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Synthesis and antimicrobial activity of novel 2-thiazolylimino-5-arylidene-4-thiazolidinones

Paola Vicini,[a,*] Athina Geronikaki,[b] Kitka Anastasia,[b] Matteo Incerti[a] and Franca Zani[a]

[a]*Dipartimento Farmaceutico, Università degli Studi di Parma, Parco Area delle Scienze 27/A, Parma 43100, Italy*
[b]*Department of Pharmaceutical Chemistry, School of Pharmacy, Aristotelian University of Thessaloniki, Thessaloniki 54006, Greece*

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Abstract—New 2-thiazolylimino-5-arylidene-4-thiazolidinones (compounds **4a**–**j**), unsubstituted or carrying hydroxy, methoxy, nitro and chloro groups on the benzene ring, were synthesized and assayed in vitro for their antimicrobial activity against Gram positive and Gram negative bacteria, yeasts and mould. The compounds were very potent towards all tested Gram positive microorganisms (MIC ranging from 0.03 to 6 μg/mL in most of the cases) and Gram negative *Haemophilus influenzae* (MIC 0.15–1.5 μg/mL), whereas no effectiveness was exhibited against Gram negative *Escherichia coli* and fungi up to the concentration of 100 μg/mL. The 5-arylidene derivatives showed an antibacterial efficacy considerably greater than that of the parent 2-(thiazol-2-ylimino)thiazolidin-4-one **3**, suggesting that the substituted and unsubstituted 5-arylidene moiety plays an important role in enhancing the antimicrobial properties of this class of compounds. The remarkable inhibition of the growth of penicillin-resistant staphylococci makes these substances promising agents also for the treatment of infections caused by microorganisms resistant to currently available drugs.
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1. Introduction

The treatment of infectious diseases still remains an important and challenging problem because of a combination of factors including emerging infectious diseases and the increasing number of multi-drug resistant microbial pathogens with particular relevance for Gram positive bacteria.[1–5] The therapeutic problem has achieved increasing importance in hospitalised patients, in immuno suppressed patients with AIDS or undergoing anticancer therapy and organ transplants. In spite of a large number of antibiotics and chemotherapeutics available for medical use, at the same time the emergence of old and new antibiotic resistance created in the last decades a substantial medical need for new classes of antibacterial agents. A potential approach to overcome the resistance problem is to design innovative agents with a different mode of action so that no cross-resistance with the present therapeuticals can occur.[6] Recently linezolid, a totally synthetic molecule of the oxazolidinone class, has been approved for human use and, consistent with its activity against resistant Gram positive organism, appeared to have a unique mechanism of antibacterial activity.[7–9]

As part of our ongoing studies in developing new active antimicrobials[10–14] against resistant bacteria too, we report the synthesis of a new class of structurally novel 4-thiazolidinone derivatives incorporating two known bioactive heterocyclic nuclei such as thiazole and thiazolidinone.[15–23] The structural variations were selected by introducing, at the 5 position of thiazolidinone moiety, different arylidene subtituents that we recently exploited as bioactive arms on heterocyclic scaffolds useful to encompass certain physico-chemical properties as hydrophobic and steric.[13,14,24]

Ten new 2-thiazolylimino-5-arylidene-4-thiazolidinones (Scheme 1, **4a**–**j**) were synthetized and tested together with starting 2-(thiazol-2-ylimino)thiazolidin-4-one (**3**) for their in vitro antimicrobial properties against Gram positive and Gram negative bacteria, yeasts and mould. The 5-arylidene derivatives were further evaluated against a wider range of pathogens including penicillin-resistant staphylococci with the aim to detect the spectrum of their inhibitory activity.

