead of 2,3,4-trifluorobenzylidene, resulted in compound **2** (*c* Log *P* = 1.1) which was active against *S. aureus* (MIC = 8 μg/mL) and MRSA (MIC = 128 μg/mL). In contrast, replacement of the rhodanine nucleus by thiazolidine-2,4-dione (compounds **3**–**4**) led to significantly weaker antibacterial activity. Thiazolidine-2,4-dione-based compound **3**, possessing the 2,3,4-trifluorobenzylidene moiety, was 16-times less active against *S. aureus* (MIC = 32 μg/mL) and 2-times less active against MRSA (MIC = 64 μg/mL) than its rhodanine counterpart **1**, while the (*Z*)-5-(3,4,5-trimethoxybenzylidene)thiazolidine-2,4-dione (**4**) was inactive against *S. aureus* and other bacterial strains, although the *c* Log *P* values for compounds **3** (*c* Log *P* = 2.0) and **4** (*c* Log *P* = 1.0) are comparable to those of compounds **1** and **2**, respectively. Considering the comparable lipophilicity of the thiocarbonyl- and carbonyl-based compounds (compound **1** and **2** versus compounds **3** and **4**, respectively), the thiocarbonyl group seems to play a very important role for the potent antibacterial activity of these compounds. The difference between the activities of the rhodanines and thiazolidine-2,4-diones tested confirms reports that various rhodanines possess antibacterial activity and inhibit bacterial enzymes, while their thiazolidine-2,4-dione counterparts usually display weaker activity or are inactive [4]. However, the inability of **1** to inhibit MurC-F ligases [12b] indicates that other targets may be involved in the antibacterial action of compounds **1**–**3** against *S. aureus*. Moreover, in our series of compounds, the antibacterial activity was also dependent on the substitution of the rhodanine nitrogen at position 3. Both rhodanine-based compounds **5** and **6** that incorporate an acetic acid group at this position 3 were inactive against all tested bacterial strains, with MIC values greater than 128 μg/mL.

Replacing the rhodanine ring in compounds **1** and **2** with thiobarbituric or barbituric acid rings gave compounds **7**–**9**. The results of the antibacterial activity of compounds **7** and **8** further support the importance of the rhodanine moiety for potent antibacterial activity, since replacement of the rhodanine ring in compound **2** by the thiobarbituric or barbituric acid rings (compounds **7** and **8**) resulted in inactivity against *S. aureus* (MIC = 8 μg/mL compared to MIC >128 μg/mL). Although the incorporation of the thiocarbonyl group (compound **7**) instead of the carbonyl group (compound **8**) increased the lipophilicity of the compound (*c* Log *P* = 0.96 versus 0.09, respectively), it did not improve antibacterial activity of compound **7**. On the other hand, replacement of the 3,4,5-trimethoxybenzylidene moiety (compound **8** with *c* Log *P* = 0.09) by a more lipophilic 2,3,4-trifluorobenzylidene moiety (compound **9** with *c* Log *P* = 1.1) had an important influence on the antibacterial activity, since 2,3,4-trifluorobenzylidenebarbituric acid (**9**) possessed activity against *S. aureus* (MIC = 32 μg/mL) and MRSA (MIC = 128 μg/mL).

In the course of our studies towards the discovery of novel Mur ligase inhibitors, recently described 2*H*-1,4-benzoxazin-3(4*H*)-one-based inhibitors of bacterial histidine protein kinase [18,19] attracted our attention due to similarities in the structure of Mur enzymes and various kinases. Thus, compounds **10** and **11**, in which the rhodanine moiety is connected to the 2*H*-1,4-benzoxazin-3(4*H*)-one scaffold *via* an ethylidene linker, were prepared as rhodanine analogues of histidine protein kinase inhibitors **II** and **III** (Fig. 2), for which docking experiments had predicted promising interaction also with bacterial enzymes MurC and MurD (unpublished results). While N^4-(4-chlorobenzyl)-substituted compound **II** showed 8-times more potent antibacterial activity against *S. aureus* (MIC = 8 μg/mL) than N^4-(4-cianobenzyl)-substituted compound **III** (MIC = 64 μg/mL) [11], the effect of N^4-substituent of the rhodanine-based analogues **10** and **11** on the antibacterial activity was less pronounced, since both compounds inhibited the growth of *S. aureus* at 32 μg/mL. Moreover, compound **10** lost its ability to inhibit the growth of *E. faecalis* (MIC > 128 μg/mL) as compared to its analogue **II** (MIC = 16 μg/mL), while compound **11** (MIC = 32 μg/mL) displayed 2-times lower MIC values against *E. faecalis* than its counterpart **III** (MIC = 64 μg/mL).

