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Design, synthesis and biological evaluation of a series of iron and copper chelating deferiprone derivatives as new agents active against *Candida albicans*

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ABSTRACT

Candida albicans, in specific conditions, is responsible of severe invasive systemic candidiasis that are related to its ability to produce biofilm on biological and artificial surfaces. Many studies reported the role of iron in fungal growth and virulence and the ability of metal chelating agents to interfere with *C. albicans* metabolism, virulence and biofilm formation.

Here we report the activity of 3-hydroxy-1,2-dimethyl-4(1*H*)-pyridinone (deferiprone) derivatives against *C. albicans* planktonic cells and biofilm. Some of the studied compounds (**2b** and **3b**) were able to chelate Fe(III) and Cu(II), and showed an interesting activity on planktonic cells (MIC_{50} of 32 μg/mL and 16 μg/mL respectively) and on biofilm formation ($BMIC_{50}$ of 32 μg/mL and 16 μg/mL respectively) in cultured ATCC 10,231*C. albicans*; this activity was reduced, in a concentration dependent way, by the addition of Fe(III) and Cu (II) to the culture media. Furthermore, the most active compound **3b** showed a low toxicity on *Galleria mellonella* larvae.

Candida albicans is normally present in human microbiota as commensal organism, colonizing skin, oral cavity, gastrointestinal and genital tracts, but in some conditions, such as impairment of the host's immune system or alterations of its microbiota, it can cause different infections, ranging from superficial infections to invasive systemic candidiasis.[1,2] The majority of clinical manifestations of candidiasis are related to biofilm formation on biological and artificial surfaces; *C. albicans* biofilms are formed by cells at different stages of growth surrounded by self-produced extracellular matrix and their production represents one of the major virulence factors of *C. albicans*. Indeed, *C. albicans* cells within the biofilms are intrinsically resistant to antifungal drugs, in particular to azole and polyene, and are protected from the host's immune response.[1–4] The low efficacy or the toxicity of some antifungal drugs have led to the need for new molecules capable of acting towards alternative *C. albicans* targets.[5–6]

For *C. albicans*, as well as for others fungi and bacteria, environmental iron is fundamental for survival, growth and virulence.[7–11] For this reason, in recent years, various studies have evaluated the effects of different iron chelators, alone or in association with antifungal drugs, towards *C. albicans*; these studies revealed that iron chelating agents are able to inhibit the growth of the fungus,[12–15] to exert a synergism with azole or with caspofungin,[14–18] to reduce virulence, modulating the expression of genes involved in iron metabolism, in adhesion and in the response to host innate immunity,[19] to inhibit biofilm formation or to affect its structural integrity.[20–22] Among these molecules there are some non-selective iron chelators and their effects may also depend on the interference with other metals fundamental for *C. albicans*. Indeed, some compounds reported in the literature are able to exert antifungal

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effects by targeting the homeostasis of different metal ions, either by decreasing the metals bioavailability or by acting as ionophores, leading to excessive accumulation of metals into the cells.[23–27] Moreover, Polvi *et al.* have demonstrated that the chelator DTPA (diethylenetriamine pentaacetic acid) enhances caspofungin activity against an echinocandin-resistant clinical isolate of *C. albicans* by depleting magnesium and induces filamentation in wild-type cells of *C. albicans* by chelation of zinc.[28] Overall these results show that metal chelation could have a potential role in the treatment of *C. albicans* infections.[29]

In this work we present the activity of some deferiprone (DFP) derivatives against *C. albicans* planktonic cells and biofilm. DFP (3-hydroxy-1,2-dimethyl-4(1*H*)-pyridinone, Chart 1) is a small molecule approved by FDA as iron chelator for the treatment of patients with transfusional iron overload due to thalassemia syndromes. DFP was already studied towards different strains of *C. albicans* and it resulted a weak inhibitor of fungal growth; differently, DIBI, a hydroxypyridinone iron-chelating polymer, inhibits *C. albicans* growth at very low concentration in comparison to chemical related DFP and its activity can be reversed by iron addition.[13,14] We decided to modify DFP moiety in N1 position, in order to increase its lipophilia, introducing aryl–alkyl groups of different sizes and polarity. We also connected DFP moiety with ketoprofen, ibuprofen or ibufenac respectively, as some authors reported the inhibitory activities of non-steroidal anti-inflammatory drugs (NSAIDs) against *C. albicans.*[30–32] In particular ibuprofen showed interestingly activity on different strains of *Candida*, both alone and in association with antifungal drugs.[33–40] Moreover, in our previous work we studied some hybrids between tryptamine and NSAIDs, which showed interesting activities against *C. albicans* biofilm.[41] Finally, we also tested compounds obtained by molecular duplication of DFP group, that have been selected from some previously synthesized molecules (Chart 1).[42]

The synthesis of all compounds required the protection of maltol with a benzyl group to obtain the intermediate **1**, as shown in Scheme 1. The benzyl-protected maltol **1** was reacted in water / ethanol mixture, in presence of sodium hydroxide (pH = 13), with the suitable alkyl-aryl amine or alkyl-imidazole amine to give the compounds **2a-5a** that were debenzylated by treatment with aqueous 6 M HCl to obtain the salts **2b-5b**.