Keywords: 2-Thiazolylimino-5-arylidene-4-thiazolidinones; Thiazole derivatives; Thiazolidinone derivatives; Antimicrobial activity.
* Corresponding author. Tel.: +39 521 905051; fax: +39 521 905006; e-mail: pvicini@ipruniv.cce.unipr.it

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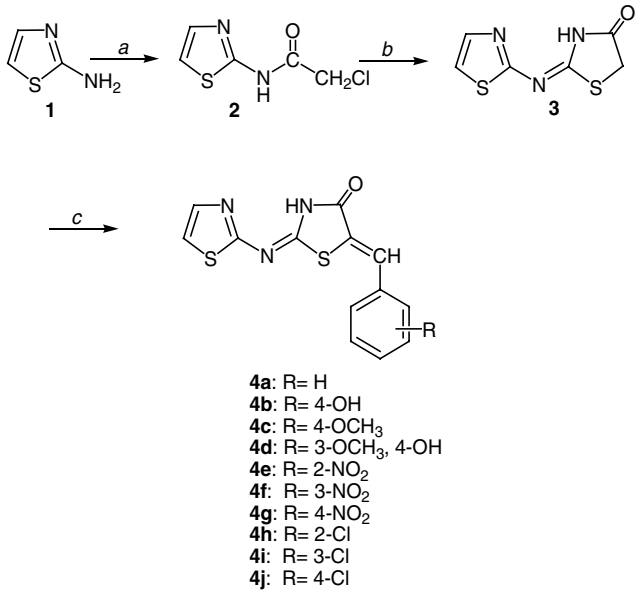


Scheme 1. Reagents and conditions: (a) $ClCOCH_2Cl$, *N*,*N*-DMF, rt, 2 h; (b) NH_4SCN, EtOH, reflux, 1 h; (c) RC_6H_4CHO, CH_3COOH, CH_3COONa, reflux, 2–4 h.

2. Results and discussion

2.1. Chemistry

2-Chloro-*N*-(thiazol-2-yl)acetamide (**2**), synthetized using procedures reported earlier[25] starting from 2-aminothiazole (**1**), upon heterocyclization in the presence of ammonium thiocyanate in refluxing ethanol, efficiently produced 2-(thiazol-2-ylimino)thiazolidin-4-one (**3**).[26] The 2-thiazolylimino-5-arylidene-4-thiazolidinones **4a**–**j** were obtained by refluxing **3** with appropriate aldehydes in buffered glacial acetic acid (Scheme 1).

All the new compounds **4a**–**j** were characterized by mp, elemental analyses and spectroscopic data (^{1}H NMR, MS and IR). Compounds **4a**–**j** exist as potential *E* and *Z* geometrical isomers; the *Z* conformation of the 5 exocyclic C=C double bond was assigned on the basis of ^{1}H NMR spectroscopy and on the basis of literature data for analogous 4-thiazolidinones and 2,4-thiazolidinediones.[27,28] The ^{1}H NMR spectra of compounds **4a**–**j** showed only one kind of methine proton that, deshielded by the adjacent C=O, was detected at 7.63–7.97 ppm, at higher chemical shift values than the expected ones for *E* isomers that have a methine proton with a lesser deshielding effect.

The yields, the physicochemical properties and the spectroscopic data of **4a**–**j** are given, respectively, in Tables 1 and 2. The substitution position in the cyclocondensation step and the tautomeric structure of the 2-imino-5-arylidene-4-thiazolidinones **4a**–**j** were determined through the analysis of IR and ^{1}H NMR spectral data. The suggested mechanism of the reaction and the theoretically tautomeric forms of key intermediate **3** are shown in Scheme 2.

