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Original article

Synthesis and antimicrobial properties of 2-(benzylidene-amino)-benzo[*d*]isothiazol-3-ones

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Abstract

The in vitro antimicrobial activity of 2-amino-benzo[*d*]isothiazol-3-one and of several 2-arylideneamino derivatives carrying in the second position a substituted or unsubstituted aromatic ring or an arylalkenylidene moiety was determined by the broth dilution method against several strains selected to define their spectrum and potency. All the compounds demonstrated good antibacterial properties against *Bacillus subtilis*, streptococci, enterococci and staphylococci including penicillin-resistant clinical isolates. Several compounds showed excellent inhibitory properties against *Streptococcus pyogenes*, which is the most sensitive microorganism tested. Many benzisothiazolones exhibited good activity against Gram-negative *Haemophilus influenzae*. As regards antifungal activity, several of the tested compounds inhibited *Saccharomyces cerevisiae* at concentrations of 3–6 μg ml^{-1}. In all cases the parent 2-amino-benzo[*d*]isothiazol-3-one was the most effective agent, with minimum inhibitory concentration (MIC) values ranging from 0.07 to 6 μg ml^{-1}. The results obtained indicate that most of these compounds are wide-spectrum antimicrobial substances and promising agents against penicillin-resistant staphylococci.

Keywords: 2-Amino-benzo[*d*]isothiazol-3-one; Benzylidene-amino derivatives; Antibacterial activity; Antifungal activity

1. Introduction

Over the past decades, the frequency of resistance in antimicrobial agents has increased dramatically [1], and this places new emphasis on the search for alternative substances effective against organisms resistant to currently available drugs.

Previous studies [2–4] have shown that benzo[*d*]isothiazole derivatives are a class of antimicrobial agents active against bacteria and fungi. Recently, in the course of our research on biologically active benzo[*d*]isothiazoles [5–7], we prepared five series of hydrazones of benzo[*d*]isothiazole hydrazides and tested them in vitro for their potential antimicrobial properties [8]. We found a very good spectrum of activity for compounds **1** and **1a–m** (Fig. 1) against both *Bacillus subtilis* and *Staphylococcus aureus* Gram-positive organisms and yeasts.

As an extension of these studies, we synthesised and tested for their antimicrobial effect the unknown fluorinated and hydroxylated analogues **1n–r** shown in Fig. 2. The new compounds have been designed with a fluorine atom or hydroxy group as functional substituents at the different positions of the benzylidene moiety, taking into account that the selective introduction of a fluorine atom into pharmaceuticals has emerged as an extremely effective tool for modifying their physicochemical properties and consequently their biological behaviour [9–11]. In view of the fluorine-hydroxy bioisosterism and because of the well-known importance of the effects of hydroxylation [12], we decided to synthesise the unknown hydroxybenzylidene derivatives. In addition, the presence of a fluorine and phenolic groups has sometimes been shown to improve antimicrobial properties by inducing bacterial lysis [13] and inactivating essential enzyme systems [14], respectively.

The present paper describes the synthesis of new compounds **1n–r** and the in vitro estimation of their potential antimicrobial activity against microorganisms such as Gram-positive and Gram-negative bacteria, yeasts and moulds.

In order to evaluate in a better way the spectrum of activity of the benzisothiazolones under study, in this work, more strains belonging to different species than in the previous one [8] were included and a higher inoculum size was used to check their antimicrobial potency more thoroughly. In view

