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Laboratory note

Hybrid molecules between benzenesulfonamides and active antimicrobial benzo[*d*]isothiazol-3-ones

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ABSTRACT

Novel hybrid molecules between benzenesulfonamides and active antimicrobial 2-amino-benzo[*d*]isothiazol-3-ones were synthesized and characterised and their in vitro antimicrobial activity was evaluated by the minimal inhibitory concentration (MIC). The compounds exhibit moderate antibacterial properties against Gram-positive bacteria (MIC 6–100 $\mu g\,ml^{-1}$) such as several bacilli, staphylococci and streptococci, including methicillin-resistant *Staphylococcus aureus* and *Staphylococcus epidermidis* strains, while no inhibition of Gram-negative *Escherichia coli* is detected up to the concentration of 100 $\mu g\,ml^{-1}$. Synergistic inhibitory activity occurs when sulfanilamides **4a** and **4c** are tested in combination with trimethoprim against *S. aureus*. Concerning antifungal properties, only compound **4c** is able to inhibit the growth of *Saccharomyces cerevisiae* and *Cryptococcus neoformans* yeasts and several dermatophytes. Structure–activity relationships are discussed.

1. Introduction

The emergence of microorganisms resistant to drugs which are utilised as therapeutic agents stimulates our interest in the search for novel and more efficient antimicrobial substances.

Benzo[*d*]isothiazol-3-ones are a class of heterocyclic compounds endowed with a broad spectrum of biological activity [1,2]. They have been reported to possess antiplatelet and spasmolytic effects [3,4] and they attracted a great deal of interest particularly on the antimicrobial activity. Their inhibitory properties as regards several Gram-positive and Gram-negative bacteria, yeasts and moulds have been extensively demonstrated [1,5–9]. Recently, in the course of our research on biologically active benzo[*d*]isothiazoles, we discovered that 2-amino-benzo[*d*]isothiazol-3-one derivatives are wide spectrum antimicrobial substances exhibiting a good inhibition of the growth of penicillin-resistant staphylococci and, in some cases, possessing activity equal to or superior to the reference drugs ampicillin and miconazole [10,11].

On the other hand, sulfonamides aroused considerable interest in biology and medicine as antimicrobial antifolic agents [12–19]. Their antibacterial effect is considerably enhanced by combination with trimethoprim through a well known synergistic effect. Thus, sulfamethoxazole is a very active pharmaceutical compound useful in combination with trimethoprim (Co-trimoxazole) as first choice treatment of pneumonia and urinary tract bacterial infections, toxoplasmosis [20] and pneumocystosis in HIV infected patients [21].

Examples of hybrid drugs, incorporating moieties both of a sulfonamide and a different antibacterial agent, i.e. fluoroquinolone, are reported in the literature [22–24] as being very active. Moreover, in our previous paper [25] the antibacterial and antifungal activities of some *N*-(benzo[*d*]isothiazolyl)benzenesulfonamides have been described.

According to Wermuth [26], the approach to drug design based on the preparation of dual- or multiple-ligands, on almost rational basis, can be expected to yield drugs of superior clinical value, compared with monotargeted ones. Among antibacterial agents, "dual targeting" compounds may be a preferred objective also because they would be less prompted to develop bacterial resistance. Thus, to prove the impact of the sulfonamide group on the biological activity of benzisothiazolones, we synthesized and evaluated for antibacterial and antifungal properties a number of unsubstituted and 5-methyl substituted 2-(benzenesulfonyl)amino-benzo[*d*]isothiazol-3-ones (Fig. 1, compounds **4a**–**4d**) and 2-(bisbenzenesulfonyl)amino-benzo[*d*]isothiazol-3-ones (Fig. 1, compounds **5a**–**5d**) bearing both the benzo[*d*]isothiazol-3-one and the sulfonamido pharmacophoric moieties.

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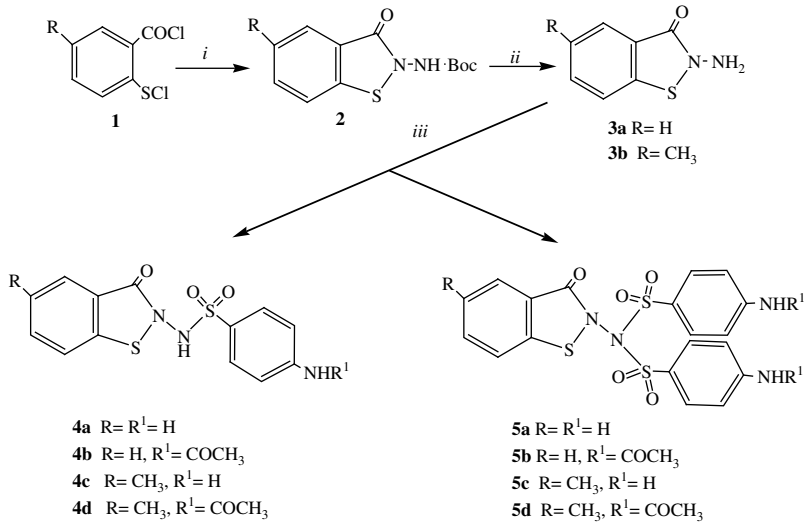

Fig. 1. Scheme of synthesis and structure of compounds **4a**–**4d** and **5a**–**5d**. Reagents and conditions: (*i*) NH_2-NH-Boc $(C_2H_5)_2O$/pyridine, 60 min 10 °C, (*ii*) TCA/H_2O, 150 min, RT, (*iii*) 4-$COCH_3$-phenyl-SO_2-Cl, pyridine, 120 min, −10 to −5 °C; for $R^1 = H$, the following: EtOH/H_2O/HCl, 120 min, reflux.