In ^{1}H NMR spectra of compounds **3** and **4a**–**j**, NH proton observed at 12.00 and 12.53–12.87 ppm showed that the substitution is on 2-position rather than on 3-position in agreement with a lactam proton, since an imine proton appears at a much higher field (about 9.70).[22,26,29]

3-Nonsubstituted 2-thiazolylimino-4-thiazolidinones display prototropic amino–imino tautomerism. The possibility of the tautomerism involving a hydroxylic group was excluded by the evident absence of typical signals of a OH group in IR and ^{1}H NMR spectra. The IR absorption of the lactam NH group at 3072–3174 cm^{-1} together with a strong band at about 1690–1714 cm^{-1}, in agreement with a γ-lactam, confirms the $\mathbf{3}_{iii}$ tautomeric form in the solid state. Concerning the ^{1}H NMR data, the low field NH signals of compounds **4a**–**j** account for the amino–imino tautomeric equilibrium shown in Scheme 2. It is worthwhile mentioning that in the biological systems the amino–imino equilibrium is of great importance in the binding process with the target biomolecule.

2.2. Antimicrobial activity

The results of antimicrobial testing of 2-(thiazol-2-ylimino)thiazolidin-4-one **3** and its 5-arylidene derivatives **4a**–**j** against a panel of selected Gram positive and Gram negative bacteria, yeasts and mould are reported in Table 3, in comparison with those of the reference drugs ampicillin and miconazole. Compound **3** showed moderate activity, expressed as minimal inhibitory concentrations (MIC), against *Bacillus subtilis* (MIC 50

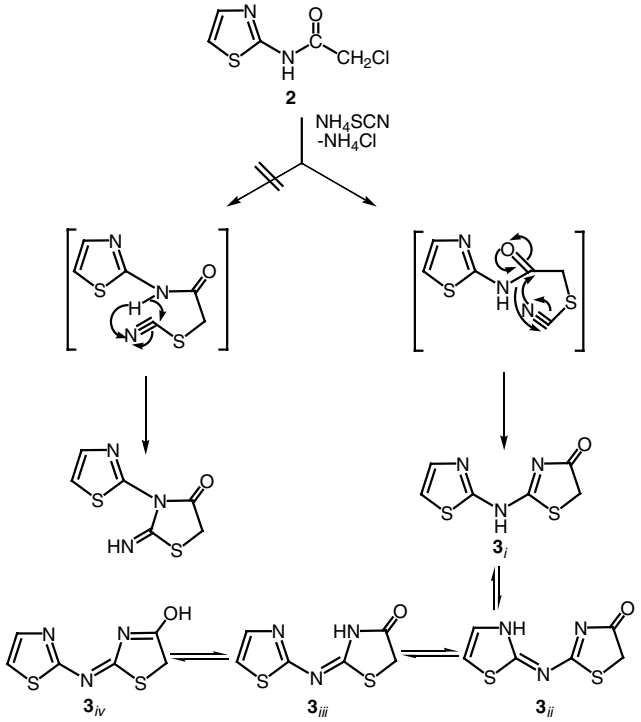
Table 1. Yields, structural and physicochemical data of new 2-thiazolylimino-5-arylidene-4-thiazolidinones

Compound	Yield (%)	Rf[a]	Mp (°C) from dioxane	Molecular formula (MW)	MS
4a	44.4	0.534	191–193	$C_{13}H_9N_3OS_2$ (287,36)	287
4b	90.7	0.58	278	$C_{13}H_9N_3O_2S_2$ (303,36)	303
4c	94.5	0.723	236–268	$C_{14}H_{11}N_3O_2S_2$ (317,39)	317
4d	58.9	0.369	195–197	$C_{14}H_{11}N_3O_3S_2$ (333,39)	333
4e	39	0.62	234–235.5	$C_{13}H_8N_4O_3S_2$ (332,36)	332
4f	96	0.39	231–233	$C_{13}H_8N_4O_3S_2$ (332,36)	332
4g	86	0.42	266–267	$C_{13}H_8N_4O_3S_2$ (332,36)	332
4h	65	0.216	194.5–196	$C_{13}H_8N_3OS_2Cl$ (321,85)	321.5
4i	68.8	0.71	193–194	$C_{13}H_8N_3OS_2Cl$ (321,85)	321.5
4j	48	0.711	257.5–258.5	$C_{13}H_8N_3OS_2Cl$ (321,85)	321.5

[a] Chromatographic conditions: silica-gel TLC plates (60 F_{254} Merck, Darmstadt, Germany); eluent: benzene/ethanol 8/2.