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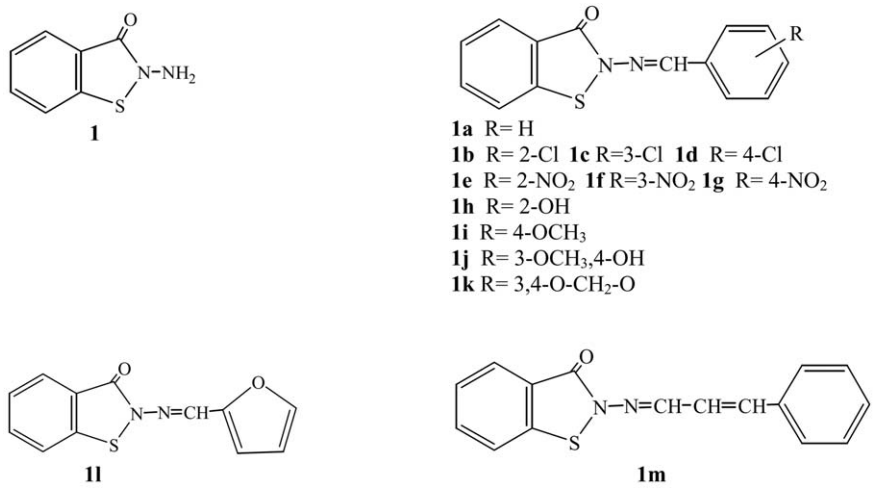
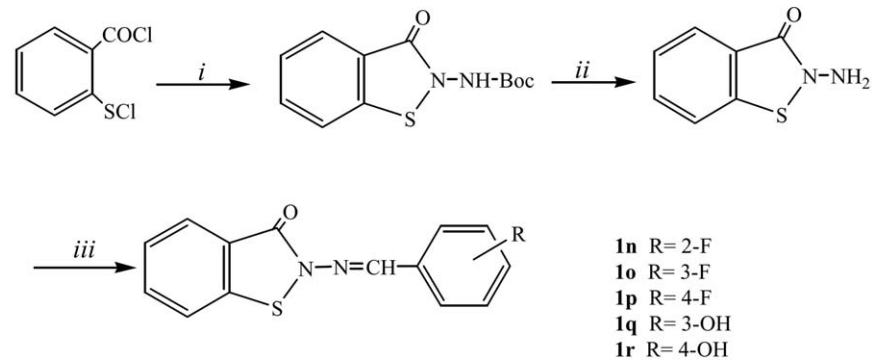


Fig. 1. Chemical structures of compounds **1** and **1a–m.**



i) NH_2-NH-Boc $(C_2H_5)_2O$/pyridine, 60 min,10°C
ii) TCA/H_2O, 150 min, RT
iii) R-C_6H_4CHO EtOH/H_2O/HCl/CH_3COONa, 60 min, 70°C.

Fig. 2. Scheme of synthesis and structure of compounds **1n–r.**

of these observations, it was thought worthwhile to evaluate again, in the new experimental conditions, the antibacterial effect of the parent 2-amino-benzo[*d*]isothiazol-3-one (**1**) and of its derivatives **1a–m**.

In addition, in consideration of the aforementioned increase in the number of Gram-positive bacteria resistant to ampicillin and other primary antimicrobial agents, isolates of both *S. aureus* and *Staphylococcus epidermidis* penicillin-resistant strains have also been included in our study.

2. Chemistry

The benzylidene derivatives **1n–r**, as well as the previously described analogues **1a–m**, were synthesised starting from chlorocarbonylphenylsulfenylchloride by treatment with *N*-Boc protected hydrazine. The *N*-Boc group was then removed by mild acidic hydrolysis with trichloroacetic acid (TCA) to yield 2-amino-benzo[*d*]isothiazol-3-one, according to the method previously described by Vicini et al. [15]. The target compounds were obtained through condensation of the amino intermediate with the appropriate aldehyde, as shown in Fig. 2.

All new compounds gave satisfactory elemental analyses (C, H, N, S) within ±0.4% of the theoretical values and the structures were in accordance with their spectroscopic data, reported in Section 6.

3. Biology

2-Amino-benzo[*d*]isothiazol-3-one derivatives were tested in vitro in order to evaluate their antimicrobial effect. The minimum inhibitory concentration (MIC) was determined by exposing the test microorganisms to increasing doses of compounds in the concentration range of 0.01–100 μg ml^{-1}.

The antibacterial activity of compounds **1** and **1a–r** was assayed against Gram-positive bacteria (including *B. subtilis*, staphylococci, enterococci, streptococci) and Gram-negative bacteria (*Escherichia coli, Haemophilus influenzae*). The susceptibility testing results are listed in Table 1

Table 1
Antibacterial activity, expressed as MIC (μg ml^{-1})