2. Results and discussion

2.1. Chemistry

The synthetic process for the preparation of the target compounds is outlined in Fig. 1. Chlorocarbonylphenylsulfenyl-chloride **1**, prepared according to the method previously described [3], upon cyclization with *N*-Boc-protected hydrazine afforded the Boc-protected derivative **2** that, after removal of the *N*-Boc group by mild acidic hydrolysis with trichloroacetic acid, gave the key intermediate **3** [3,27]. The 2-amino-benzo[*d*]isothiazol-3-ones **3** were then reacted with 4-acetamidobenzene-1-sulfonyl chloride, in pyridine, in a cooling bath, to afford 2-(benzenesulfonyl)amino-benzo[*d*]isothiazol-3-ones **4** and 2-(bisbenzenesulfonyl)amino-benzo[*d*]isothiazol-3-ones **5**. Indeed once the sulfonamide **4** is formed it readily undergoes, by the action of the electrophilic sulfonyl chloride, a subsequent sulfonylation yielding the disulfonyl derivative **5**. Products **4** and **5** can anyway be simply separated because of the acidic character of the former.

The structures of the synthesized compounds were supported by the spectral IR and ^{1}H NMR data and by the results of elemental analysis which are in agreement with the proposed structures.

2.2. Microbiology

The antimicrobial activity of benzo[*d*]isothiazol-3-one sulfonamides **4a**–**4d** and **5a**–**5d** was evaluated by determination of the minimal inhibitory concentration (MIC) against various bacteria and fungi, each referred to standard collection strains or fresh clinical isolates. The results are summarized in Table 1 and are compared with those of standards ampicillin and sulfamethoxazole as antibacterial agents and miconazole as an antifungal drug. Previously reported data concerning the parent 2-amino-benzisothiazolones **3a** and **3b** have also been included in the table in order to provide structure–activity relations.

Apart from a few exceptions, the new compounds exhibit moderate antibacterial activity against representative Gram-positive bacteria *Bacillus subtilis* and *Staphylococcus aureus* with MICs ranging from 12 to 100 $\mu g\,ml^{-1}$. Among the active compounds, **5c** shows the highest inhibition of the above bacteria (MIC 12 $\mu g\,ml^{-1}$ and 50 $\mu g\,ml^{-1}$, respectively). Compound **4b** and compounds **4d** and **5d** have no effect up to the dose of 100 $\mu g\,ml^{-1}$ against both bacteria and *S. aureus* only, respectively.

No activity is detected for all of the tested compounds against Gram-negative *Escherichia coli* as well as any of the yeasts and moulds tested (MIC > 100 $\mu g\,ml^{-1}$). Only compound **4c** exhibits a certain antifungal activity inhibiting the growth of *Saccharomyces cerevisiae* at the concentration of 100 $\mu g\,ml^{-1}$.

In all the cases, MIC values are higher than those of the reference drugs sulfamethoxazole, ampicillin and miconazole and those of the starting compounds **3a** and **3b**.

With the aim of gaining deeper insights into the antimicrobial activity of the new compounds, the minimal bactericidal concentrations (MBCs) and the minimal fungicidal concentrations (MFCs) were determined. As a general trend, these values are higher than MICs, pointing out that the effectiveness of these substances is endowed with a bacteriostatic and fungistatic character.

The mode of action of the new benzisothiazolone derivatives here under study was also investigated by detecting the possible reaction with essential thiol groups of enzymes. In fact, the target of the class of benzisothiazolones as antimicrobials was identified with susceptible enzymes bearing accessible thiols at the interactive centres since the growth inhibitory potency of these compounds was rapidly quenched by the presence of thiol-containing materials such as glutathione or cysteine [28,29]. So the effect on the growth inhibitory activity of a known concentration of cysteine, added to the media before inoculation, was estimated. In all cases MICs increased by a factor two or more (Table 1, values in italic). That is consistent with the previously cited reports [28,29] and supports the binding of these compounds to sulphurated proteins as if they would exert their biological action through a benzisothiazolone-like mechanism of action.

To clarify the contribution of the sulfonamide moiety to the antibacterial activity of these compounds, the effect of *p*-amino-benzoic acid (PABA) on the MICs was tested by including it in the medium (Table 1, values underlined). Only for compounds **4a** and **4c** this supplementation caused a two-fold decrease in the inhibition of *S. aureus* growth, while no changes in the MIC values of the other compounds were observed, in accordance with the suggestion that **4a** and **4c** against *S. aureus* behave as dual inhibitors both by binding to thiol groups and by inhibiting the synthesis of folic acid.