Table 2. Spectral data (IR, ^{1}H NMR)

Compound	IR (ν, cm^{-1})	^{1}H NMR (δ, ppm)
4a	3149 (NH lactam); 3075 (CH arom); 1702 (C=O lactam); 1589 (C=N)	12.68 (s, 1H, NH); 7.73 (d, 1H, J = 3.3 H-4); 7.72 (s, 1H, CH); 7.65 (d, 2H, J = 7.8 H-2′, H-6′); 7.57 (t, 2H, J = 7.3 H-3′, H-5′); 7.50–7.46 (m, 2H, H-5, H-4′)
4b	3448 (OH); 3084 (NH lactam);1697 (C=O lactam); 1594 (C=N)	12.55 (s, 1H, NH); 10.31 (s, 1H, OH); 7.71 (d, 1H, J = 3.6 H-4); 7.63 (s, 1H, CH); 7.53–7.47 (m, 3H, H-5, H-2′, H-6′); 6.95 (d, 2H, J = 9.0 H-3′, H-5′)
4c	3110 (NH lactam);1697 (C=O lactam); 1597 (C=N)	12.57 (s, 1H, NH); 7.71 (d, 1H, J = 3.3 H-4); 7.68 (s, 1H, CH); 7.61 (d, 2H, J = 8.7, H-2′, H-6′); 7.47 (d, 1H, J = 3.3, H-5); 7.14 (d, 2H, J = 8.7 H-3′, H-5′); 3.84 (s, 3H, CH_3)
4d	3454 (OH); 3087 (NH lactam); 1704 (C=O lactam); 1589 (C=N)	12.53 (s, 1H, NH); 9.90 (s, 1H, OH); 7.69 (d, 1H, J = 3.6 H-4); 7.65 (s, 1H, CH); 7.47 (d, 1H, J = 3.6 H-5); 7.27 (d, 1H, J = 1.5 H-2′); 7.13 (dd, 1H, J = 1.8, J = 8.4 H-6′); 6.99 (d, 1H, J = 8.4 H-5′); 3.83 (s, 3H, CH_3)
4e	3085 (NH lactam); 1718 (C=O lactam); 1614 (C=N); 1522, 1343 (NO_2)	12.82 (s, 1H, NH) 8.21 (d, 1H, J = 8.1); 7.97–7.92 (m, 2H, CH, H-4′); 7.79 (d, 1H, J = 7.8 H-6′); 7.71 (t, 1H, J = 8.1, H-5′); 7.64 (d, 1H, J = 3.6, H-4); 7.48 (d, 1H,J = 3.6 H-5)
4f	3085 (NH lactam); 1714 (C=O lactam); 1604 (C=N); 1527, 1349 (NO_2)	12.86 (s, 1H, NH); 8.47 (s, 1H, H-2′); 8.27 (d, 1H, J = 7.8.1 H-4′); 8.03 (d, 1H, J = 7.5 H-6′); 7.87–7.82 (m, 2H, H-5′, CH); 7.70 (d, 1H, J = 3.3 H-4); 7.49 (d, 1H, J = 3.3 H-5)
4g	3080 (NH lactam); 1710 (C=O lactam); 1597 (C=N); 1518, 1340 (NO_2)	12.87 (s, 1H, NH); 8.39 (d, 2H, J = 8,7 H-3′, H-5′); 7.89 (d, 2H, J = 8.4 H-2′, H-6′); 7.81 (s, 1H, CH); 7.73 (d, 1H, J = 3.6 H-5); 7.53 (d, 1H, J = 3.6 H-4)
4h	3091(NH lactam); 1716 (C=O lactam); 1597 (C=N)	12.81 (s, 1H, NH); 7.85 (s, 1H, CH); 7.71–7.48 (m, 6H, H-4, H-5, H-3′, H-4′, H-5′, H-6′)
4i	3082 (NH lactam); 1713 (C=O lactam); 1603 (C=N)	12.74 (s, 1H, NH) 7.74–7.70 (m, 3H, CH, H-2′, H-6′); 7.62–7.50 (m, 4H, H-4, H-5, H-4′, H-5′)
4j	3077 (NH lactam); 1697 (C=O lactam); 1595 (C=N)	12.73 (s, 1H, NH); 7.71–7.65 (m, 6H, CH, H-4, H-2′, H-3′, H-6′); 7.50 (d, 1H, J = 3.3, H-5)



