Full Paper

4H-1,4-Benzothiazine, Dihydro-1,4-benzothiazinones and 2-Amino-5-fluorobenzenethiol Derivatives: Design, Synthesis and *in vitro* Antimicrobial Screening

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As part of our studies focused on the design and synthesis of new antimicrobial agents a series of 7-fluoro-3,4-dihydro-2*H*-1,4-benzothiazine derivatives (**4a–4f**, **4h**) and 7-fluoro-2*H*-1,4-benzothiazin-3(4*H*)-one analogues (**4j–4o**) were synthesized and evaluated for their *in vitro* inhibitory activity against a representative panel of Gram-positive and Gram-negative bacteria strains and also toward selected fungi species. These compounds were prepared in one step from chloro-substituted-2-amino-5-fluorobenzenethiol **6a–6c**. The biological screening identified in compounds **4a**, **4j** and **4l** the most promising results of both series showing an interesting antimicrobial activity. Our antibiotic investigation was also completed by testing the key intermediates **6a–6c**. Surprisingly, **6a–6c** emerged as the compounds exhibiting the highest antimicrobial activity by possessing a remarkable antibacterial effect against the Gram-positive strains with MIC (minimal inhibitory concentration) values between 2 and 8 μg/mL and the fungi panel with MIC values between 2 and 8 μg/mL. These results may prove useful in the design of a novel pool of antimicrobial agents.

Keywords: *4H*-1,4-Benzothiazine / Antibacterial activity / Antifungal activity

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Introduction

Bacterial resistance to antibiotics has become an increasingly serious problem in recent years. Drug-resistant pathogens have evolved the MRSA (methicillin-resistant *Staphylococcus aureus*) and the VRSA (vancomycin-resistant *Staphylococcus aureus*) phenotype. Unfortunately, bacterial resistance has also emerged against newer agents such as linezolid and daptomycin shortly after their use in clinic [1]. Therefore the development of new and different antimicrobial drugs is a very important goal and most of the research program efforts in this field are directed towards the design of new agents.

It is well documented that the benzothiazine template is generally recognized as a privileged structure in medicinal chemistry to investigate both potentially antibacterial [2–9] and anticancer [10–18] molecules. Looking at the importance of the benzothiazine nucleus, in the recent past, we reported several examples of benzothiazine derivatives bearing substituents on the heterocyclic ring able to exhibit both antibacterial and antifungal activity (**1**, **2**, **3**, Figure 1) [2–9].

However to our knowledge, few data [2, 9] have been collected to obtain comprehensive SAR indications about the effects that the substituents on the benzothiazine nucleus exert on the antibacterial and antifungal biological profile.

In this regard, several questions were raised: (*i*) how such a significant effect was produced in the antibacterial activity by the aromatic substituents (**1**, Figure 1) and (*ii*) what significant effect could a C=O moiety exert on the antimicrobial activity in comparison with the R_2 and R_3 substituents? (**1** *vs.* **2**, Figure 1). In addition, (*iii*) could dihalogenated derivatives play a role in the antibacterial activity (**3**, Figure 1)?

In continuation to extend our research on the synthesis and biological behaviour of *4H*-1,4-benzothiazine analogues as potential drugs for antimicrobial management, here we have been interested in the preparation of new compounds containing modified benzothiazine. In detail, in the present investigation, three new different *4H*-1,4-benzothiazine series

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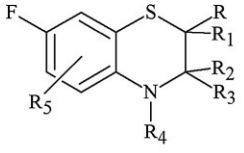
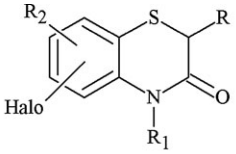


[1]



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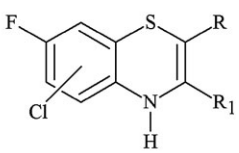


[3]

Figure 1. Initial 3,4-dihydro-2*H*-1,4-benzothiazines and their derivatives.

(**4a–4f**, **4h**, Table 1, Schemes 1 and 2) and two series of dihydro-1,4-benzothiazinones (**4j–4o**, Table 2, Scheme 2) were synthesized and evaluated. Although compounds **4b**, **4c**, **4k** and **4l** are reported in the literature [2] they have been included in our research program to screen their antimicrobial profile in order to have more data for the SAR proposal.

In relation with the above considerations and because in the recent literature many benzimidazole, benzoxazole and benzothiazole derivatives were regarded as promising classes of bioactive heterocyclic compounds able to exhibit a range of biological activities [19–22], we recently reported a series of 2-mercapto-1,3-benzothiazole derivatives and their *in vitro* antibacterial evaluation (Figure 2). [23] It is disclosed that the SH moiety at the C2 position of the heterocyclic nucleus led to a remarkable antibacterial activity.

Taking the above points in consideration, to confirm this preliminary hypothesis we decided to include the isosteric (NH_2 instead of SH) derivatives (**5a–5c**, Table 3, Scheme 3) and their reaction products (**6a–6c**, Table 4, Scheme 3), containing the SH moiety, in our antibacterial screening.

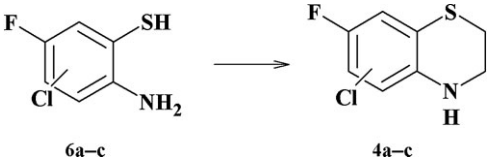
The structures herein reported are easily prepared, retained all main characteristics mentioned above and elucidated by spectral data.

In order to enhance selectivity and potency for addressing the pathological diversity of microbial human disorders, the MIC (minimal inhibitory concentration) of the benzothiazine derivatives (**4a–4o**) and of compounds **5a–5c** and **6a–6c** against different Gram-positive, Gram-negative and fungi strains belonging to the American Type Culture Collection (ATCC) was reported (Table 5) and the structure-activity relationship (SAR) was also discussed. A molecular modeling study was, at the end, also carried out to update insights into the molecular determinants most largely affecting the activity of our newly synthesized antimicrobial agents.