Table 1
Antibacterial activity of compounds **1**–**11** and **I**–**III** against *S. aureus* and *E. faecalis* (MIC, μg/mL) and their *c* Log *P* values [17].

Compound	*S. aureus* ATCC 29213	*E. faecalis* ATCC 29212	*c* Log *P*
1	0.5	>128	2.1
2	8	>128	1.1
3	32	>128	2.0
4	>128	>128	1.0
5	>128	>128	1.5
6	>128	>128	0.47
7	>128	>128	0.96
8	>128	>128	0.09
9	32	>128	1.1
10	32	>128	3.3
11	32	32	2.0
I	>128	>128	−0.13
II	8	16	4.7
III	64	64	3.4

4. Conclusion

The synthesis and antibacterial activity are reported for a series of rhodanine-, rhodanine-*N*-acetic acid-, thiazolidine-2,4-dione-,

Table 2
Antibacterial activity of compounds **1**–**3** against MRSA, *S. pneumoniae* and *S. pyogenes* (MIC, μg/mL).

Compound	MRSA ATCC 43300	*S. pneumoniae*[a] ATCC 49619	*S. pyogenes*[a] ATCC 19615
1	32	64[a]	>128[a]
2	128	>128[a]	>128[a]
3	64	ND	ND
9	128	ND	ND

[a] MBC, μg/mL. ND – not determined.

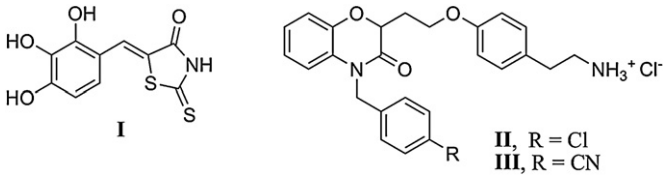


Fig. 2. (*Z*)-5-(2,3,4-Trihydroxybenzylidene)rhodanine (**I**) and 2*H*-1,4-benzoxazin-3(4*H*)-one-based inhibitors of histidine protein kinase **II** and **III**.

barbituric- and thiobarbituric acid-based compounds bearing an ylidene substituent at position 5. The results of antibacterial activity of the synthesized compounds against selected Gram-positive and Gram-negative bacteria show that the 2,3,4-trifluorobenzylidene moiety and the thiocarbonyl group on the thiazolidin-4-one ring are important for potent inhibition of *S. aureus* and MRSA growth. The most potent compound of the series, (*Z*)-5-(2,3,4-trifluorobenzylidene)rhodanine (**1**), inhibited the growth of *S. aureus* at 0.5 μg/mL and MRSA at 32 μg/mL. When 2,3,4-trifluorobenzylidene moiety was replaced by a 3,4,5-trimethoxybenzylidene moiety or when thiazolidine-2,4-dione, rhodanine-*N*-acetic acid, barbituric or thiobarbituric acid ring was used instead of the rhodanine ring, thus obtained compounds **2**–**9** always possessed weaker antibacterial activity than compound **1**. 2*H*-1,4-Benzoxazin-3(4*H*)-one-based compound **11** displayed activity also against *E. faecalis* (MIC = 32 μg/mL), but all of the synthesized compounds were inactive against Gram-negative bacteria. Potent antibacterial activity makes compound **1** promising starting point for further optimization. However, additional studies regarding the mechanism of action are necessary for a complete understanding of the antimicrobial activity of these compounds.