Compounds **4a**, **4c** and **5a**–**5d**, demonstrated to be the most active against Gram-positive bacteria, were subjected to a screening against a wide spectrum of bacteria such as *Sarcina lutea*, several bacilli, staphylococci and streptococci, including

Table 1
Antimicrobial activity, expressed as MIC (μg ml^{-1}) and, in brackets, as MBC and MFC (μg ml^{-1})

Compounds	Bacteria[a]			Fungi[b]		
	BS	SA	EC	SC	CT	AN
3a[c]	0.7 (1.5)	6 (12)	6 (50)	6 (25)	6 (25)	100 (>100)
3b[c]	0.7 (1.5)	6 (25)	25 (100)	6 (12)	12 (25)	50 (>100)
4a	50 (>100) *100*[d] 50[e]	50 (>100) *>100* 100	>100	>100	>100	>100
4b	>100	>100	>100	>100	>100	>100
4c	25 (100) *50* 25	50 (>100) *100* 100	>100	100 (>100) *>100*	>100	>100
4d	100 (>100) *>100* 100	>100	>100	>100	>100	>100
5a	25 (50) *>100* 25	50 (>100) *>100* 50	>100	>100	>100	>100
5b	50 (>100) *>100* 50	100 (>100) *>100* 100	>100	>100	>100	>100
5c	12 (100) *>100* 12	50 (100) *>100* 50	>100	>100	>100	>100
5d	50 (>100) *>100* 50	>100	>100	>100	>100	>100
Sulfamethoxazole	6 (25)	25 (100)	>100	>100	>100	>100
Ampicillin	0.007 (0.15)	0.07 (1.5)	3 (25)	–[f]	–	–
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).
[b] Yeasts: *Saccharomyces cerevisiae* ATCC 9763 (SC) and *Candida tropicalis* ATCC 1369 (CT); mould: *Aspergillus niger* ATCC 6275 (AN).
[c] Values reported in a previous paper [10].
[d] MIC obtained in the presence of cysteine.
[e] MIC obtained in the presence of PABA.
[f] Not tested.

methicillin-resistant *S. aureus* and *Staphylococcus epidermidis* strains (Table 2). Comparison tests were performed by using sulfamethoxazole as standard drug. The compounds exert a moderate inhibition of all the tested microorganisms, methicillin-resistant bacteria included. *S. lutea* appears to be the most sensitive strain. In general, MIC values range from 6 to 100 μg ml^{-1} and in the case of both methicillin-susceptible and methicillin-resistant *S. epidermidis*, *Streptococcus faecalis* and *Streptococcus faecium* benzisothiazolone sulfonamides are either more active or equipotent to sulfamethoxazole. MBCs (Table 2) increase by a factor of two or more when compared with MICs, confirming the bacteriostatic effect of the tested compounds.

Table 3 presents the antifungal effectiveness of compound **4c** against various yeasts and moulds. This compound acts towards *Cryptococcus neoformans* at 100 μg ml^{-1} in a fungistatic manner (MFC > 100 μg ml^{-1}) and shows significant inhibition of mould *Madurella mycetomatis* at 12 μg ml^{-1} and dermatophytes *Epidermothyton floccosum*, *Microsporum gypseum*, *Trichophyton mentagrophytes*, *Trichophyton rubrum* and *Trichophyton soudanense* at concentrations of 6–50 μg ml^{-1}. However, its antifungal activity is less potent than that of miconazole standard drug.

The analysis of the results reported in Table 1 shows that the activity of the chemicals tested can be correlated to the substituents both in the sulfonamide and in the heterocyclic nucleus.

Table 2
Antibacterial activity, expressed as MIC (μg ml^{-1}) and, in brackets, as MBC (μg ml^{-1}), against Gram-positive bacteria

Microorganisms	Compounds						
	4a	**4c**	**5a**	**5b**	**5c**	**5d**	SMZ[a]
Bacillus cereus ATCC 11966	100 (>100)	50 (>100)	100 (>100)	>100	50 (>100)	>100	12 (50)
Bacillus circulans BGSC 16A1	50 (>100)	25 (100)	50 (>100)	25 (>100)	25 (50)	100 (>100)	1.5 (12)
Bacillus megaterium BGSC 7A2	25 (>100)	25 (50)	50 (>100)	100 (>100)	25 (50)	50 (100)	1.5 (6)
Bacillus pumilus BGSC 8E2	50 (>100)	50 (>100)	50 (100)	50 (100)	25 (50)	100 (>100)	1.5 (12)
Bacillus subtilis var. *sotto* BGSC 27A1	50 (>100)	50 (100)	25 (50)	100 (>100)	25 (50)	100 (>100)	1.5 (12)
Bacillus thuringiensis var. *kurstak*, BGSC 4D1	50 (>100)	25 (>100)	50 (>100)	>100	50 (>100)	>100	1.5 (12)
Sarcina lutea ATCC 9341	6 (12)	12 (50)	25 (50)	100 (>100)	25 (50)	50 (>100)	0.7 (3)
Staphylococcus aureus methicillin-resistant[b]	100 (>100)	50 (>100)	50 (>100)	100 (>100)	25 (>100)	100 (>100)	12 (50)
Staphylococcus epidermidis[b]	100 (>100)	50 (>100)	25 (100)	100 (>100)	25 (>100)	>100	50 (>100)
Staphylococcus epidermidis methicillin-resistant[b]	100 (>100)	50 (>100)	25 (>100)	>100	25 (>100)	>100	>100
Staphylococcus haemolyticus[b]	50 (>100)	50 (>100)	50 (>100)	>100	25 (>100)	>100	25 (>100)
Streptococcus agalactiae[b]	50 (>100)	25 (>100)	50 (>100)	100 (>100)	25 (>100)	100 (>100)	6 (50)
Streptococcus faecalis[b]	100 (>100)	50 (>100)	50 (>100)	100 (>100)	50 (>100)	100 (>100)	>100
Streptococcus faecium[b]	50 (>100)	25 (>100)	50 (>100)	100 (>100)	50 (>100)	>100	>100
Streptococcus pyogenes[b]	50 (100)	25 (50)	50 (100)	50 (100)	25 (100)	50 (>100)	3 (25)

[a] Sulfamethoxazole.
[b] Isolate of clinical origin.