Results and discussion

Chemistry

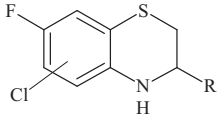
The synthetic routes used to synthesize compounds **4a–f, 4h,** and **4j–o** are reported in Schemes 1 and 2. Starting materials **6a–6c** were prepared by hydrolytic basic cleavage of chloro-6-fluoro-2-aminobenzothiazoles (**5a–5c**) commercially available by following the synthetic pathway described in the litera-



6a–c **4a–c**

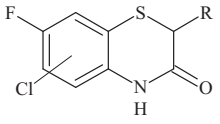
Scheme 1. 1,2-Dibromoethane, anhyd. CH_3OH or anhyd. EtOH, 0.4 N CH_3ONa or 0.65 N CH_3ONa, N_2, reflux.

Table 1. 7-Fluoro-3,4-dihydro-2*H*-1,4-benzothiazine derivatives.

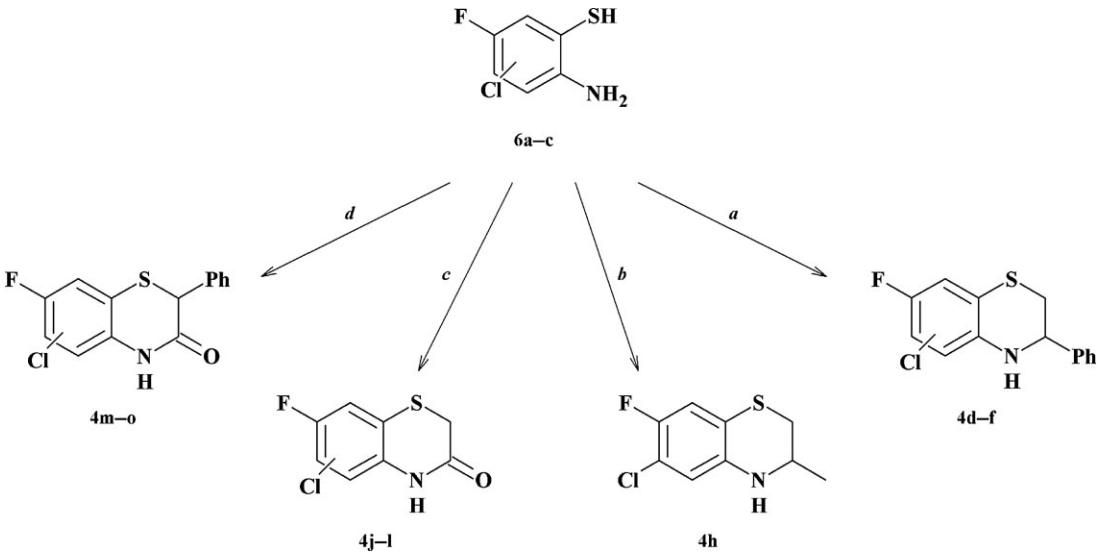


Compd	5	6	8	R
4a	Cl			H
4b[2]		Cl		H
4c[2]			Cl	H
4d	Cl			Ph
4e		Cl		Ph
4f			Cl	Ph
4g	Cl			CH_3
4h		Cl		CH_3
4i			Cl	CH_3

Table 2. 7-Fluoro-2*H*-1,4-benzothiazin-3(4*H*)-one derivatives.



Compd	5	6	8	R
4j	Cl			H
4k[2]		Cl		H
4l[2]			Cl	H
4m	Cl			Ph
4n		Cl		Ph
4o			Cl	Ph



Scheme 2. *a) Step 1*: 2-bromo-1-phenylethanone, anhyd. Et_2O, N_2, reflux, *step 2*: acetic acid, CH_3OH, $NaBH_4$, 0°C; *b) step 1*: 1-chloroacetone, anhyd. Et_2O, N_2, reflux, *step 2*: acetic acid, CH_3OH, $NaBH_4$, 0°C; *c)* chloroacetic acid, 2.5 N NaOH, H_2O, reflux; *d)* bromo(phenyl)acetic acid, 1.87 N EtONa, reflux.

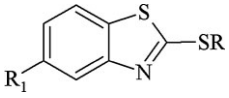
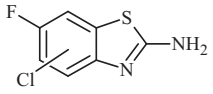
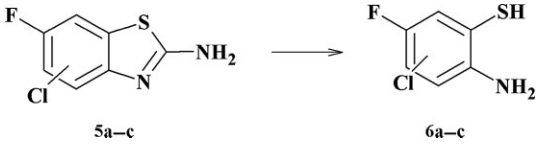


Figure 2. 2-Mercapto-1,3-benzothiazole derivatives [23].

Table 3. Structural formulae for Cl-substituted-6-fluoro-1,3-benzothiazol-2-amine.

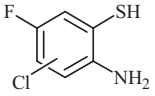


Compd	4	5	7
5a	Cl		
5b		Cl	
5c			Cl



Scheme 3. Reagents and conditions: 50% NaOH for 24 h; reflux.

Table 4. Structural formulae for Cl-substituted-2-amino-5-fluorobenzenethiol.



Compd	3	4	6
6a	Cl		
6b		Cl	
6c			Cl

ture (Scheme 3) [9]. In detail, **4a–4c** (Scheme 1) were prepared according to the method reported in the literature [9] by using a different starting reagent. In fact, in order to improve the yield, Cl-substituted-2-amino-5-fluorobenzenethiol **6a–6c** were converted to the corresponding Cl-substituted-7-fluoro-3,4-dihydro-2*H*-1,4-benzothiazine **4a–4c** starting from 1,2-dibromoethane rather than 1-bromo-2-chloroethane. As expected the cyclization reaction afforded heterocyclic derivatives **4a–4c** in higher yields than in the literature [9] (54% *vs.* 28% for **4b**, 50% *vs.* 20% for **4c**).

To obtain compounds **4d–4f** (Scheme 2), compounds **6a–6c**, in anhyd. Et_2O at reflux, were combined with 2-bromo-1-phenylethanone. After work the crude reaction was first treated with CH_3COOH in MeOH, then with $NaBH_4$ and finally

Table 5. Antimicrobial activity[a)] results (MIC μg/mL) of *4H*-1,4-benzothiazine (**4a–4f**, **4h**), dihydro-1,4-benzothiazinones (**4j–4o**) and 2-amino-5-fluorobenzenethiol derivatives (**6a–6c**).