5. Experimental protocols

Chemicals were obtained from Acros, Aldrich Chemical Co. and Fluka and used without further purification. Yields refer to purified products and were not optimized. Analytical thin-layer chromatography (TLC) was performed using silica gel $60F_{254}$ pre-coated plates (0.25 mm thick) with a fluorescent indicator from Merck (Germany). Flash column chromatography was carried out on silica gel 60 (particle size 0.040–0.063 mm; Merck, Germany). The components of chromatographic eluents are given as volume-to-volume ratios (v/v). Melting points were determined on a Reichert hot stage microscope and are uncorrected. Structures of compounds were confirmed by routine spectrometric analysis. ^{1}H NMR spectra were recorded at 300 MHz on a Bruker AVANCE DPX_{300} spectrometer in DMSO-d_6 solution with TMS as the internal standard. IR spectra were recorded on a Perkin–Elmer 1600 FT-IR spectrometer. Elemental analyses were performed on a Perkin–Elmer C, H, N analyzer 240C and were within ±0.4% of the theoretical values. Mass spectra were obtained using a VG-Analytical Autospec Q mass spectrometer. Microwave-assisted reactions were performed using a focused microwave reactor (Discover™, CEM Corporation, Matthews, NC). Reactions were carried out in septum-sealed glass vials (10 mL) which enable high-pressure reaction conditions (max 20 bar). The temperature of the reaction mixture was monitored using a calibrated infrared temperature controller mounted under the reaction vessel.

5.1. Chemistry

5.1.1. General procedure for microwave-assisted synthesis of (Z)-2-thioxo-5-benzylidenethiazolidin-4-ones *1* and *2*

To a suspension of 2-thioxo-thiazolidin-4-one (0.200 g, 1.50 mmol, 1.0 equiv) in dry ethanol (5 mL), the corresponding aldehyde (1.50 mmol, 1.0 equiv), a catalytic amounts of piperidine (0.150 mmol, 0.1 equiv) and glacial acetic acid (0.150 mmol, 0.1 equiv) were added. The reaction mixture was heated with microwave irradiation (max. power of 30 W) to 140 °C and the heating continued for 30 min to maintain the temperature. The pressure limit was set at 18 bar. The reaction vessel was cooled in an ice-bath and the precipitate was filtered off, washed with ice-cooled ethanol and dried under vacuum.

*5.1.1.1. (Z)-2-Thioxo-5-(2,3,4-trifluorobenzylidene)thiazolidin-4-one (**1**).* Orange crystalline solid. Yield: 87.8%. Melting range 198–200 °C. IR (KBr): ν_{max} = 3552, 3474, 3413, 3231, 1718, 1637, 1615, 1590, 1515, 1466, 1438, 1302, 1216, 1052, 1010, 976, 798, 671, 638, 601 cm^{-1}. ^{1}H NMR (DMSO-d_6): δ = 7.35–7.43 (m, 1H, Ar-H^5), 7.46–7.55 (m, 2H, CH, Ar-H^6), 13.93 (br s, 1H, CONHCS) ppm. MS (ESI+): *m*/*z* (%) = 276 ([M + H]$^+$, 85), 327 (100). Anal. calcd for $C_{10}H_4F_3NOS_2$: C, 43.63%; H, 1.46%; N, 5.09%; found C, 43.88%; H, 1.49%; N, 5.12%.

*5.1.1.2. (Z)-2-Thioxo-5-(3,4,5-trimethoxybenzylidene)thiazolidin-4-one (**2**).* Orange crystalline solid. Yield: 71.3%. Melting range 196–198 °C (202–203 °C [20]). IR (KBr): ν_{max} = 3546, 3003, 1686, 1596, 1572, 1499, 1442, 1332, 1300, 1250, 1216, 1154, 1128, 1068, 991, 824, 712, 668, 614, 550 cm^{-1}. ^{1}H NMR (DMSO-d_6): δ = 3.74 (s, 3H, 4-OCH_3), 3.85 (s, 6H, 3-OCH_3, 5-OCH_3), 6.92 (s, 2H, Ar-H^2, H^6), 7.61 (s, 1H, CH), 13.82 (s, 1H, CONHCS) ppm. MS (ESI+): *m*/*z* (%) = 312 ([M + H]$^+$, 60), 334 (100). Anal. calcd for $C_{13}H_{13}NO_4S_2$: C, 50.11%; H, 4.12%; N, 4.45%; found C, 50.14%; H, 4.21%; N, 4.50%.