Table 3
Antifungal activity of compound **4c** against yeasts and moulds (MIC and, in brackets, MFC expressed in $\mu g\,ml^{-1}$)

Fungi	4c	Miconazole
Yeasts		
Candida albicans ATCC 10231	>100	6
Candida guilliermondii[a]	>100	0.15
Candida parapsilosis[a]	>100	3
Cryptococcus neoformans[a]	100 (>100)	0.3
Moulds[a]		
Epidermophyton floccosum	12	0.01
Madurella mycetomatis	12	0.007
Microsporum gypseum	50	12
Pseudoallescheria boydii	100	100
Sporothrix schenckii	>100	50
Trichophyton interdigitalis	>100	1
Trichophyton mentagrophytes	50	1
Trichophyton rubrum	6	1
Trichophyton soudanense	12	0.1

[a] Clinical isolates.

Compounds **4a**, **4c**, **5a** and **5c**, having a free amino group at the 4 position of the sulfonamide moiety, are more potent than their corresponding acetylamino parent compounds.

Moreover, the inhibition activity seems to be driven to a certain degree by the lipophilicity, because the 5-methyl substituted benzothiazolones **4c**, **4d**, **5c** and **5d** are more active than their 5-unsubstituted analogs **4a**, **4b**, **5a** and **5b**. Similarly, 2-(bisbenzenesulfonyl)amino-benzo[*d*]isothiazol-3-ones exhibit, in general, higher inhibitory activity than the corresponding monobenzenesulfonyl derivatives. This trend supports the hypothesis that these compounds can penetrate the microbial membranes of sensitive Gram-positive bacteria and interact with their cellular targets at a higher extent.

Clearly, the insertion of the sulfonamido group plays a negative role on the antimicrobial activity of the 2-aminobenzisothiazolones **3a** and **3b** as it produces a remarkable decrease of the inhibitory potency towards all the tested microorganisms. This suggests that a sulfa-like mode of action would make no contribution to the in vitro activity of the compounds under study and that the steric hindrance due to the sulfonamido group partially prevents the interaction of the benzisothiazolone derivatives with their target thiol groups.

In addition, to investigate if the antibacterial effectiveness of the new sulfonamides increases in the presence of trimethoprim, synergism studies were carried out. So, the inhibition profile of the sulfonamides **4a**, **4c**, **4d** and **5a**–**5d** active against *B. subtilis* and of the sulfonamides **4a**, **4c** and **5a**–**5c** active against *S. aureus* was evaluated in combination with trimethoprim against the respective sensible strains. The results were, then, compared with the antibacterial activity of the substances used individually. Sulfamethoxazole–trimethoprim mixture was taken as positive control.

At first the paper strip diffusion determinations on agar medium were performed. The antibacterial activity of the sulfanilamides **4a** and **4c** was potentiated by trimethoprim when *S. aureus* was assayed. In this case the size of inhibition zones was greatest where the two drugs overlapped (data not shown), as observed for trimethoprim and sulfamethoxazole. On the contrary, no synergistic action was presented by combination of trimethoprim and compounds **5a**–**5c** for the same strain and by the interaction of trimethoprim and all the sulfonamides against *B. subtilis*.

Since the paper strip test has a qualitative character, the antimicrobial synergism was then quantitatively assessed by the two-fold dilution method in the checkerboard titration. Sulfonamides and trimethoprim were tested together in a large number of dilutions to determine the concentrations of each drug in combination with trimethoprim that produce an endpoint of no growth towards *B. subtilis* and *S. aureus*. The interactions were detected in comparison with sulfamethoxazole–trimethoprim mixture and are summarized by the isobolograms of Figs. 2 and 3. With regard to *B. subtilis*, the graphical representation (Fig. 2) displays straight lines which connect the individual fractional inhibitory concentrations of the drugs and the fractional inhibitory concentration indices range from 1 (for compounds **4a**, **4c**, **4d**, **5a** and **5c**) to 1.5 (for compounds **5b** and **5d**). It suggests that only additivity or indifference occurs from the combined action of trimethoprim with compounds **4a**, **4c**, **4d**, **5a** and **5c** and with compounds **5b** and **5d**, respectively. On the contrary, subinhibitory concentrations of trimethoprim potentiates the antibacterial activity of mono-sulfanilamides **4a** and **4c** against *S. aureus* cells by reducing their MIC values. Fig. 3A, that refers to the latter compounds, exhibits concave isobols and fractional inhibitory concentration indices lower than 0.5. This synergistic effect is comparable, even if less marked, to that produced by the reference sulfamethoxazole–trimethoprim mixture and supports a sulfa-like mechanism of action. On the contrary, combinations of bis-sulfonamides **5a**–**5c** have shown to be additive (Fig. 3B), FIC indices being >0.5 and <1. Thus, in accordance with the observations done when the compounds were tested in the presence of PABA, it can be pointed out that only **4a** and **4c** act as "dual targeting" compounds, both by binding to thiol groups and by inhibiting the synthesis of folic acid. Nevertheless, their antibacterial efficacy against *S. aureus* is not higher than that of the corresponding bis-sulfanilamides **5a** and **5c**, which probably owe their quantitatively similar activity to the favourable increase of lipophilicity with respect to **4a** and **4c**.