	Microorganism								
	Gram positive			*Gram negative*		*Fungi*			
Compd	***S. a.* 6538P**	***E. f.* 29212**	***B. s.* 6633**	***E. c.* 25922**	***A. b.* 19606**	***C. a.* 10231**	***C. p.* 22019**	***C. t.* 750**	***C. k.* 6258**
5a	256	256	512	R	R	256	64	256	256
5b	512	256	256	256	512	128	64	128	128
5c	512	256	256	512	256	128	64	128	128
6a	8	16	4	R	512	8	2	4	8
6b	4	4	4	R	64	4	2	4	16
6c	8	8	2	R	16	16	4	8	16
4a	32	64	16	R	R	32	8	32	32
4b	R	R	R	R	R	R	500	500	n.e.
4c	64	128	64	128	32	500	500	500	n.e.
4d	R	R	R	R	R	R	R	R	n.e.
4e	128	256	64	R	R	128	64	256	256
4f	R	R	R	R	R	128	32	128	256
4h	125	250	64	125	n.e.	n.e.	n.e.	n.e.	n.e.
4j	16	32	16	R	R	500	500	500	n.e.
4k	512	512	R	R	R	512	512	512	512
4l	250	250	250	125	32	250	64	64	n.e.
4m	125	64	128	250	250	250	250	250	n.e.
4n	250	250	250	250	250	250	250	250	n.e.
4o	500	500	500	500	500	R	R	R	n.e.
NRF	0.5	4	0.250	0.03	4				
OXA	0.250	16	0.250	R	512				
AMB						0.5	1	1	1
FLU						0.5		1	

[a)] Antimicrobial activity was estimated by using NCCLS assay [24]. Abbreviations: *S. a., S. aureus; E. f., E. faecalis; B. s., B. subtilis; E. c., E. coli; A. b., A. baumannii; C. a., C. albicans; C. p., C. parapsilosis; C. t., C. tropicalis; C. k., C. krusei;* NRF, norfloxacin; OXA, oxacillin; AMB, amphotericin B; FLU, fluconazole; R, resistant; n.e., not evaluated.

basified with NaOH pellets to yield the desired compounds **4d–4f** in good overall yields. We next attempted to transfer the synthetic route described above to obtain the **4g–4i** (Scheme 2) series using 1-chloroacetone as starting material. This reaction pathway gave only the **4h** derivative in very low yield (11%). 7-Fluoro-2*H*-1,4-benzothiazin-3(4*H*)-one analogues **4j–4l** and **4m–4o** were respectively synthesized in good yields by reaction of the respective chloro-substituted-2-amino-5-fluorobenzenethiol **6a–6c** with chloroacetic acid in NaOH or with chloro(phenyl)acetic acid in EtONa (Scheme 2).

The structures of the obtained compounds were fully supported through their spectroscopic data (IR, ^{1}H NMR, and MS). Further details about the synthetic procedures are reported in the Experimental section.

Biological evaluation

According to NCCLS guidelines [24] these compounds were tested against an assortment of Gram-positive and Gram-negative bacteria belonging to the ATCC collection (*Staphylococcus aureus* 6538P, *Enterococcus faecalis* 29212, *Bacillus subtilis* 6633, *Escherichia coli* 25922, *Acinetobacter baumannii* 19606) to evaluate their antibacterial profile. In addition, the selected molecules were also tested against a panel of fungi strains (*Candida albicans* 10231, *Candida parapsilosis* 22019, *Candida tropicalis* 750 and *Candida krusei* 6258). Norfloxacin, oxacillin, amphothericin B and fluconazole were used as references drugs. The results expressed as MIC (μg/mL) are listed in Table 5.

The combined data showed that the screened compounds exerted inhibitory activity against the tested bacterial strains with MIC values between 2 and 512 μg/mL. The obtained results generally indicate that the majority of these series of compounds are more active against Gram-positive than Gram-negative bacteria. It is worth noting that among the selected fungi, *Candida parapsilosis* growth seems to be especially affected by the tested molecules, ranging from 2 to 512 μg/mL.

Among both mentioned series **4a–4o**, the most promising results were recorded for **4a**, **4j** and **4l** derivatives as showed in Table 5.

In detail, among 7-fluoro-3,4-dihydro-2*H*-1,4-benzothiazine derivatives (**4a–4f**, **4h**), **4a** resulted in a significant potency against both Gram-positive and fungi species. In particular, **4a** revealed its best antimicrobial activity against

Staphylococcus aureus (MIC: 32 μg/mL), *Bacillus subtilis* (MIC: 16 μg/mL) and *Candida parapsilosis* (MIC: 8 μg/mL). Interestingly, the **4b** analogue did not demonstrate an antimicrobial profile, while isomer **4c** only slightly affected *Acinetobacter baumannii* growth (MIC: 32 μg/mL). The above results suggest that a chlorine atom at the C5 position of the 7-fluoro-3,4-dihydro-2*H*-1,4-benzothiazine nucleus could influence the antimicrobial activity. A comparison of 7-fluoro-3,4-dihydro-2*H*-1,4-benzothiazine derivatives **4d–4f** and **4h** with the **4a–4c** series revealed that the introduction of a methyl or a phenyl group at the C3 position of the heterocycle results in a significant decrease of the antimicrobial activity.

Next, antimicrobial profile was measured for 7-fluoro-2*H*-1,4-benzothiazin-3(4*H*)-one analogues (**4j–4o**). Among this series, **4j**, bearing a chlorine atom at the C5 position of the heteronucleus, exhibited a good antibacterial activity against *Staphylococcus aureus* (MIC: 16 μg/mL), and *Bacillus subtilis* (MIC: 16 μg/mL). Moreover, compound **4l**, having a chlorine atom at the C8 position of the heterocycle displayed a moderate activity against Gram-negative *Acinetobacter baumannii* (MIC: 32 μg/mL) and the fungi *Candida parapsilosis* and *Candida tropicalis* with MIC values of 64 μg/mL. Interestingly, introduction of a phenyl group at the C2 position of the 7-fluoro-2*H*-1,4-benzothiazin-3(4*H*)-one analogues (**4m–4o**) led to a decrease of the antimicrobial profile.