5.1.2. General procedure for microwave-assisted synthesis of (Z)-5-benzylidenethiazolidine-2,4-diones *3* and *4*

To a suspension of thiazolidine-2,4-dione (0.200 g, 1.71 mmol, 1.0 equiv) in dry ethanol (5 mL), the corresponding aldehyde (1.71 mmol, 1.0 equiv), a catalytic amounts of piperidine (0.171 mmol, 0.1 equiv) and glacial acetic acid (0.171 mmol, 0.1 equiv) were added. The reaction mixture was heated with microwave irradiation (max. power of 30 W) to 140 °C and the heating continued for 30 min to maintain the temperature. The pressure limit was set at 18 bar. The reaction vessel was cooled in an ice-bath and the precipitate was filtered off, washed with ice-cooled ethanol and dried under vacuum.

*5.1.2.1. (Z)-5-(2,3,4-Trifluorobenzylidene)thiazolidine-2,4-dione (**3**).* Yellow crystalline solid. Yield: 85.8%. Melting range 120–122 °C. IR (KBr): ν_{max} = 3414, 3034, 2769, 1745, 1690, 1616, 1509, 1466, 1343, 1304, 1291, 1176, 1153, 1047, 1013, 974, 896, 808, 728, 676, 634, 602, 536, 503, 474 cm^{-1}. ^{1}H NMR (DMSO-d_6, 300 MHz): δ = 7.36–7.55 (m, 2H, Ar-H^5, H^6), 7.70 (s, 1H, CH), 12.79 (br s, 1H, CONHCO) ppm. MS (ESI+): *m*/*z* (%) = 260 ([M + H]$^+$, 60), 164 (100). Anal. calcd for $C_{10}H_4F_3NO_2S$: C, 46.34%; H, 1.56%; N, 5.40%; found C, 46.43%; H, 1.70%; N, 5.65%.

*5.1.2.2. (Z)-5-(3,4,5-Trimethoxybenzylidene)thiazolidine-2,4-dione (**4**).* The precipitate was recrystallized from ethyl acetate to obtain compound **4** as a pale yellow crystalline solid. Yield: 39.0%. Melting range 170–172 °C (184–186 °C [21]). IR (KBr): ν_{max} = 3165, 2371, 1742, 1708, 1609, 1508, 1313, 1252, 1132, 999, 621, 556 cm^{-1}. ^{1}H NMR (DMSO-d_6): δ = 3.73 (s, 3H, 4-OCH_3), 3.83 (s, 6H, 3-OCH_3, 5-OCH_3), 6.92 (s, 2H, Ar-H^2, H^6), 7.75 (s, 1H, CH), 12.58 (s, 1H, CONHCO) ppm.

MS (ESI−): m/z (%) = 294 ([M − H]$^-$, 100). HRMS (ESI−): calcd for $C_{13}H_{14}NO_5S$: 294.0436, found: 294.0440.

*5.1.3. General procedure for microwave-assisted synthesis of (Z)-2-(4-oxo-2-thioxo-5-benzylidenethiazolidin-3-yl)acetic acids **5** and **6***

To a suspension of rhodanine-3-acetic acid (0.200 g, 1.05 mmol, 1.0 equiv) in dry ethanol (5 mL), aldehyde (1.05 mmol, 1.0 equiv), a catalytic amounts of piperidine (0.105 mmol, 0.1 equiv) and glacial acetic acid (0.105 mmol, 0.1 equiv) were added. The reaction mixture was heated with microwave irradiation (max. power of 30 W) to 110 °C and the heating continued for 40 min to maintain the temperature. The pressure limit was set at 18 bar. The reaction vessel was cooled in an ice-bath, the precipitate was filtered off, washed with ice-cooled ethanol and dried under vacuum.