Scheme 2. Mechanistic pathway for compound **3** and its tautomers.

μg/mL), whereas it exhibited a strong effectiveness towards *Haemophilus influenzae* (MIC 1.5 μg/mL); furthermore, it was inactive against *Staphylococcus aureus*, *Escherichia coli* and fungi up to the concentration of 100 μg/mL. More significant inhibitory properties were detected for 5-arylidene derivatives **4a**–**j** against *Bacillus subtilis* (MICs 0.3–6 μg/mL) and *Haemophilus influenzae* (MICs 0.15–1.5 μg/mL) as well as towards *Staphylococcus aureus* at concentrations ranging from 1.5 to 25 μg/mL. None of the tested compounds showed any activity against Gram negative *Escherichia coli* and fungi (MIC > 100 μg/mL). All of the novel 2-thiazolylimino-5-arylidene-4-thiazolidinones exhibited activity lower than those of the reference drugs ampicillin and miconazole.

The kind of the exerted antibacterial activity was investigated by determining the minimal bactericidal concentrations (MBCs). The experimental data (values in brackets) presented in Table 3 show that 2-(thiazol-2-ylimino)thiazolidin-4-one **3** and its 5-arylidene derivatives **4a**–**j** possess bacteriostatic properties, being MBCs twofold or more higher than the corresponding MICs.

In addition, compounds **4a**–**j** were evaluated for antibacterial activity against a wide number of Gram positive bacteria including penicillin-resistant strains of *Staphylococcus aureus* and *Staphylococcus epidermidis*. The MIC values are given in Table 4 in comparison with those of ampicillin used as the standard. The novel 2-thiazolylimino-5-arylidene-4-thiazolidinones demonstrated a high inhibition of all the tested Gram positive microorganisms and a lot of compounds have MIC values in the range of 0.03–6 μg/mL. Most of the compounds demonstrated an excellent antimicrobial activity against bacilli (MICs 0.03–1.5 μg/mL), *Sarcina lutea* (MICs 0.15–6 μg/mL), *Staphylococcus epidermidis*

Table 3. Antimicrobial activity of compounds **3** and **4a–j** expressed as MIC (μg/mL) and, in brackets, as MBC (μg/mL)

Compound	Bacteria[a]				Fungi[b]		
	BS	SA	EC	HI	SC	CT	AN
3	50 (>100)	>100	>100	1.5 (3)	>100	>100	>100
4a	0.7 (3)	3 (12)	>100	0.7 (1.5)	>100	>100	>100
4b	3 (50)	25 (>100)	>100	1.5 (3)	>100	>100	>100
4c	1.5 (12)	12 (>100)	>100	0.3 (0.7)	>100	>100	>100
4d	3 (50)	12 (100)	>100	1.5 (3)	>100	>100	>100
4e	6 (>100)	6 (>100)	>100	0.7 (1.5)	>100	>100	>100
4f	3 (50)	6 (50)	>100	0.7 (1.5)	>100	>100	>100
4g	1.5 (12)	3 (25)	>100	0.3 (0.7)	>100	>100	>100
4h	0.7 (6)	1.5 (25)	>100	0.15 (0.3)	>100	>100	>100
4i	0.3 (6)	1.5 (25)	>100	0.3 (0.7)	>100	>100	>100
4j	0.3 (3)	1.5 (100)	>100	0.15 (12)	>100	>100	>100
Ampicillin	0.007 (0.15)	0.07 (1.5)	3 (25)	0.07 (0.3)	—	—	—
Miconazole	—	—	—		12 (25)	6 (25)	3 (12)