On the basis of the biological data until here discussed, due both to the small numbers of evaluated series and to the low chemical diversity, an attempt to analyse the structure activity relationships does not seem obvious. Anyway, several comparison on the results led us to hypothesize that the insertion of a steric bulk as methyl or phenyl group on the heteronucleus could be detrimental to antimicrobial activity. On the contrary, the chlorine atom could play a role in the antimicrobial activity even if its effect does not depend on the relative position on the heterocyclic ring. Generally among the analyzed series, compared with 7-fluoro-2*H*-1,4-benzothiazin-3(4*H*)-one analogues (**4j–4o**, Table 2), 7-fluoro-3,4-dihydro-2*H*-1,4-benzothiazine derivatives (**4a–4f**, **4h**, Table 1) showed a higher antimicrobial profile suggesting that a C=O moiety exerts a low ability to inhibit bacterial and fungal growth.

Then, these preliminary results led us to include compounds **5a–5c** and intermediates **6a–6c** in our biological screening with the aim to generate a new pool of antimicrobial molecules. Interesting, the 2-mercapto-1,3-benzothiazole derivatives (Figure 2) isosters **5a–5c** essentially lost antimicrobial activity. These data are consistent with our previous findings [23] that the SH moiety is clearly essential for the antibacterial profile.

Surprisingly, as shown in Table 5, **6a–6c** emerged as the compounds exhibiting the highest antimicrobial activity by possessing a remarkable antibacterial effect against Gram-positive strains with MIC values between 2 and 8 μg/mL and against the fungi panel with MIC values between 2 and 8 μg/mL. It must be underlined that we found significant antibacterial activity of **6c** against *Bacillus subtilis* (MIC: 2 μg/mL) and Gram-negative *Acinetobacter baumannii* (MIC: 16 μg/mL). Moreover, the broad-spectrum antifungal activity registered for **6b** with MIC values between 2 and 16 μg/mL is noteworthy.

In summary, comparing the overall antimicrobial trend of the **6a–6c** series with the **4a–4o** series it could be hypothesized that the lesser steric hindrance associated to the SH moiety could be more favourable for inducing both antibacterial and antifungal activity.

Molecular modelling

As a final task of this study, a molecular modelling study was carried out to better perceive and evaluate the biological profile of most interesting compounds emerged from the experimental assays. In detail, the remarkable antifungal activity of **4a**, but especially **6b**, was interpreted through docking experiments carried out on an assumed target for our antifungal agents represented by the cytochrome P450-dependent lanosterol 14α-demethylase (CYP51) of *Candida albicans*.

The sterols-biosynthesis pathway, and therefore the enzymes specifically involved, are indeed already known to be targeted by compounds such as azoles (i.e. fluconazole and miconazole), but also by other drugs characterized by a different molecular scaffold (i.e. benzothiazines and benzoxazine) [25–27], responsible for the cell growth inhibition determined by the depletion of ergosterol due to CYP51 inhibition.

In order to accomplish our task, **4a** and **6b** were docked into the catalytic site of a homology based model of *Candida albicans* CYP51. The choice of this theoretical structure was supported by the evidence that the same has been proved to be a robust tool for understanding the molecular basis for antifungal activity of a previously published series of 1,4-benzothiazines [6–7].

The binding poses achieved throughout the docking simulations highlighted some interesting features that might be in charge of the antifungal activity of our newly synthesized compounds: as it might be perceived from Figure 3, both **4a** and **6b** are capable to accommodate the active site of CA-CYP51 mainly anchoring to the heme group with the fluorine atom. For both the examined compounds, the same atom is placed at 1.9 Å distance from the iron atom generating a highly favorable coordination bond (−27.59 and −26.97 kcal/mol respectively), with the rest of the molecular skeleton being almost perpendicular to the plane defined by the heme group. It has to be pointed out that SAR already outlined the value of this kind of substitution, since the unsubstituted derivatives showed a very low antifungal profile [3], moreover

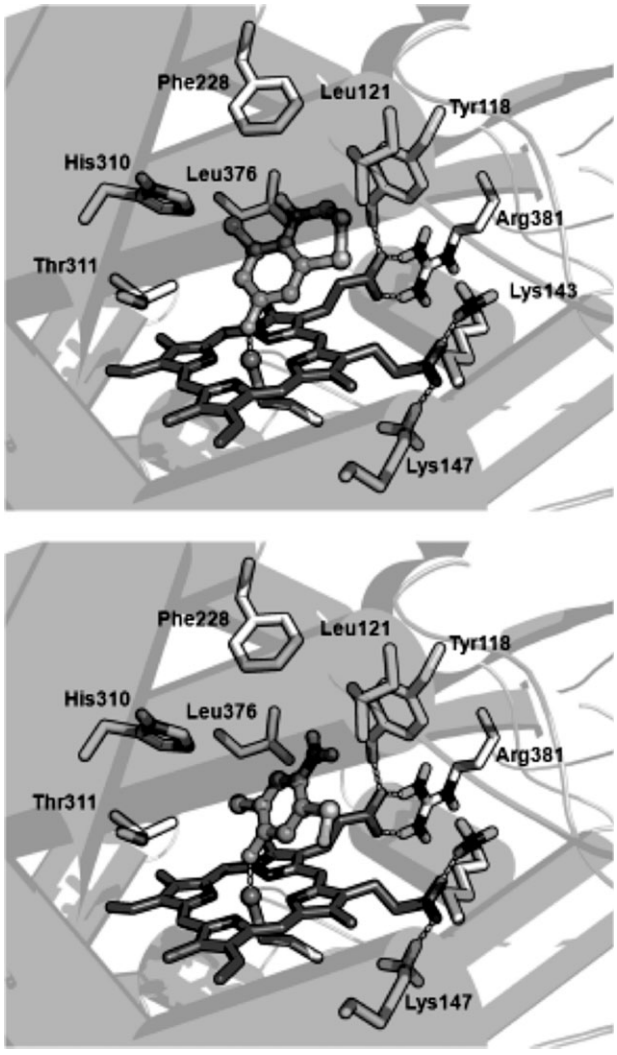


Figure 3. Docking poses for **4a** (top) and **6b** (bottom) in the active site of CA-CYP51. Ligands and iron atom are displayed as ball and stick to help interpretation.

a similar interaction pattern has been also experimentally observed in the X-ray structure of fluconazole with CYP51 of *Mycobacterium tuberculosis* (PDB code: 1EA1, 1E9X) selected as template for the comparative building of CA-CYP51 [28].