*5.1.3.1. (Z)-2-(4-Oxo-2-thioxo-5-(2,3,4-trifluorobenzylidene)thiazolidin-3-yl)acetic acid (**5**).* Yellow crystalline solid. Yield: 40.5%. Melting range 169–171 °C. IR (KBr): ν_{max} = 3431, 3033, 2982, 1721, 1634, 1614, 1591, 1512, 1468, 1436, 1411, 1374, 1329, 1308, 1249, 1215, 1118, 1060, 1042, 985, 945, 904, 796, 749, 724, 664, 619, 600, 564, 550, 531, 501, 476 cm^{-1}. ^{1}H NMR (DMSO-d_6): δ = 4.72 (s, 2H, CH_2-COOH), 7.45–7.58 (m, 2H, Ar-H^5, H^6), 7.76 (s, 1H, CH) ppm, broad signal for COOH not seen. MS (EI): m/z (%) = 333 (M, 41), 188 (100). HRMS (EI): calcd for $C_{12}H_6F_3NO_3S_2$: 332.9741, found: 332.9745.

*5.1.3.2. (Z)-2-(4-Oxo-2-thioxo-5-(3,4,5-trimethoxybenzylidene)thiazolidin-3-yl)acetic acid (**6**).* Dark yellow crystalline solid. Yield: 35.4%. Melting range 217–219 °C. IR (KBr): ν_{max} = 3446, 1706, 1603, 1577, 1503, 1465, 1417, 1322, 1250, 1149, 1128, 1053, 1003, 830, 764, 739, 629, 575 cm^{-1}. ^{1}H NMR (DMSO-d_6): δ = 3.76 (s, 3H, 4-OCH$_3$), 3.86 (s, 6H, 3-OCH$_3$, 5-OCH$_3$), 4.66 (s, 2H, CH_2-COOH), 6.99 (s, 2H, Ar-H^2, H^6), 7.82 (s, 1H, CH) ppm, broad signal for COOH not seen. MS (ESI−): m/z (%) = 368 ([M − H]$^-$, 20), 324 (100). HRMS (ESI−): calcd for $C_{15}H_{14}NO_6S_2$: 368.0263, found: 368.0270.

*5.1.4. General procedure for the synthesis of compounds **7** and **8***

A suspension of 3,4,5-trimethoxybenzaldehyde (200 mg, 1.02 mmol) and barbituric or 2-thiobarbituric acid (1.02 mmol) in water (20 mL) was refluxed for 10 h, after which the reaction mixture was cooled to room temperature, the product filtered off and washed with diethyl ether.

*5.1.4.1. 2-Thioxo-5-(3,4,5-trimethoxybenzylidene)dihydropyrimidine-4,6(1H,5H)-dione (**7**).* Orange crystalline solid. Yield: 84.1%. Melting range 238–250 °C. IR (KBr): ν_{max} = 3524, 3104, 3062, 2994, 2907, 1646, 1573, 1556, 1539, 1495, 1423, 1302, 1123, 998 cm^{-1}. ^{1}H NMR (DMSO-d_6): δ = 3.81 (s, 3H, 4-OCH$_3$), 3.84 (s, 6H, 3-OCH$_3$, 5-OCH$_3$), 7.89 (s, 2H, Ar-H^2, H^6), 8.28 (s, 1H, CH), 12.31 (s, 1H, NH), 12.43 (s, 1H, NH) ppm. MS (ESI+): m/z (%) = 345 ([M + Na]$^+$, 2), 323 ([M + H]$^+$, 29), 77 (100). HRMS (ESI+): calcd for $C_{14}H_{15}N_2O_5S$: 323.0702, found 323.0697.