[a] Gram positive bacteria: *Bacillus subtilis* ATCC 6633 (BS) and *Staphylococcus aureus* ATCC 25923 (SA); Gram negative bacteria: *Escherichia coli* SPA 27 (EC) and *Haemophilus influenzae* clinical isolate (HI).
[b] Yeasts: *Saccharomyces cerevisiae* ATCC 9763 (SC) and *Candida tropicalis* ATCC 1369 (CT); mould: *Aspergillus niger* ATCC 6275 (AN).

Table 4. Spectrum of inhibitory activity of compounds **4a–j** against Gram positive bacteria, expressed as MIC (μg/mL) and, in brackets, as MBC (μg/mL)

Microorganism	Compound										
	4a	**4b**	**4c**	**4d**	**4e**	**4f**	**4g**	**4h**	**4i**	**4j**	Ampicillin
Bacillus megaterium	1.5 (12)	6 (25)	1.5 (6)	12 (25)	3 (12)	1.5 (6)	0.7 (3)	0.3 (1.5)	0.15 (0.7)	0.03 (0.7)	0.07 (0.15)
Bacillus thuringiensis var. *kurstaki*	3 (25)	6 (50)	3 (25)	12 (100)	6 (100)	3 (25)	1.5 (25)	0.7 (12)	1.5 (12)	0.3 (6)	50 (>100)
Sarcina lutea	3 (>100)	12 (25)	6 (>100)	12 (>100)	12 (>100)	25 (>100)	3 (>100)	3 (>100)	1.5 (25)	0.15 (6)	0.0015 (0.007)
Staphylococcus aureus methicillin-resistant	25 (>100)	12 (>100)	3 (>100)	6 (100)	3 (100)	1.5 (25)	1.5 (25)	0.7 (>100)	0.7 (25)	0.7 (12)	25 (>100)
Staphylococcus epidermidis	6 (25)	25 (>100)	6 (>100)	12 (100)	12 (>100)	12 (50)	6 (50)	3 (6)	1.5 (25)	0.7 (6)	3 (25)
Staphylococcus epidermidis methicillin-resistant	50 (>100)	25 (>100)	12 (>100)	12 (>100)	25 (>100)	12 (>100)	6 (>100)	3 (>100)	1.5 (50)	1.5 (50)	25 (100)
Staphylococcus haemolyticus	6 (>100)	25 (>100)	12 (>100)	12 (>100)	12 (>100)	25 (>100)	6 (>100)	3 (>100)	3 (>100)	3 (>100)	0.03 (0.7)
Streptococcus agalactiae	3 (50)	12 (>100)	3 (>100)	6 (>100)	12 (>100)	6 (25)	1.5 (>100)	3 (>100)	1.5 (25)	0.3 (>100)	0.03 (0.07)
Streptococcus faecalis	25 (>100)	25 (>100)	>100	50 (>100)	50 (>100)	50 (>100)	>100	12 (>100)	50 (>100)	3 (>100)	0.7 (6)
Streptococcus faecium	25 (>100)	25 (>100)	>100	25 (>100)	25 (>100)	50 (>100)	>100	3 (>100)	3 (>100)	3 (>100)	100 (>100)
Streptococcus pyogenes	>100	25 (>100)	12 (>100)	25 (>100)	25 (>100)	>100	>100	12 (>100)	12 (>100)	3 (>100)	0.007 (1.5)