Additional non-bonded interactions between the cyclic moieties of our antifungal agents and a cluster of nonpolar residues, comprising Tyr118, Leu121, Phe228 and Leu376, defining a hydrophobic dome placed above the heme group, are also detected, nonetheless with relatively low contribution to the binding energy (−16.51 and −13.60 kcal/mol of **4a** and **6b** respectively) while no significant weight can be ascribed to the chlorine atoms, since in both dockings no direct involvement was observed. On the other hand, it might be postulated that the valence of this molecular element should be basically referred to its lipophilicity, and therefore to the capability of passing the cell membrane, as suggested by the calculated partition coefficient values. In fact, correspondingly CLogP for **4a** and **6b** are 2.41 and 2.85, while the deschloro analogues data are well below an optimal value of 2.0, especially for the latter compounds.

Then, from this modeling study it emerged a plausible mechanism of inhibition of CA-CYP51 elicited by our compounds, suggesting a mandatory role of a fluorine properly placed on the aromatic ring of the molecule, but at the same time showing the need of additional functional groups for a better antifungal activity. In this context, the synthesis of novel derivatives with a correct volume and hindrance required to fulfill the binding site cavity is our ongoing project.

Conclusions

In summary in this study we report on the synthesis and antimicrobial activity of 7-fluoro-3,4-dihydro-2*H*-1,4-benzothiazine derivatives (**4a–4f**, **4h**, Table 1) and 7-fluoro-2*H*-1,4-benzothiazin-3(4*H*)-one analogues (**4j–4o**, Table 2). Additional investigation was realized on Cl-substituted-6-fluoro-1,3-benzothiazol-2-amine (**5a–5c**) and Cl-substituted-2-amino-5-fluorobenzenethiol (**6a–6c**).

Among the 7-fluoro-3,4-dihydro-2*H*-1,4-benzothiazine derivatives (**4a–4f**, **4h**), **4a** exhibited highly potent antimicrobial activity against Gram-positive bacteria and fungi strains. Its MIC values toward *Bacillus subtilis* (MIC: 16 μg/mL) and *Candida parapsilosis* (MIC: 8 μg/mL) are very interesting.

Derivatives **4d–f** and **4h** containing the sterically bulky phenyl and methyl, respectively, at the C3 position of the heterocyclic ring showed weak antimicrobial activity compared to the **4a–4c** analogues.

Among series **4j–4o**, the most significant data were obtained for **4j** and **4l**. Significant antibacterial activity against *Bacillus subtilis* (MIC: 16 μg/mL) was recorded for compound **4j**. **4l** showed an interesting activity against Gram-negative *Acinetobacter baumannii* (MIC: 32 μg/mL). Compounds **4m–4o**, bearing a phenyl at the C2 position of the heterocycle, are only weakly active against the bacteria and fungi panels.

Rather surprisingly, Cl-substituted-2-amino-5-fluorobenzenethiol derivatives (**6a–6c**) are the most promising compounds of all series described in this study, having a strong activity against both bacteria and fungi strains (MIC values between 2 and 16 μg/mL). The high antibacterial effect of **6c** toward *Bacillus subtilis* (MIC: 2 μg/mL) and the antifungal activity of **6b** against *Candida parapsilosis* (MIC: 2 μg/mL) are worthy to note.

Then, taking into account that this preliminary study does not produce conclusive evidences about a structure-antibacterial relationship the results presented in this work suggest

that *a)* the 7-fluoro-3,4-dihydro-2*H*-1,4-benzothiazine nucleus rather than 7-fluoro-2*H*-1,4-benzothiazin-3(4*H*)-one, *b)* the SH moiety and *c)* the chlorine atom on the aromatic ring may positively favor an antimicrobial profile. On the contrary, an increase of the steric hindrance on the heterocycle probably could lead to a loss of antimicrobial activity.

In light of the data herein discussed we focussed our attention on the most promising series **6a–6c**. Then, in our ongoing research program to the synthesis of new antimicrobial agents, several synthetic routes are in progress to acquire more data for guiding the further rational design of 2-amino-5-fluorobenzenethiol analogues to serve as a new *cluster* for the antibacterial and antifungal investigation.

Experimental section

Chemistry

Melting points were recorded on a Gallenkamp melting point apparatus in open glass capillary tubes. The IR spectra were recorded on a Perkin-Elmer Spectrum One FT spectrophotometer and band positions were given in reciprocal centimeters (cm^{-1}). ^1H NMR spectra were recorded on a FT Bruker Aspect 3000 spectrometer using $CDCl_3$ as the solvent, unless otherwise indicated. Chemical shifts were reported in part per million (ppm) relative to solvent resonance: $CDCl_3$, δ 7.26 (^1H NMR). Amino proton assignments were confirmed by D_2O exchange. *J* values are given in Hz. EIMS spectra were recorded with a Hewlett-Packard 6890-5973 MSD gas chromatograph/mass spectrometer at low resolution. Silica gel chromatographic separations were performed by chromatography with silica gel (Kieselgel 60, 40–63 μm, Merck) packed in glass columns, using the technique described in the literature [29]. The weight of the silica gel was approximately 100 times that of the substance. The eluting solvent indicated in parentheses for each purification was determined by TLC, which was performed on precoated silica gel on aluminum sheets (Kieselgel 60, F_{254}, Merck). TLC plates were visualized with UV light and/or in an iodine chamber.

All chemicals were purchased from Aldrich Chemical Co. in the highest quality commercially available. The structures of the compounds were confirmed by routine spectrometric and spectroscopic analyses. Only spectra for compounds not previously described are given.