Compound	Gram-positive bacteria [a]										Gram-negative bacteria [b]	
	BS	SA	SAR	SE	SER	SH	EF	EFU	SAG	SP	EC	HI
1	0.7	6	6	3	3	3	1.5	1.5	0.3	0.07	6	0.7
1a	6	12	6	6	6	12	6	12	6	3	>100	6
1b	6	12	12	12	6	12	6	6	6	1.5	>100	>100
1c	6	>100	>100	>100	50	>100	25	>100	25	0.3	>100	>100
1d	6	12	12	6	6	12	6	6	6	6	>100	12
1e	12	>100	12	6	6	12	6	6	6	1.5	>100	12
1f	6	50	100	12	6	12	6	6	6	0.7	>100	>100
1g	6	>100	>100	6	12	25	25	>100	12	3	>100	>100
1h	6	25	12	12	12	12	12	12	12	1.5	>100	>100
1i	6	12	12	12	6	25	6	6	6	6	>100	25
1j	6	25	6	6	6	6	6	6	6	6	>100	>100
1k	6	12	12	6	6	12	6	25	12	3	>100	12
1l	6	12	12	12	6	12	6	12	12	3	50	3
1m	12	25	12	12	12	12	12	12	12	1.5	>100	6
1n	6	12	12	6	6	6	12	6	6	6	>100	25
1o	6	12	12	6	12	12	12	6	6	3	>100	100
1p	6	12	6	6	6	6	12	6	6	3	>100	6
1q	6	12	6	6	6	12	12	12	12	3	>100	6
1r	6	12	6	6	6	6	12	6	6	6	>100	6
A [c]	0.15	0.15	100	25	100	0.15	0.7	100	0.07	0.03	3	0.3

[a] BS, *B. subtilis* ATCC 6633; SA, *S. aureus* ATCC 25923; SAR, penicillin-resistant *S. aureus*; SE, *S. epidermidis*; SER, penicillin-resistant *S. epidermidis*; SH, *S. haemolyticus*; EF, *E. faecalis*; EFU, *E. faecium*; SAG, *S. agalactiae*; SP, *S. pyogenes*.
[b] EC, *E. coli* SPA 27; HI, *H. influenzae*.
[c] Ampicillin.

and are compared to those of ampicillin, which was used as reference drug. The antifungal potency of compounds **1n–r** is summarised in Table 2 in comparison with that of miconazole antifungal control.

The microbiological studies were completed by the evaluation of the minimum fungicidal concentrations (MFC) and the minimum bactericidal concentrations (MBC) (Tables 2 and 3, respectively) which allow for the establishment of the microbistatic or microbicidal type of exerted inhibition.

4. Results

Table 1 lists the antimicrobial susceptibility results for both Gram-positive and Gram-negative bacteria.

All benzisothiazoles tested in this study exhibited good antibacterial activity against Gram-positive bacteria. *B. subtilis* was inhibited by compound **1** at 0.7 μg ml^{-1} and by its derivatives **1a–r** at 6–12 μg ml^{-1}. Against all staphylococci, compound **1** and its derivatives **1a–b** and **1d–r** were generally effective at concentrations of 3–12 μg ml^{-1}. Additionally, a remarkable potency was always exhibited against both *S. aureus* and *S. epidermidis* penicillin-resistant isolates. In this specific case, the MIC values were similar to or even lower than those obtained with the same penicillin-susceptible strains. Enterococci and streptococci were generally slightly more susceptible than staphylococci. *Streptococcus pyogenes* resulted as being the most sensitive microorganism tested: it was notably inhibited at concentrations of 0.07–6 μg ml^{-1}. In particular, compound **1** was strongly active, with MIC results at 0.07 μg ml^{-1}. Compounds **1b, 1c, 1e, 1f, 1h** and **1m** were also endowed with high activity (MIC 0.3–1.5 μg ml^{-1}).

Against Gram-negative bacteria, the assayed substances showed an activity level lower than that reported against

Table 2
Antifungal activity (μg ml^{-1})

Compound	SC [a]		CT		AN	
	MIC	MFC	MIC	MFC	MIC	MFC
1n	6	25	>100	– [b]	>100	–
1o	3	>100	>100	–	>100	–
1p	6	12	12	25	>100	–
1q	6	50	>100	–	>100	–
1r	6	25	>100	–	>100	–
Miconazole	12	nt [c]	6	nt	3	nt

[a] SC, *S. cerevisiae* ATCC 9763; CT, *C. tropicalis* ATCC 1369; AN, *A. niger* ATCC 6275.
[b] Not tested because MIC value is higher than 100 μg ml^{-1}.
[c] Not tested.