(MICs 0.7–6 μg/mL) and *Streptococcus agalactiae* (MICs 0.3–6 μg/mL). It is worth noting that compounds **4a–j** are very potent also towards clinical isolates of penicillin-resistant *Staphylococcus aureus* and *Staphylococcus epidermidis* that are inhibited by many thiazolidinones at concentrations of 0.7–3 and 1.5–12 μg/mL, respectively. Furthermore, the data obtained indicate that the antibacterial activity against methicillin-resistant *Staphylococcus epidermidis* is comparable to that exhibited against the methicillin-susceptible one, whereas the growth inhibition of methicillin-resistant *Staphylococcus aureus* is 1- to 2-fold stronger than that exerted towards methicillin-susceptible strain. MBC values reported in Table 4 are always higher than the corresponding MIC ones, in agreement with the bacteriostatic effect of these compounds. It is also interesting to note that *Bacillus thuringiensis* var. *kurstaki*, methicillin-resistant *Staphylococcus aureus*, methicillin-resistant *Staphylococcus epidermidis* and *Streptococcus faecium* were more susceptible to **4a–j** than to standard drug ampicillin used as positive control.

As regards the relationships between the structure and the detected antibacterial activity, the 5-arylidene derivatives **4a–j** showed a significant antibacterial efficacy greater than that of the parent 2-(thiazol-2-ylimino)thiazolidin-4-one **3**, suggesting that the unsubstituted (**4a**) and substituted (**4b–j**) 5-arylidene moiety plays an important role in enhancing the antimicrobial properties of this class of compounds.

Among the 5-arylidene derivatives the inhibitory effect appears to be dependent on the substitution at the benzene ring. The introduction of a chloro atom in the isomeric substances **4h–j**, containing 2, 3- or 4-chlorobenzene ring, respectively, improved antibacterial activity in respect to compound **4a**. Chloro derivatives are generally more effective against all tested microorganisms than arylidene derivatives carrying hydrophilic hydroxy or methoxy group (**4b–d**) or nitro substituted compounds (**4e–g**). At concentrations comparable to or lower than that of ampicillin, compounds **4h–j** act as the most potent inhibitors of the growth of some microorganisms as *Haemophilus influenzae*, *Bacillus megaterium*, *Bacillus thuringiensis* var. *kurstaki*, methicillin-resistant *Staphylococcus aureus*, methicillin-susceptible and -resistant *Staphylococcus epidermidis* and *Streptococcus faecium*.

It is interesting to point out that for the isomeric nitro substituted compounds **4e–g** as well as for the chloro substituted ones **4h–j**, the *para* substituted derivatives (**4g** and **4j**) are mostly endowed with higher activity with respect to *ortho* (**4e** and **4h**) and *meta* (**4f** and **4i**) derivatives.

3. Conclusion

The newly synthesized 2-thiazolylimino-5-arylidene-4-thiazolidinones **4a–j** exhibit a remarkable inhibition of the growth of a wide spectrum of Gram positive bacteria and Gram negative *Haemophilus influenzae*, whereas *Escherichia coli* (Gram negative microorganism as well) and fungi are not susceptible. The 5-arylidene derivatives have an antibacterial efficacy greater than that of the starting 2-(thiazol-2-ylimino)thiazolidin-4-one **3**, suggesting the important role of the arylidene ring in increasing the antimicrobial properties of this class of compounds. All the compounds **4a–j** showed excellent antibacterial activity indicating that the diverse substitutions were well tolerated on the benzylidene moiety for proper fit at the potential receptor site. The excellent effectiveness against Gram positive bacteria, Gram negative *Haemophilus influenzae* and the spectrum of activity extended to penicillin-resistant staphylococci make these substances attractive antibacterial candidates also for the treatment of infections caused by microoganisms resistant to currently available drugs. The outstanding properties of this new class of antibacterial substances deserve further investigation in order to clarify the mode of action at molecular level, responsible for the activity observed. More extensive study is also warranted to determine additional physicochemical and biological parameters to have a deeper insight into structure–activity relationship and to optimize the effectiveness of this series of molecules.