*Synthesis of 5-chloro-7-fluoro-3,4-dihydro-2H-1,4-benzothiazine (**4a**)*

2-Amino-3-chloro-5-fluorobenzenethiol (**6a**) (1 mmol) in methanol (10 mL) and 1,2-dibromoethane (8.8 mmol) were stirred at reflux under N_2 atmosphere for 15 min. Then 0.4 N CH_3ONa (20 mL) was added dropwise over 10 min. After cooling to room temperature, the reaction mixture was quenched with 2 N $NaHCO_3$ and extracted with EtOAc. The organic layer was separated, dried over anhyd. Na_2SO_4 and evaporated under reduced pressure. The residue was purified by silica gel column-chromatography with petroleum ether/EtOAc (8:2 v/v) to provide compound **4a** as a brown oil. Yield: 56%; ^1H NMR (300 MHz, $CDCl_3$): δ 3.03–3.08 (m, 2H, SCH_2), 3.65–3.68 (m, 2H, NCH_2), 3.9 (s, br, 1H, NH), 6.68 and 6.70 (dd, 1H, $J = 3.2$ Hz, H_6), 6.78 and 6.80 (dd, 1H, $J = 3.2$ Hz, H_8). MS (70 eV) *m/z* (%) 203 (M^+), 188 (100).

*Synthesis of 6-chloro-7-fluoro-3,4-dihydro-2H-1,4-benzothiazine (**4b**) [2]*

2-Amino-4-chloro-5-fluorobenzenethiol (**6b**) (1 mmol) in ethanol (10 mL) and 1,2-dibromoethane (4.3 mmol) was heated at reflux under nitrogen, for 1.5 h. Then 0.65 N CH_3ONa (10 mL) was added and heating was continued for 2 h. After cooling to room temperature, the reaction mixture was diluted with 2 N $NaHCO_3$ (10 mL) and extracted with CH_2Cl_2. The organic layer was separated, dried over anhyd. Na_2SO_4 and then evaporated under reduced pressure. The crude product was separated by column-chromatography on silica gel by using light petroleum ether and EtOAc (7:3 v/v) as eluent to obtain the pure compound **4b** as a grey solid.

Mp is in agreement with the published data [2].

Yield: 54%; IR (nujol mull): 3410 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 3.00–3.06 (m, 2H, SCH_2), 3.55–3.61 (m, 2H, NCH_2), 3.92 (s, br, 1H, NH), 6.46 (d, 1H, $J = 6.3$ Hz, H_5), 6.77 (d, 1H, $J = 9.0$ Hz, H_8); MS (70 eV) *m/z* (%) 203 (M^+), 188 (100), 135, 113.

*Synthesis of 8-chloro-7-fluoro-3,4-dihydro-2H-1,4-benzothiazine (**4c**) [2]*

The title compound was prepared starting from 6-amino-2-chloro-3-fluorobenzenethiol (**6c**) according to the synthetic protocol described for **4b**. After standard workup, the desired product was isolated as a brown oil.

Yield: 49%; IR (nujol mull): 3377 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 3.08–3.15 (m, 2H, SCH_2), 3.52–3.60 (m, 2H, NCH_2), 3.70 (s, br, 1H, NH), 6.34–6.42 (m, 1H, H_5), 6.72 (t, 1H, $J = 8.2$ Hz, H_6); MS (70 eV) *m/z* (%) 203 (M^+), 188 (100).

*General procedure for the synthesis of 7-fluoro-3,4-dihydro-2H-1,4-benzothiazine derivatives (**4d–f**)*

The preparation of 5-chloro-7-fluoro-3-phenyl-3,4-dihydro-2*H*-1,4-benzothiazine (**4d**) can be taken as reference for the synthesis of 7-fluoro-3,4-dihydro-2*H*-1,4-benzothiazine derivatives (**4d–f**).

A mixture of **6a** (1 mmol) and 2-bromo-1-phenylethanone (1 mmol) in anhydrous Et_2O (30 mL) was heated at reflux under N_2 atmosphere for 30 min. After cooling to room temperature, 2 N $NaHCO_3$ (10 mL) was added in one portion. Then the reaction mixture was extracted with CH_2Cl_2. The obtained organic layer was separated, dried over anhyd.

Na_2SO_4 and concentrated under reduced pressure. A mixture of CH_3COOH and methanol (30 mL, 1:1) at 0°C was carefully added to the crude residue. Subsequently, $NaBH_4$ (4.84 mmol) was added portionwise under stirring. The mixture was diluted with H_2O and then basified with NaOH pellets (pH ≅ 11). The basic layer was extracted with $CHCl_3$ (3 × 30 mL). Organic layers were dried over anhyd. Na_2SO_4 and concentrated in vacuum. The residue was purified by silica gel column-chromatography (petroleum ether/EtOAc, 8:2) to afford the desired compound **4d** as a brown oil.

Yield: 10%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.12 (d, 2H, J = 4.7 Hz, SCH_2), 4.35 (s, l, 1H, NH), 4.63 (s, 1H, CH-Ph), 6.75 and 6.95 (dd, 1H, J = 8.3 Hz, H_6), 6.84 and 6.87 (dd, 1H, J = 10.7 Hz, H_8), 7.35–7.44 (m, 5H, H-Arom); MS (70 eV) *m/z* (%) 279 (M^+), 264, 211, 188 (100).

*6-Chloro-7-fluoro-3-phenyl-3,4-dihydro-2H-1,4-benzothiazine (**4e**)*

The title compound was prepared by using the corresponding benzenethiol derivative **6b** as starting material. The synthetic procedure provided **4e** as a brown oil.

Yield: 17%; IR (nujol mull): 3379, 1645 cm^{-1}; ^{1}H NMR (300 MHz, DMSO-*d6*): δ 2.9–3.3 (m, 2H, CH_2), 4.0–4.2 (m, 1H, CH_2–C*H*), 4.07 (s, br, 1H, NH), 6.53 (d, 1H, J = 6.0 Hz, H_5), 6.86 (d, 1H, J = 8.8 Hz, H_8), 7.4–7.6 (m, 5H, H-Arom); MS (70 eV) *m/z* (%) 279 (M^+), 264, 211, 188 (100).