Table 3
Antibacterial activity, expressed as MBC (μg ml^{-1})

Compound	Gram-positive bacteria [a]										Gram-negative bacteria [b]	
	BS	SA	SAR	SE	SER	SH	EF	EFU	SAG	SP	EC	HI
1	3	50	25	25	25	25	25	25	25	3	50	1.5
1a	12	100	100	100	100	100	100	100	50	6	– [c]	12
1b	100	>100	>100	100	>100	>100	>100	>100	>100	6	–	–
1c	>100	–	–	–	>100	–	>100	–	100	50	–	–
1d	50	100	>100	>100	25	100	>100	>100	100	12	–	100
1e	50	–	100	50	50	100	>100	100	50	3	–	>100
1f	50	>100	>100	50	100	>100	100	100	100	12	–	–
1g	100	–	–	100	>100	>100	>100	–	>100	12	–	–
1h	12	>100	100	100	100	>100	100	>100	100	25	–	–
1i	25	>100	100	100	100	>100	100	100	100	12	–	50
1j	25	>100	100	50	50	100	50	50	50	25	–	–
1k	25	>100	50	50	25	50	50	>100	50	25	–	>100
1l	12	100	100	50	100	100	100	50	50	12	>100	6
1m	25	>100	50	50	50	50	50	100	50	6	–	12
1n	25	100	100	100	100	100	100	100	100	12	–	100
1o	25	>100	100	>100	>100	>100	>100	100	100	12	–	>100
1p	25	100	100	25	25	100	100	100	100	12	–	12
1q	12	>100	>100	100	25	100	100	100	100	12	–	12
1r	50	100	>100	25	>100	>100	>100	100	100	12	–	12

[a] BS, *B. subtilis* ATCC 6633; SA, *S. aureus* ATCC 25923; SAR, penicillin-resistant *S. aureus*; SE, *S. epidermidis*; SER, penicillin-resistant *S. epidermidis*; SH, *S. haemolyticus*; EF, *E. faecalis*; EFU, *E. faecium*; SAG, *S. agalactiae*; SP, *S. pyogenes*.
[b] EC, *E. coli* SPA 27; HI, *H. influenzae*.
[c] Not tested because MIC value is higher than 100 μg ml^{-1}.

Gram-positive ones. The compounds displayed no significant effect against *E. coli*, since they exhibited, in general, MIC > 100 μg ml^{-1}; **1** and **1l** were the only ones which were fairly effective, with MIC values of 6 and 50 μg ml^{-1}, respectively. *H. influenzae* was found to be more sensitive than *E. coli*: compound **1** was the most active agent (MIC 0.7 μg ml^{-1}), while MIC values of most derivatives ranged from 3 to 25 μg ml^{-1}.

On the whole, the most potent in vitro antibacterial effect was demonstrated by compound **1**, having MIC values ranging from 0.07 to 6 μg ml^{-1} against the tested strains. It showed strong activity against *B. subtilis* and *H. influenzae* (MIC 0.7 μg ml^{-1}), and against streptococci (MIC 0.07–1.5 μg ml^{-1}), and good activity against staphylococci and *E. coli* (MIC 3–6 μg ml^{-1}).

In general, 2-amino-benzo[*d*]isothiazol-3-one derivatives displayed inhibitory properties against bacteria which were lower than that of the ampicillin reference drug. Only the parent 2-amino-benzo[*d*]isothiazol-3-one had an effectiveness level comparable to that of ampicillin in the case of *B. subtilis, Enterococcus faecalis, S. pyogenes, E. coli* and *H. influenzae*. However, most of the 2-arylideneamino derivatives were more active than ampicillin against *S. epidermidis* (MIC 3–12 and 25 μg ml^{-1}, respectively) and *Enterococcus faecium* (MIC 1.5–25 and 100 μg ml^{-1}, respectively); it is noteworthy that the inhibitory effect shown by all the compounds against *S. aureus* and *S. epidermidis* was not affected by the resistance to penicillins. In fact, both *S. aureus* and *S. epidermidis* penicillin-resistant isolates had a higher degree of susceptibility to these benzisothiazolones (MIC range 6–12 and 3–12 μg ml^{-1}, respectively) in contrast to ampicillin (MIC 100 μg ml^{-1}).

The newly synthesised compounds **1n–r** showed noticeable antifungal properties (Table 2) against *Saccharomyces cerevisiae* (MIC 3–6 μg ml^{-1}), displaying a growth inhibition higher than that of the reference drug miconazole. Compound **1p** also showed in vitro effectiveness against *Candida tropicalis* (MIC 12 μg ml^{-1}). None of the tested compounds inhibited the mould *Aspergillus niger* (MIC > 100 μg ml^{-1}). These results indicate the significantly high sensitivity of *S. cerevisiae* compared with other tested fungi.