3. Conclusion

Unsubstituted and 5-methyl substituted 2-(benzenesulfonyl)amino-benzo[*d*]isothiazol-3-ones and 2-(bisbenzenesulfonyl)amino-benzo[*d*]isothiazol-3-ones exhibit moderate antimicrobial properties against Gram-positive bacteria, including methicillin-resistant strains. The inhibition activity seems to be controlled to a certain degree by the facility of entering the bacterial membrane and blocking the essential enzymes involved in the cell metabolism. Although the newly synthesized compounds, carrying the

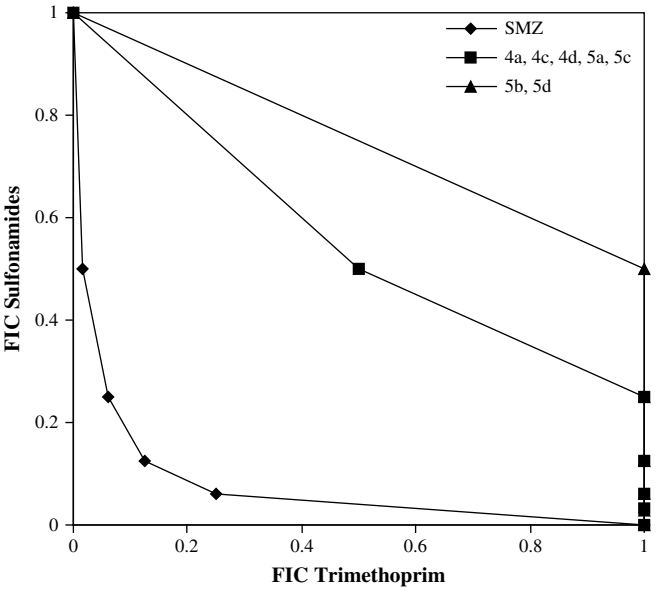

Fig. 2. Assessment of combinations of sulfamethoxazole (SMZ) and sulfonamides **4a**, **4c**, **4d**, **5a**–**5d** with trimethoprim against *Bacillus subtilis* ATCC 6633 in the checkerboard broth dilution method. The concentrations of the drugs are expressed as fractions of the minimal inhibitory concentrations (FIC, fractional inhibitory concentration).

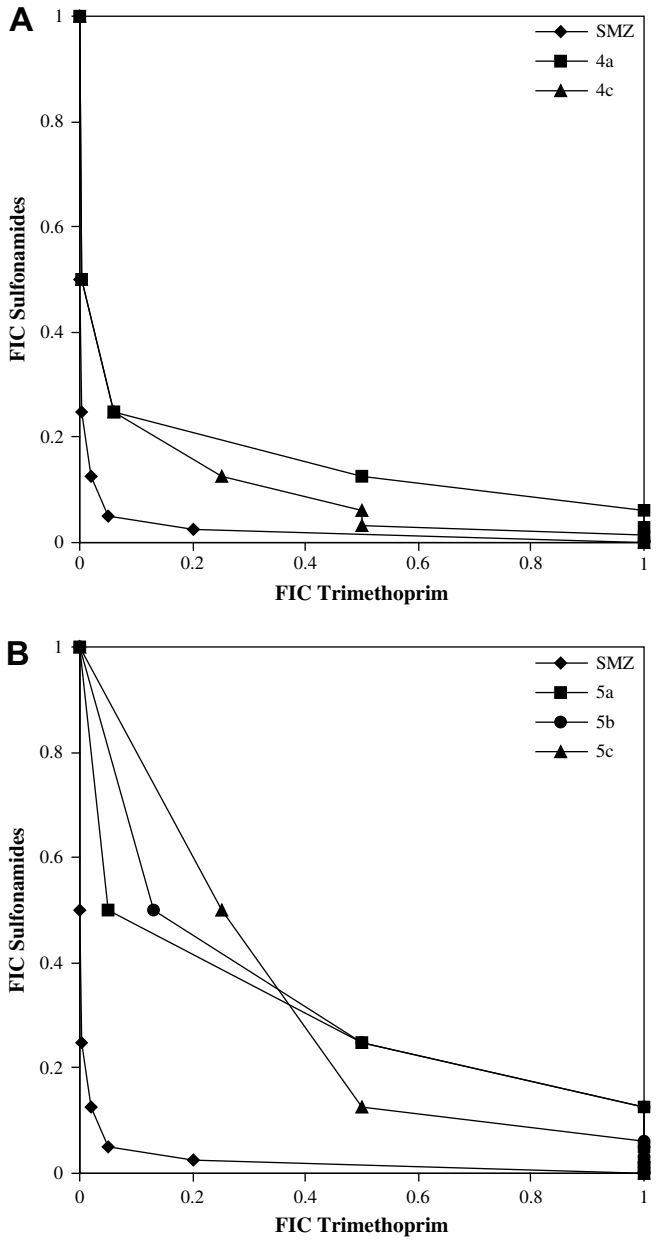

Fig. 3. Assessment of combinations of sulfamethoxazole (SMZ) and sulfonamides **4a** (A), **4c** (A), **5a**–**5c** (B) with trimethoprim against *Staphylococcus aureus* ATCC 25923 in the checkerboard broth dilution method. The concentrations of the drugs are expressed as fractions of the minimal inhibitory concentrations (FIC, fractional inhibitory concentration).

benzisothiazolone moiety and the sulfonamide portion, could be considered hybrid drugs from a structural point of view, notwithstanding two of them act also by a sulfa-like mechanism, it can be stated the sulfa-like mode of action would make no or little contribution to the detected antimicrobial activity.