*5.1.4.2. 5-(3,4,5-Trimethoxybenzylidene)pyrimidine-2,4,6(1H,3H,5H)-trione (**8**).* Yellow crystalline solid. Yield: 78.3%. Melting range 274–276 °C. IR (KBr): ν_{max} = 3506, 3241, 3139, 3016, 2839, 1753, 1734, 1667, 1654, 1550, 1507, 1418, 1301, 1125, 996 cm^{-1}. ^{1}H NMR (DMSO-d_6): δ = 3.80 (s, 3H, 4-OCH$_3$), 3.83 (s, 6H, 3-OCH$_3$, 5-OCH$_3$), 7.84 (s, 2H, Ar-H^2, H^6), 8.26 (s, 1H, CH), 11.22 (s, 1H, NH), 11.53 (s, 1H, NH) ppm. MS (ESI+): m/z (%) = 329 ([M + Na]$^+$, 5), 307 ([M + H]$^+$, 38), 77 (100). HRMS (ESI+): calcd. for $C_{14}H_{15}N_2O_6$: 307.0930, found 307.0919.

*5.1.5. 5-(2,3,4-Trifluorobenzylidene)pyrimidine-2,4,6(1H,3H,5H)-trione (**9**)*

2,3,4-Trifluorobenzaldehyde (71.2 μL, 0.625 mmol) was added to a solution of barbituric acid (80 mg, 0.625 mmol) in water (5 mL) at 100 °C and the mixture stirred at 100 °C for 1 h. After cooling to room temperature, the product was filtered off and washed with petroleum ether. Compound **9** was obtained as a white crystalline solid. Yield: 68.0%. Melting range 320–325 °C. IR (KBr): ν_{max} = 3524, 3256, 3124, 3092, 2819, 1770, 1673, 1583, 1516, 1439, 1348, 1300, 1040 cm^{-1}. ^{1}H NMR (DMSO-d_6): δ = 7.36–7.45 (m, 1H, Ar-H), 7.71–7.80 (m, 1H, Ar-H), 8.17 (s, 1H, CH), 11.33 (s, 1H, NH), 11.50 (s, 1H, NH) ppm. MS (ESI−): m/z (%) = 269 ([M − H]$^-$, 83), 249 (100). Anal. calcd for $C_{11}H_5F_3N_2O_3$: C, 48.90%; H, 1.87%; N, 10.37%; found C, 48.89%; H, 2.09%; N, 10.32%.

5.2. Determination of antibacterial activity

The susceptibilities of four standard strains (*E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212) to compounds **1**–**11** were determined by the macrodilution method. Compounds (10 mg) were dissolved in 5 mL of DMSO to give a stock solution of 2 mg/mL concentration. Working solutions were made by serially diluting stock solution in cation-adjusted Mueller–Hinton broth (CAMHB), as described by Amsterdam and Barry [22,23]. 34 mL of CAMHB was added to 5 mL stock to give a 256 μg/mL concentration, then filter sterilized. The compound was further serially diluted to provide 14 dilutions down to the lowest concentration of 0.031 μg/mL [22] that were stored frozen for a maximum of two weeks. 0.5 mL of these dilutions were pipetted into 13 × 100 mm screw cap tubes just prior to bacterial inoculation.

Four colonies of a fresh overnight culture on a non-selective agar plate were inoculated into saline. The turbidity was adjusted to match that of 0.5 McFarland standard (approx. 10^8 CFU/mL). A portion of the standardized suspension was diluted approx. 1:1000 (to 10^5 CFU/mL). 0.5 mL of this dilution was then added within 30 min to each tube containing 0.5 mL of test compound diluted in CAMHB and incubated at 35 °C for 18–24 h. Dilutions from 0.016 to 128 μg/mL of the compound were achieved on addition of the inoculum. Broth not containing any compound was inoculated as a growth control. The lowest concentration of antimicrobial agent that resulted in complete inhibition of visible growth was the minimal inhibitory concentration (MIC). In the case of agar dilution, an inoculum of 10^6 CFU/ml was prepared. 20 μl, containing 10^4 CFU/spot was inoculated on sheep blood agar plates, each containing different concentrations of test compounds, and incubated at 35 °C for 18–24 h in CO_2 atmosphere. Quality control of the methods was performed by testing *S. aureus* ATCC 29213 and gentamicin. Dilutions of antibiotic were made in the same way as for tested compound and the MICs obtained were in the range proposed by the Clinical Laboratory Standards Institute [24].

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