All the compounds studied showed fungistatic and bacteriostatic activity, MFC (Table 2) and MBC (Table 3) values being higher than MIC values.

5. Discussion

This work addresses a novel class of potent, wide-spectrum antimicrobial compounds. The parent 2-amino-benzo[*d*]isothiazol-3-one and several substituted or unsubstituted 2-arylideneamino derivatives are good antibacterial substances against several strains, including *B. subtilis*, streptococci, enterococci, penicillin-susceptible and -resistant staphylococci and *H. influenzae*.

The results obtained are in agreement with the previous work [8], where compounds **1** and **1a–m** were tested against *B. subtilis, S. aureus* and *E. coli* using an inoculum size of 10^4 bacteria/ml. The MIC values observed in the present experimental conditions are similar to those reported in [8], confirming the potent inhibitory effect of these compounds

even in the presence of high inoculum densities (5×10^5 bacteria/ml).

Interestingly, the good activity of these compounds was observed for both penicillin-susceptible and penicillin-resistant staphylococci, irrespective of the resistance level against penicillin. The present results are worth noting because in recent years increasing rates of antimicrobial resistance among community and nosocomial pathogens has severely limited the therapeutic options for treating infections caused by such organisms [16].

Among all the tested compounds, the parent 2-amino-benzo[*d*]isothiazol-3-one proved to be the most effective agent; in all cases the studied derivatives showed comparable or lower antimicrobial properties. This demonstrates that every substitution at the amino group in the second position of the benzisothiazole ring is ineffective in improving the intensity of the antimicrobial activity of compound **1**.

As regards the structure–activity relationships of the derivatives, the introduction of fluorine or hydroxy as substituent in the *ortho, meta* or *para* position of the benzylidene moiety did not noticeably enhance the growth inhibition displayed by the unsubstituted analogue **1a**. However, a positive effect of both substituents can be recognised for *Staphylococcus haemolyticus* and *E. faecium*; for *S. pyogenes* and *S. cerevisiae* the improvement is restricted to *ortho* hydroxy and *meta* fluorine isomer, respectively.

The substitution of fluorine or hydroxy group for chlorine or nitro group yields compounds exhibiting lower effectiveness against both bacteria and fungi. This behaviour evidences that the introduction of bulky substituents significantly decreases the antimicrobial potency. It is worth noting that only in the case of *S. pyogenes* does the introduction of chlorine or nitro group in *ortho* or *meta* position give new compounds with very potent antibacterial activity. However, of the chlorosubstituted substances, *ortho* and *para* derivatives were in general endowed with the best antibacterial properties. The *meta* derivative **1c** had the poorest activity of all the assayed compounds.

As regards the hydroxy derivatives, in the case of *meta* and *para* substitution an inhibition higher than that exhibited by the *ortho* position was observed against all tested microorganisms, except *S. pyogenes*.

In conclusion, taking into account that 2-amino-benzo[*d*]isothiazol-3-one and most of the tested 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, it can be stated that they are promising new agents in treating microbial infections.

6. Experimental protocols

6.1. Chemistry

Melting points (m.p., °C) were determined with a Buchi 512 apparatus and are uncorrected. New compounds were analysed in the analytical laboratory of the Dipartimento Farmaceutico, Università di Parma, on a ThermoQuest (Italia) FlashEA 1112 Elemental Analyser, for C, H, N, and S. The values found for C, H, N, S were always ±0.4% of the theoretical ones. IR spectra, such as KBr pellets, were recorded on a JASCO FT-IR 300E spectrophotometer (Jasco Ltd., Tokyo, Japan); wave numbers in the IR spectra are given in cm^{-1}. ^{1}H-NMR spectra of the newly synthesised compounds, 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; coupling constants J are expressed in Hz. The reactions were followed by TLC on F_{254} silica-gel precoated sheets (Merck) and the purified compounds each showed a single spot.

Solvents, unless otherwise specified, were of analytical reagent grade or of the highest quality commercially available. Synthetic starting material, reagents and solvents were purchased from Aldrich Chemical Co.