4. Experimental protocols

4.1. Chemistry

Synthetic starting material, reagents and solvents were purchased from Aldrich or Fluka. Solvents, unless otherwise specified were reagent grade or of the highest quality commercially available. Melting points (°C) were determined with a Buchi 512 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 for C, H, N, S 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. The progress of the reactions was monitored by thin layer chromatography with F_{254} silica-gel precoated sheets (Merck, Darmstadt, Germany) and UV light was used for detection.

*4.1.1. General procedure for synthesis of 2-(4′-acetylaminobenzenesulfonyl)amino-benzo[*d*]isothiazol-3-ones (**4b**, **4d**) and of 2-(bis-4′-acetylaminobenzenesulfonyl) amino-benzo[*d*]isothiazol-3-ones (**5b**, **5d**)*

Small portions of 4-acetylaminobenzene-1-sulfonyl chloride (10 mmol) were added to a stirred mixture of the suitable 2-amino-benzo[*d*]isothiazol-3-one **3** (9 mmol) in pyridine (9 ml) at 0–5 °C for 1 h and then kept at room temperature for 2 h. The reaction mixture was poured into water and crushed ice (20 ml/20–30 g), acidified with conc. HCl and stirred at room temperature overnight. The solid obtained was filtered and washed thoroughly with water (3 × 10 ml), then poured again into water (200 ml), added with 10% Na_2CO_3 (5 ml) and kept under stirring for 4 h. The residue (product **5**) was filtered off and the filtrate, treated with conc. HCl, afforded compound **4** that was collected by filtration and purified by column chromatography (CH_2Cl_2/EtOH, 95:5). Compounds **4** and **5** were then recrystallised from suitable solvents.

*4.1.1.1. 2-(4′-Acetylaminobenzenesulfonyl)amino-benzo[*d*]isothiazol-3-one (**4b**).* Yield: 35%; mp 189–190 °C (ethanol). IR (KBr): 3379, 3163 (NH), 1691 (CO), 1336, 1157 (SO_2) cm^{-1}; ^1H NMR (DMSO-d_6, δ, ppm): 11.10 (s, 1H, $NHSO_2$); 10.36 (s, 1H, NHCO); 7.88–7.78 (m, 6H, H-4, H-7, H-2′, H-3′, H-5′, H-6′); 7.69 (t, 1H, $J = 7.2$, H-5); 7.40 (t, 1H, $J = 7.2$, H-6); 2.10 (s, 3H, CH_3). Anal. Calcd. for $C_{15}H_{13}N_3O_4S_2$ (363.41): C, 49.57; H, 3.61; N, 11.56; S, 17.65. Found: C, 49.52; H, 3.66; N, 11.44; S, 17.53.

*4.1.1.2. 2-(4′-Acetylaminobenzenesulfonyl)amino-5-methyl-benzo[*d*]isothiazol-3-one (**4d**).* Yield: 32%; mp 175–177 °C (dichloromethane). IR (KBr): 3373, 3170 (NH), 1687 (CO), 1338, 1157 (SO_2) cm^{-1}; ^1H NMR (DMSO-d_6, δ, ppm): 11.11 (s, 1H, $NHSO_2$); 10.37 (s, 1H, NHCO); 7.77–7.74 (m, 5H, H-7, H-2′, H-3′, H-5′, H-6′); 7.63 (s, 1H, H-4); 7.52 (dd, 1H, $J = 8.4$, $J = 1.8$, H-6); 2.37 (s, 3H, CH_3); 2.10 (s, 3H, $COCH_3$). Anal. Calcd. for $C_{16}H_{15}N_3O_4S_2$ (377.44): C, 50.91; H, 4.01; N, 11.13; S, 16.99. Found: C, 50.73; H, 4.06; N, 10.93; S, 16.79.

*4.1.1.3. 2-(Bis-4′-acetylaminobenzenesulfonyl)amino-benzo[*d*]isothiazol-3-one (**5b**).* Yield: 36%; mp 216–218 °C (ethyl acetate/petroleum ether). IR (KBr): 3316 (NH), 1680 (CO), 1375, 1169 (SO_2) cm^{-1}; ^1H NMR (DMSO-d_6, δ, ppm): 10.53 (s, 2H, NH); 7.96 (d, 1H, $J = 8.1$, H-4); 7.92–7.86 (m, 9H, H-2′, H-3′, H-5′, H-6′, H-2″, H-3″, H-5″, H-6″, H-7); 7.78 (t, 1H, $J = 7.2$, H-5); 7.47 (t, 1H, $J = 7.2$, H-6); 2.13 (s, 6H, CH_3). Anal. Calcd. for $C_{23}H_{20}N_4O_7S_3$ (560.63): C, 49.27; H, 3.60; N, 9.99; S, 17.16. Found: C, 49.48; H, 3.93; N, 9.66; S, 16.81.