*8-Chloro-7-fluoro-3-phenyl-3,4-dihydro-2H-1,4-benzothiazine (**4f**)*

The title compound was prepared by using the corresponding benzenethiol derivative **6c** as starting material. The synthetic procedure provided **4f** as a brown oil.

Yield: 25%; °C; IR (nujol mull): 3414, 1598 cm^{-1}; ^{1}H NMR (300 MHz, DMSO-*d6*): δ 3.16 (d, 2H, J = 8.6 Hz, CH_2), 4.10 (s, br, 1H, NH), 4.52 and 4.56 (dd, 1H, J = 2.4 Hz, CH_2–C*H*), 6.38–6.42 (d, 1H, J = 5.0 Hz, H_5), 6.75 (t, 1H, J = 8.8 Hz, H_6), 7.3–7.4 (m, 5H, H-Arom); MS (70 eV) *m/z* (%) 279 (M^+), 264, 211, 188 (100).

*6-Chloro-7-fluoro-3-methyl-3,4-dihydro-2H-1,4-benzothiazine (**4h**)*

A mixture of **6b** (1 mmol) and 1-chloropropan-2-one (1.5 mmol) in anhydrous Et_2O (30 mL) were heated under reflux in an N_2 atmosphere for 30 min. After cooling to room temperature, 2 N $NaHCO_3$ (10 mL) were added in one portion. Then the reaction mixture was extracted with CH_2Cl_2. The obtained organic layer was separated, dried over anhyd. Na_2SO_4 and concentrated under reduced pressure. A mixture of CH_3COOH and methanol (30 mL, 1:1) at 0°C was carefully added to the crude residue. Subsequently, $NaBH_4$ (4.84 mmol) was added portionwise under stirring. The mixture was diluted with H_2O and then basified with NaOH pellets (pH ≅ 11). The basic layer was extracted with $CHCl_3$ (3 × 30 mL). Organic layers were dried over anhyd. Na_2SO_4 and concentrated in vacuum. The residue was purified by silica gel column-chromatography (petroleum ether/EtOAc, 7:3) to provide compound **4h** as an orange solid.

Yield: 12%; mp 212–214°C (EtOAc); IR (nujol mull): 3351 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.30 (d, 3H, J = 6.2 Hz, CH_3), 2.7–2.9 (m, 2H, CH_2), 3.6–3.7 (m, 2H, CH), 3.77 (s, br, 1H, NH), 6.45 (d, 1H, J = 7.1 Hz, H_5), 6.81 (d, 1H, J = 9.4 Hz, H_8). MS (70 eV) *m/z* (%) 217 (M^+), 202 (100).

*General procedure for the synthesis of 7-fluoro-2H-1,4-benzothiazin-3(4H)-one derivatives (**4j–l**)*

The preparation of 5-chloro-7-fluoro-2*H*-1,4-benzothiazin-3(4*H*)-one (**4j**) can be taken as reference for the synthesis of 7-fluoro-2H-1,4-benzothiazin-3(4H)-one derivatives (**4j–l**).

2-Amino-3-chloro-5-fluorobenzenethiol (**6a**) (1 mmol) in 2.5 N NaOH (15 mL) was heated under reflux for 30 min. Then a solution of chloroacetic acid (2 mmol) in H_2O (3.5 mL) was added dropwise. The reflux was continued for 1.5 h. The reaction mixture was then cooled at room temperature and extracted with EtOAc. The organic layer was dried over anhyd. Na_2SO_4 and concentrated under reduced pressure.

The crude residue was purified by silica gel column-chromatography (petroleum ether/EtOAc, 7:3) to afford **4j** as a yellow solid.

*5-Chloro-7-fluoro-2H-1,4-benzothiazin-3(4H)-one (**4j**)*

Yield: 25%; mp 209–212°C (EtOAc); IR (nujol mull): 3183, 1666 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.42 (m, 2H, CH_2), 6.92 (d, 1H, $J_{H\text{-}F}$ = 6.11 Hz, H_5), 7.12 (d, 1H, $J_{H\text{-}F}$ = 8.33 Hz, H_8), 8.72 (s, br, 1H, NH); MS (70 eV) *m/z* (%) 217 (M^+, 100), 188, 172, 113.

*6-Chloro-7-fluoro-2H-1,4-benzothiazin-3(4H)-one (**4k**) [2]*

The title compound was prepared by using the corresponding benzenethiol derivative **6b** as starting material. The synthetic procedure provided **4k** as a yellow solid.

Mp is in agreement with the published data [2].

Yield: 25%; IR (nujol mull): 3184, 1667 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.48 (m, 2H, CH_2), 6.73 and 6.77 (dd, 1H, J = 6.10 Hz, H_6), 6.98 (t, 1H, J = 8.30 Hz, H_5), 8.60 (s, br, 1H, NH); MS (70 eV) *m/z* (%) 217 (M^+, 100), 188, 172, 113.

*8-Chloro-7-fluoro-2H-1,4-benzothiazin-3(4H)-one (**4l**) [2]*

The title compound was prepared by using the corresponding benzenethiol derivative **6c** as starting material. The synthetic procedure provided **4l** as a green solid.

Mp is in agreement with the published data [2].

Yield: 32%; IR (nujol mull): 3140, 1678 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.42 (m, 2H, CH_2), 6.90 (d, 1H, J = 7.0 Hz, H_8), 7.12 (d, 1H, J = 8.8 Hz, H_5), 8.15 (s, br, 1H, NH); MS (70 eV) *m/z* (%) 217 (M^+, 100), 188, 172, 113.