*6.1.1. General procedure for synthesis of 2-(benzylidene-amino)-benzo[*d*]isothiazol-3-ones (**1n–r**)*

2-Amino-benzo[d]isothiazol-3-one (5 mmol) was poured into water (70 ml), while stirring, and hydrochloric acid was added up to acidic pH; the suspension was then buffered with sodium acetate, and ethanol was added (20 ml). Suitable aldehydes (5.7 mmol) dissolved in ethanol (10 ml) were dropped into the mixture and the reaction was heated at 70 °C for 1 h, while stirring. After cooling to r.t., the resulting crude product was filtered, washed with water and recrystallised.

*6.1.1.1. 2-[(2-Fluoro-benzylidene)-amino]-benzo[*d*]isothiazol-3-one (**1n**).* Pale-pinkish crystalline solid from EtOH (84%); m.p. 191–192 °C; TLC: eluent = CH_2Cl_2/MeOH 9:1. Anal. Calc. for $C_{14}H_9FN_2OS$ (272.30): C, 61.75; H, 3.33; N, 10.29; S, 11.78. Found: C, 61.55; H, 3.29; N, 9.89; S, 11.71. IR(KBr) ν (cm^{-1}): 3071 (aromatic C–H), 2916, 2852, 1385 (azomethine C–H), 1662 (C=O), 1601 (N=C), 1240 (C–F). ^{1}H-NMR (300 MHz, DMSO-d_6): 8.32 (s, 1H, CH); 8.05–7.95 (m, 3H, 4 + 7 + 6′); 7.80 (t, 1H, 5, J = 8.1); 7.61–7.49 (m, 2H, 6 + 5′); 7.40–7.33 (m, 2H, 3′ + 4′).

*6.1.1.2. 2-[(3-Fluoro-benzylidene)-amino]-benzo[*d*]isothiazol-3-one (**1o**).* Ivory crystals from EtOH (79%); m.p. 200–202 °C; TLC: eluent = EtOAc/hexane 1:1. Anal. Calc. for $C_{14}H_9FN_2OS$ (272.30): C, 61.75; H, 3.33; N, 10.29; S, 11.78. Found: C, 61.75; H, 3.42; N, 10.13; S, 11.64. IR(KBr) ν (cm^{-1}): 3061 (aromatic C–H), 2920, 2861, 1381 (azomethine C–H), 1670 (C=O), 1591 (N=C), 1228 (C–F). ^{1}H-NMR (300 MHz, DMSO-d_6): 8.33 (s, 1H, CH); 8.08 (d, 1H, 4, J = 8.1); 8.01 (d, 1H, 7, J = 8.1); 7.80 (t, 1H, 5, J = 8.4); 7.75 (t, 1H, 6, J = 8.1); 7.69–7.65 (m, 1H, 6′); 7.60–7.48 (m, 2H, 2′ + 4′); 7.37–7.31 (m, 1H, 5′).

*6.1.1.3. 2-[(4-Fluoro-benzylidene)-amino]-benzo[*d*]isothiazol-3-one (**1p**).* Pale-yellow crystalline solid from EtOH (67%); m.p. 168–169 °C; TLC: eluent = EtOAc/hexane 1:1.

Anal. Calc. for $C_{14}H_9FN_2OS$ (272.30): C, 61.75; H, 3.33; N, 10.29; S, 11.78. Found: C, 62.04; H, 3.45; N, 9.78; S, 11.76. IR(KBr) ν (cm^{-1}): 3051 (aromatic C–H), 2918, 2850, 1375 (azomethine C–H), 1672 (C=O), 1605 (N=C), 1234 (C–F). ^{1}H-NMR (300 MHz, DMSO-d_6): 8.34 (s, 1H, CH); 8.03 (d, 1H, 4, J = 8.4); 8.00–7.97 (m, 3H, 7 + 2′ + 6′); 7.79 (t, 1H, 5, J = 6.9); 7.50 (t, 1H, 6 J = 7.0); 7.38–7.32 (m, 2H, 3′ +5′).