*4.1.1.4. 2-(Bis-4′-acetylaminobenzenesulfonyl)amino-5-methyl-benzo[*d*]isothiazol-3-one (**5d**).* Yield: 62%; mp 175–178 °C (ethyl acetate/petroleum ether). IR (KBr): 3322 (NH), 1685 (CO), 1371, 1165 (SO_2) cm^{-1}; ^1H NMR (DMSO-d_6, δ, ppm): 10.55 (s, 2H, NH); 7.88–7.81 (m, 9H, H-2′, H-3′, H-5′, H-6′, H-2″, H-3″, H-5″, H-6″, H-7); 7.71 (s, 1H, H-4); 7.61 (dd, 1H, $J = 8.3$, $J = 1.35$, H-6); 2.40 (s, 3H, CH_3); 2.13 (s, 6H, CH_3). Anal. Calcd. for $C_{24}H_{22}N_4O_7S_3$ (574.65): C, 50.16; H, 3.86; N, 9.75; S, 16.74. Found: C, 50.09; H, 4.13; N, 9.34; S, 16.57.

*4.1.2. General procedure for synthesis of 2-(4′-aminobenzenesulfonyl)amino-benzo[*d*]isothiazol-3-ones (**4a**, **4c**) and of 2-(bis-4′-aminobenzenesulfonyl) amino-benzo[*d*]isothiazol-3-ones (**5a**, **5c**)*

A suspension of the appropriate 4′-acetylaminobenzenesulfonylamino-benzo[*d*]isothiazol-3-one **4b**, **4d** or **5b**, **5d** (5 mmol) in ethanol (75 ml) and 1.2 N HCl (20 ml) were refluxed for 120 min. The solvent was evaporated under reduced pressure. The residue was added with water (100 ml) and sodium acetate to pH 5–6. The precipitate was filtered, washed with water, dried and crystallized from suitable solvent.

*4.1.2.1. 2-(4-Aminobenzenesulfonyl)amino-benzo[*d*]isothiazol-3-one (**4a**).* Yield: 70%; mp 172–174 °C (ethanol). IR (KBr): 3473, 3345 (NH_2), 3180 (NH), 1673 (CO), 1348, 1163 (SO_2) cm^{-1}; ^1H NMR (DMSO-d_6, δ, ppm): 11.12 (s, 1H, $NHSO_2$); 7.88–7.81 (m, 2H, H-4, H-7); 7.68 (t, 1H, $J = 7.2$, H-5); 7.47–7.38 (m, 3H, H-6, H-2′, H-6′); 6.60 (d, 2H, $J = 9$, H-3′, H-5′); 6.14 (s, 2H, NH_2). Anal. Calcd. for $C_{13}H_{11}N_3O_3S_2$ (321.38): C, 48.59; H, 3.45; N, 13.08; S, 19.05. Found: C, 48.81; H, 3.77; N, 12.78; S, 18.96.

*4.1.2.2. 2-(4-Aminobenzenesulfonyl)amino-5-methyl-benzo[*d*]isothiazol-3-one (**4c**).* Yield: 73%; mp 187–190 °C (ethyl acetate/petroleum ether). IR (KBr): 3471, 3389 (NH_2), 3037 (NH), 1670 (CO), 1345, 1155 (SO_2) cm^{-1}; ^1H NMR (DMSO-d_6, δ, ppm): 10.58 (s, 1H, $NHSO_2$); 7.74 (d, 1H, $J = 8.3$, H-7); 7.64 (s, 1H, H-4); 7.51 (dd, 1H, $J = 8.3$, $J = 1.5$, H-6); 7.41 (d, 2H, $J = 8.5$, H-2′, H-6′); 6.59 (d, 2H, $J = 8.5$, H-3′, H-5′); 6.14 (s, 2H, NH_2); 2.38 (s, 3H, CH_3). Anal. Calcd. for $C_{14}H_{13}N_3O_3S_2$ (335.41): C, 50.13; H, 3.91; N, 12.53; S, 19.12. Found: C, 50.00; H, 4.09; N, 12.22; S, 18.82.

*4.1.2.3. 2-(Bis-4′-aminobenzenesulfonyl)amino-benzo[*d*]isothiazol-3-one (**5a**).* Yield: 70%; mp 203–205 °C (ethyl acetate/petroleum ether). IR (KBr): 3494, 3394 (NH_2), 1693 (CO), 1375, 1159 (SO_2) cm^{-1}; ^1H NMR (DMSO-d_6, δ, ppm): 7.94–7.87 (m, 2H, H-4, H-7); 7.75 (t, 1H, $J = 7.2$, H-5); 7.55–7.41 (m, 5H, H-6, H-2′, H-6′, H-2″, H-6″); 6.62 (d, 4H, $J = 8.4$, H-3′, H-5′, H-3″, H-5″); 6.45 (br s, 4H, NH_2). Anal. Calcd. for $C_{19}H_{16}N_4O_5S_3$ (476.55): C, 47.89; H, 3.38; N, 11.76; S, 20.19. Found: C, 48.09; H, 3.69; N, 11.38; S, 19.87.