*General procedure for the synthesis of 7-fluoro-2H-1,4-benzothiazin-3(4H)-one derivatives (**4m–o**)*

The preparation of 5-chloro-7-fluoro-2-phenyl-2*H*-1,4-benzothiazin-3(4*H*)-one (**4m**) can be taken as reference for the synthesis of 7-fluoro-2H-1,4-benzothiazin-3(4H)-one derivatives (**4m–o**). 2-Amino-3-chloro-5-fluorobenzenethiol (**6a)** (1 mmol) in 1.87 N EtONa (5 mL) was heated under reflux for 30 min. Then, a solution of bromo(phenyl)acetic acid (1.5 mmol) was added dropwise over 10 min. The reaction mixture was stirred for 24 h at reflux and subsequently quenched with H_2O (5 mL) and then basified with NaOH pellets. The aqueous phase was extracted with $CHCl_3$ (3 × 25 mL). The combined organic layers were dried over anhyd. Na_2SO_4 and concentrated in vacuum. Purification of the crude product by silica gel chromatography (petroleum ether/EtOAc, 1:1) led to the pure compound **4m** as a yellow solid.

*5-Chloro-7-fluoro-2-phenyl-2H-1,4-benzothiazin-3(4H)-one (**4m**)*

Yield: 60%; mp 210–212°C (EtOAc); IR (nujol mull): 3188, 1668 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 4.25 (s, 1H, CH), 7.23 (d, 1H, $J_{H\text{-}F} = 8.65$ Hz, H_6), 7.32 (d, 1H, $J_{H\text{-}F} = 12$ Hz, H_8), 7.15–7.20 (m, 5H, H–Arom.), 9.2 (s, br, 1H, NH); MS (70 eV) *m/z* (%) 293 (M^+, 100), 264, 188, 172.

*6-Chloro-7-fluoro-2-phenyl-2H-1,4-benzothiazin-3(4H)-one (**4n**)*

The title compound was prepared by using the corresponding benzenethiol derivative **6b** as starting material. The synthetic procedure provided **4n** as a yellow solid.

Yield: 79%; mp 210–215°C (EtOAc); IR (nujol mull): 3130, 1668 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 4.72 (s, 1H, CH), 6.68–6.75 (m, 1H, H_5), 6.97 (t, 1H, $J_{H\text{-}F} = 8.7$ Hz, H_8), 7.28–7.40 (m, 5H, H–Arom.), 8.2 (s, br, 1H, NH); MS (70 eV) *m/z* (%) 293 (M^+, 100), 264, 188, 172.

*8-Chloro-7-fluoro-2-phenyl-2H-1,4-benzothiazin-3(4H)-one (**4o**)*

The title compound was prepared by using the corresponding benzenethiol derivative **6c** as starting material. The synthetic procedure provided **4o** as a yellow solid.

Yield: 40%; mp 189–201°C (EtOAc); IR (nujol mull): 3140, 1670 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 4.72 (s, 1H, CH), 6.7–6.9 (m, 5H, H–Arom.), 7.2–7.4 (m, 2H, $H_5 + H_6$), 9.4 (s, br, 1H, NH); MS (70 eV) *m/z* (%) 293 (M^+, 100), 264, 188, 172.

Biology

The *in-vitro* minimum inhibitory concentrations (MICs, μg/mL) of the prepared compounds were assessed by the broth microdilution method, using 96-well plates, according to the National Committee for Clinical Laboratory Standard (CLSI) formerly NCCLS.

Stock solutions of the tested compounds were obtained in DMSO. Stock solutions of lower concentrations were prepared for those substances which did not dissolve well.

Then two-fold serial dilutions in the suitable test medium between 512 and 0.5 μg/mL were plated. To be sure that the solvent had no adverse effect on bacterial growth, a control test was carried out by using ethanol at its maximum concentration along with the medium.

Bacteria strains available as freeze-dried discs, belonging to the ATCC collection, were used: Gram-positive strains such as *Staphylococcus aureus* 6538P, *Enterococcus faecalis* 29212, *Bacillus subtilis* 6633, and Gram-negative ones such as *Escherichia coli* 25922, *Acinetobacter baumannii* 19606.

To preserve the purity of cultures and to allow the reproducibility, a series of cryovials of all microbial strains in glycerolic medium was set up and stored at −80°C.

Pre-cultures of each bacterial strain were prepared in Cation Adjusted Mueller-Hinton broth (CAMHB) and incubated at 37°C until the growth ceased. The turbidity of bacterial cell suspension was calibrated to 0.5 McFarland Standard by spectrophotometric method (625 nm, range 0.08–0.10), and further the standardized suspension was diluted 1:100 with CAMHB to have 1–2 × 10^6 CFU/mL (rif. a).

All wells were seeded with 100 μL of inoculums.

A number of wells containing only inoculated broth as control growth were prepared.

The plates were incubated at 37°C for 24 h, and the MIC values were recorded as the last well containing no bacterial growth. The MIC were determined by using an antibacterial assayed repeated twice in triplicate.

Norfloxacin and oxacillin were used as standard drugs.

Antifungal activity

Four yeast strains (ATCC) were used: *Candida albicans* 10231, *Candida parapsilosis* 22019, *Candida tropicalis* 750 and *Candida krusei* 6258.

Pre-cultures of each yeast strain were prepared in Sabouraud broth 2% glucose (SAB), and incubated at 35°C until the growth ceased. The turbidity of yeast stock suspension was calibrated to 0.5 McFarland Standard by spectrophotometric method (530 nm, range 0.12–0.15), and further the standardized suspension was diluted first 1:50 with SAB ad then 1:20 in the same medium to have 1–5 × 10^6 CFU/mL (rif. b).

All wells were seeded with 100 μL of inoculums.

A number of wells containing only inoculated broth as control growth were prepared.

The plates were incubated at 35°C for 24–48 h, and the MIC values were recorded as the last well containing no fungal growth.

The MIC were determined by using an antifungal assay repeated twice in triplicates.

Amphotericin B and fluconazole were used as standard drugs.

Molecular modeling

Dockings were carried out by means of the Glue model implemented in the Grid software [30]. The binding site was defined as 10 × 10 × 10 cubic box centering on the iron atom of the heme group. Molecular interaction maps were calculated using a set of properly selected probes reproducing the molecular fragments of the examined compounds, namely CL, F, S1, STH, N1, N2 and DRY according to the Grid atoms code. The maximum number of sites and interactions was set to 10 000, while the minimum energy change was 0.001 kcal/mol. Electrostatic contribution was considered, and the pose scored with the best binding affinity was selected. CLogP were calculated with ACD/ChemSketch v.12.01.

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