*6.1.1.4. 2-[(3-Hydroxybenzylidene)-amino]-benzo[*d*]isothiazol-3-one (**1q**).* Pale-pinkish crystalline solid from THF/H_2O (76%); m.p. 225–227 °C; TLC: eluent = EtOAc/hexane 1:1. Anal. Calc. for $C_{14}H_{10}N_2O_2S$ (270.31): C, 62.21; H, 3.73; N, 10.36; S, 11.86. Found: C, 61.87; H, 3.85; N, 9.80; S, 11.37. IR(KBr) ν (cm^{-1}): 3350 (O–H), 3056 (aromatic C–H), 2920, 2852, 1383 (azomethine C–H), 1657 (C=O), 1574 (N=C). ^{1}H-NMR (300 MHz, DMSO-d_6): 9.72 (s, 1H, OH); 8.22 (s, 1H, CH); 8.06 (d, 1H, 4 J = 8.4); 7.99 (d, 1H, 7 J = 7.5); 7.78 (t, 1H, 5 J = 8.1); 7.50 (t, 1H, 6, J = 7.4); 7.33–7.27 (m, 3H, 2′ + 6′ + 3′); 6.904–6.868 (m, 1H, 4′).

*6.1.1.5. 2-[(4-Hydroxybenzylidene)-amino]-benzo[*d*]isothiazol-3-one (**1r**).* Pale-yellow crystalline solid from THF (80%); m.p. 228–230 °C; TLC: eluent = EtOAc/hexane 1:1. Anal. Calc. for $C_{14}H_{10}N_2O_2S$ (270.31): C, 62.21; H, 3.73; N, 10.36; S, 11.86. Found: C, 62.13; H, 3.79; N, 10.25; S, 11.59. IR(KBr) ν (cm^{-1}): 3354 (O–H), 3053 (aromatic C–H), 2910, 2836, 1367 (azomethine C–H), 1666 (C=O), 1597 (N=C). ^{1}H-NMR (300 MHz, DMSO-d_6): 10.08 (s, 1H, OH); 8.23 (s, 1H, CH); 8.04 (d, 1H, 4, J = 8.1); 7.98 (d, 1H, 7, J = 8.1); 7.79–7.74 (m, 3H, 5 + 2′ + 6′); 7.49 (t, 1H, 6, J = 6.9); 6.89 (d, 2H, 3′ + 5′).

6.2. Microbiology

The potential antimicrobial activity was estimated in vitro by determining the MIC against a wide-spectrum of microorganisms. Isolates of clinical origin were selected to include both susceptible and resistant strains. In particular, the following strains were used: Gram-positive bacteria (*B. subtilis* ATCC 6633; *S. aureus* ATCC 25923; clinical isolates of methicillin-resistant *S. aureus*, *S. epidermidis*, methicillin-resistant *S. epidermidis, S. haemolyticus, E. faecalis, E. faecium, Streptococcus agalactiae, S. pyogenes*), Gram-negative bacteria (*E. coli* SPA 27, clinical isolates of *H. influenzae*), yeasts (*S. cerevisiae* ATCC 9763, *C. tropicalis* ATCC 1369) and moulds (*A. niger* ATCC 6275).

The MIC (μg ml^{-1}), which denotes the lowest drug concentration that prevents the visible growth of tested microorganisms, was evaluated by using the serial double dilution method [17] in Mueller–Hinton broth (for bacteria) or Sabouraud liquid medium (for fungi). For susceptibility testing of *H. influenzae*, the Haemophilus test medium (HTM) was prepared by using Mueller–Hinton broth as the base to which 15 μg of purified bovine ematin, 15 μg of nicotinamide adenine dinucleotide and 5 mg of yeast extract per ml were added. The growth of *S. pyogenes* fastidious microorganism is improved by the addition of Supplement B (Becton Dickinson, Sparks, MA, USA) sterile enrichment. The compound solutions were prepared by dissolving the powders in dimethyl sulfoxide and were added to wells so as to achieve the concentration range of 0.01–100 μg ml^{-1}. At the same time, the sensitivity of each strain to dimethyl sulfoxide was determined. The activity was also estimated of ampicillin and miconazole used as reference antibacterial and antifungal agents, respectively. The final inoculum density was 5×10^5 bacteria and 10^3 fungi into 1 ml of medium. The wells were incubated for 24 h at 37 °C (bacteria) and for 48 h at 30°C (fungi) before interpretation of MIC endpoints.

The MBC and the MFC were determined by subculturing 100 μl of culture from each tube remaining clear on fresh medium. The wells were incubated before being examined. MBC and MFC values represent the lowest concentration of drug needed for the reduction of the initial inoculum of 99.9%.

The present results were obtained from three independent measurements.

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