*4.1.2.4. 2-(Bis-4′-aminobenzenesulfonyl)amino-5-methyl-benzo[*d*] isothiazol-3-one (**5c**).* Yield: 60%; mp 204–206 °C (ethanol/water). IR (KBr): 3480, 3379 (NH_2), 1691 (CO), 1369, 1159 (SO_2) cm^{-1}; ^1H NMR (DMSO-d_6, δ, ppm): 7.8 (d, 1H, $J = 8.4$, H-7); 7.69 (s, 1H, H-4); 7.58 (dd, 1H, $J = 8.5$, $J = 1.5$, H-6); 7.47 (d, 4H, $J = 9$, H-2′, H-6′, H-2″, H-6″); 6.61 (d, 4H, $J = 9$, H-3′, H-5′, H-3″, H-5″); 6.48 (s, 4H, NH_2); 2.39 (s, 3H, CH_3). Anal. Calcd. for $C_{20}H_{18}N_4O_5S_3$ (490.58): C, 48.97; H, 3.70; N, 11.42; S, 19.61. Found: C, 48.55; H, 3.80; N, 11.81; S, 19.31.

4.2. Microbiology

4.2.1. Antimicrobial activity evaluation

The new compounds were tested in vitro for their antimicrobial properties against a wide spectrum of microorganisms (Tables 1–3) using the serial double dilution method [30].

The compounds under investigation were dissolved in dimethyl sulfoxide and diluted in the media (Tryptose Phosphate Broth for *Streptococcus pyogenes*, Mueller Hinton broth for other bacteria and Sabouraud liquid medium for fungi) so as to achieve the concentration range of 0.001–100 μg ml^{-1}. To ensure that dimethyl sulfoxide had no effect on microbial growth, a control test with media supplemented with the solvent was performed. Ampicillin, sulfamethoxazole and miconazole were used as reference antibacterial and antifungal agents, respectively. An inoculum of 5×10^4 CFU for bacteria and 1×10^3 CFU for fungi per millilitre of medium was added. After an incubation period of 24 h at 37 °C (bacteria) and of 48 h at 30 °C (fungi), the minimum inhibitory concentrations (MIC, μg ml^{-1}) were detected as the lowest concentrations of compound that resulted in complete inhibition of the microbial growth.

To detect the mechanism of action of the tested compounds, the experiments described above were repeated by assaying the antimicrobial properties of the active chemicals in the presence of L-cysteine hydrochloride. Thus, cysteine hydrochloride was dissolved in water at the concentration of 3.5 mg ml^{-1} and sterilized by filtration. Fifty microlitres of the obtained solution were added to each millilitre of the testing suspensions before incubation. In addition, Mueller Hinton broth containing PABA at 10 μg ml^{-1} was used to test the effect of this substance on the MICs.

The minimum bactericidal concentrations (MBC) and the minimum fungicidal concentrations (MFC), both expressed in μg ml^{-1}, were determined by subculturing on fresh medium 100 μl of liquid from each suspension remaining clear and incubating the samples obtained at 37 °C for 24 h (bacteria) and at 30 °C for 48 h (fungi). MBC and MFC values represent the lowest concentration of drug needed for the reduction of the initial inoculum of 99.9%.

Compound **4c**, the only one active against fungi, was screened for its antifungal activity by mean of serial plate dilution method towards several pathogenic moulds (Table 3) freshly isolated from pathological material. A suspension of each fungal strain in physiologic saline was streaked onto the surface of Sabouraud Dextrose Agar plates containing 0.001–100 μg ml^{-1} of compound and the plates were incubated at 25 °C for 14 days. MICs were determined and the antifungal activity was compared with that of miconazole used as standard drug.

All the experiments were performed in triplicate and the reported results were obtained from three independent measurements.

4.2.2. Synergism studies

The effects of combinations of new sulfonamides with trimethoprim (Sigma, Milano, Italy) against *B. subtilis* ATCC 6633 and *S. aureus* ATCC 25923 was examined in vitro according to two procedures: the paper strip diffusion method and the checkerboard titration technique [25,31]. The antimicrobial combination of sulfamethoxazole with trimethoprim was used as positive standard control. The solutions of the standard drugs were prepared in water by adding the minimal amount of 2.5 M NaOH (for sulfamethoxazole) or 0.05 M HCl (for trimethoprim) to dissolve them.

As regards the checkerboard titration technique, sulfonamides were tested at concentrations ranging from 100 to 0.15 μg ml^{-1} and trimethoprim from 0.0003 to 3 μg ml^{-1}. The effect of the studied combinations was expressed by the fractional inhibitory concentration (FIC) of each tested compound (S, sulfonamides; T, trimethoprim):

FIC_S = MIC of each sulfonamide in combination/MIC of each sulfonamide alone

FIC_T = MIC of trimethoprim in combination/MIC of trimethoprim alone.

The FIC indices (FICI) for the most effective combinations were then calculated by summing the separate FIC values of each sulfonamide and trimethoprim:

$$FICI = FIC_S + FIC_T$$

Drug interactions are defined as synergistic when the FIC index is ≤ 0.5, additive when the FIC index is >0.5 and ≤ 1, while values >1

and <4 are classified as indifference and values ≥4 as antagonism [32].

These results were summarized graphically on the isobolograms constructed taking along the *x*-axis the FIC of trimethoprim and along the *y*-axis the corresponding FIC values of each sulfonamide. The isobolograms were constructed by connecting with a line (isobol) the series of points generated for each drug combination. Synergistic interaction is diagrammed as concave isobol, additive effect as straight line and antagonism as a convex isobol.

All tests were performed in triplicate and the results were confirmed by three separate experiments.

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