4. Experimental

4.1. Chemistry

Synthetic starting material, reagents and solvents were purchased from Aldrich or Fluka. All the solvents were of reagent grade and dried prior to use. Melting points °C were determined with a Boetius apparatus and are uncorrected. Elemental analysis were performed in the analytical laboratory of Dipartimento Farmaceutico, Università di Parma, on a ThermoQuest (Italia) FlashEA 1112 Elemental Analyzer, for C H N and S. The found values were always ±0.4% of the theoretical ones. IR spectra were recorded, as KBr pellets, on a Jasco FT-IR 300E spectrophotometer (Jasco Ltd., Tokyo, Japan) and the reported wavenumbers are given in cm^{-1}. ^1H NMR spectra, in DMSO-d_6 solutions, were recorded on a Bruker AC 300 instrument at 298 K. Chemical shifts are reported as δ (ppm) relative to TMS as internal standard. Mass spectra were recorded on a VG-250 spectrometer (VG Labs., Tritech England) at 70 eV. The progress of the reactions was monitored by thin layer chromatography with F_{254} silica-gel precoated sheets (Merck, Darmstadt, Germany) using benzene/ethanol 8/2 as eluent; UV light was used for detection.

4.1.1. General procedure for synthesis of 2-thiazolylimino-5-arylidene-4-thiazolidinones 4a–j. A well-stirred solution of 0.8 g of 2-(thiazol-2-ylimino)thiazolidin-4-one (4 mmol) in 35 mL of acetic acid was buffered with sodium acetate (8 mmol) and added with the appropriate arylaldehyde (6 mmol). The solution was refluxed for 4 h and then poured into ice-cold water. The precipitate was filtered and washed with water and the resulting crude product was purified by recrystallisation from dioxane.

4.2. Microbiology

The antimicrobial susceptibility testing was performed in vitro by the twofold broth dilution technique.[30] The Gram positive bacteria utilized in this study consisted of *Bacillus megaterium* BGSC 7A2, *Bacillus subtilis* ATCC 6633, *Bacillus thuringiensis* var. *kurstaki* BGSC 4D1, *Sarcina lutea* ATCC 9341, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228 and clinical isolates of methicillin-resistant *Staphylococcus aureus*, methicillin-resistant *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Streptococcus agalactiae*, *Streptococcus faecalis*, *Streptococcus faecium* and *Streptococcus pyogenes*. The Gram negative bacteria included *Escherichia coli* SPA 27 and a clinical isolate of *Haemophilus influenzae*. The antifungal activity was assayed against yeasts (*Candida tropicalis* ATCC 1369 and *Saccharomyces cerevisiae* ATCC 9763) and moulds (*Aspergillus niger* ATCC 6275). The minimal inhibitory concentrations (MIC, μg/mL) were defined as the lowest concentrations of compound that completely inhibited the growth of each strain. Test compounds were dissolved in dimethylsulfoxide and then diluted in culture medium (Haemophilus Test Medium for *Haemophilus influenzae*, Tryptose Phosphate Broth for *Streptococcus pyogenes*, Mueller–Hinton Broth for other bacteria and Sabouraud Liquid Medium for fungi) to obtain final concentrations ranging from 100 to 0.0015 μg/mL. Dimethylsulfoxide never exceeded 1% v/v. The amount of inocula was 5×10^4 bacteria/mL and 1×10^3 fungi/mL. The MICs were read after incubation at 37 °C for 24 h (bacteria) and at 30 °C for 48 h (fungi). Growth controls consisting of media and media with

1% v/v dimethylsulfoxide were employed. Ampicillin and miconazole were used as reference antibacterial and antifungal substances, respectively.

The minimal bactericidal concentrations (MBC, μg/mL) were measured by subculturing 100 μL of each sample remaining clear in tubes containing 1 mL of fresh medium. The tubes were then incubated at 37 °C for 24 h.

All experiments were performed in duplicate and repeated three times.

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