The syntheses of the compounds **2b** and **3b** were previously reported in literature, but with very low yields;[43] here we described new synthetic procedure that allows to obtain them in higher yields. A different synthetic procedure to give the compound **5b** was previously reported in literature.[44]

Compounds **7b-10b** were synthesized as illustrated in Scheme 2; benzylated maltol **1** and 1,4-diaminobutane were reacted in a mixture of ethanol and aqueous sodium hydroxide (pH = 13) to give the double

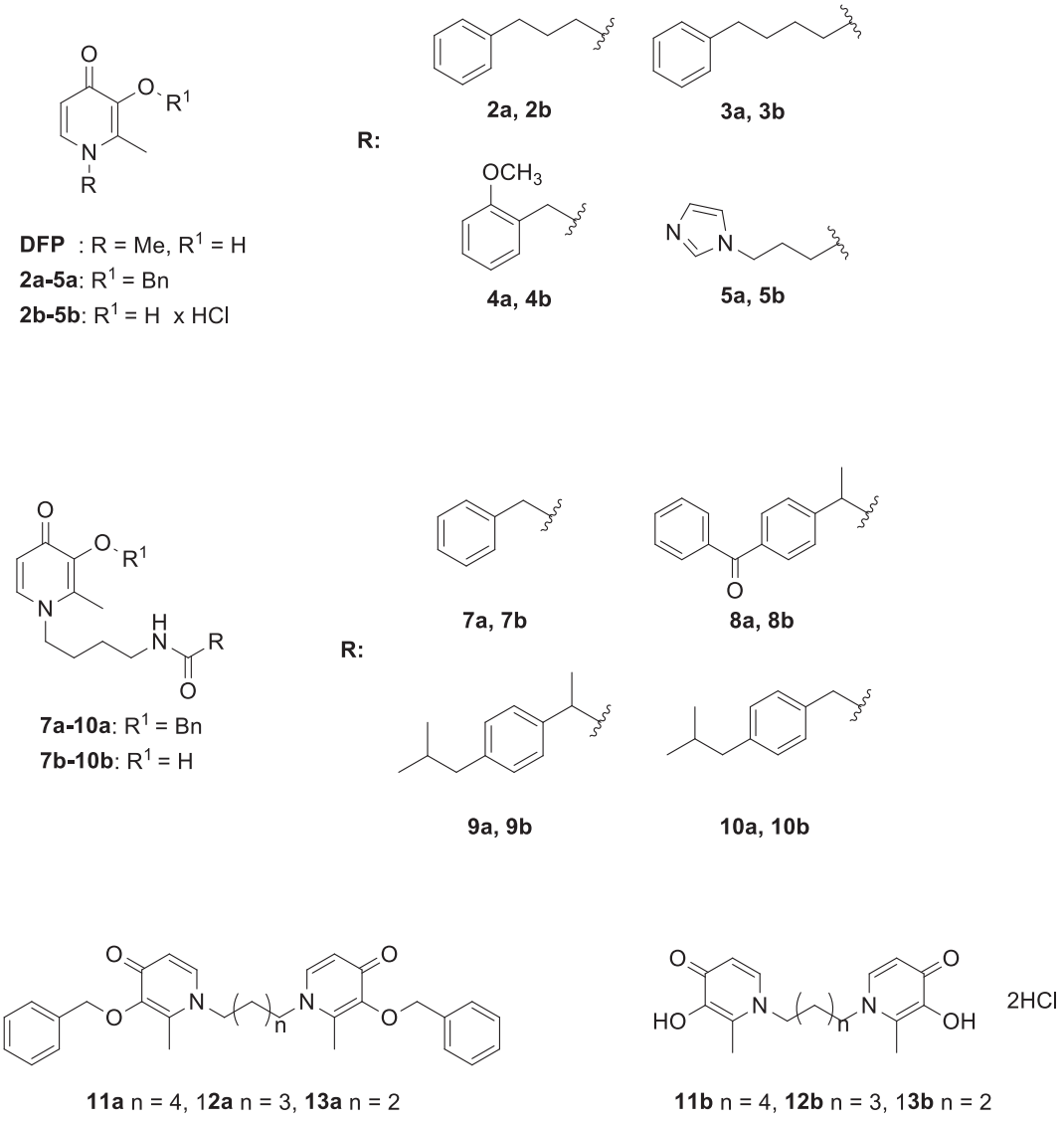
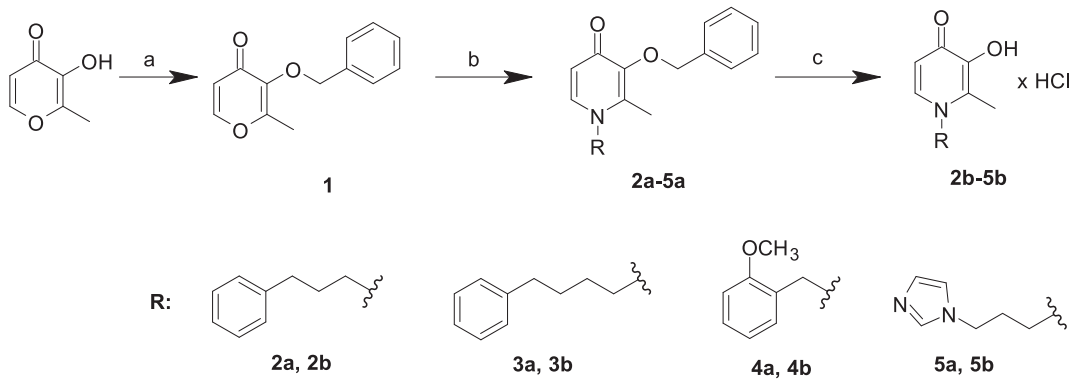
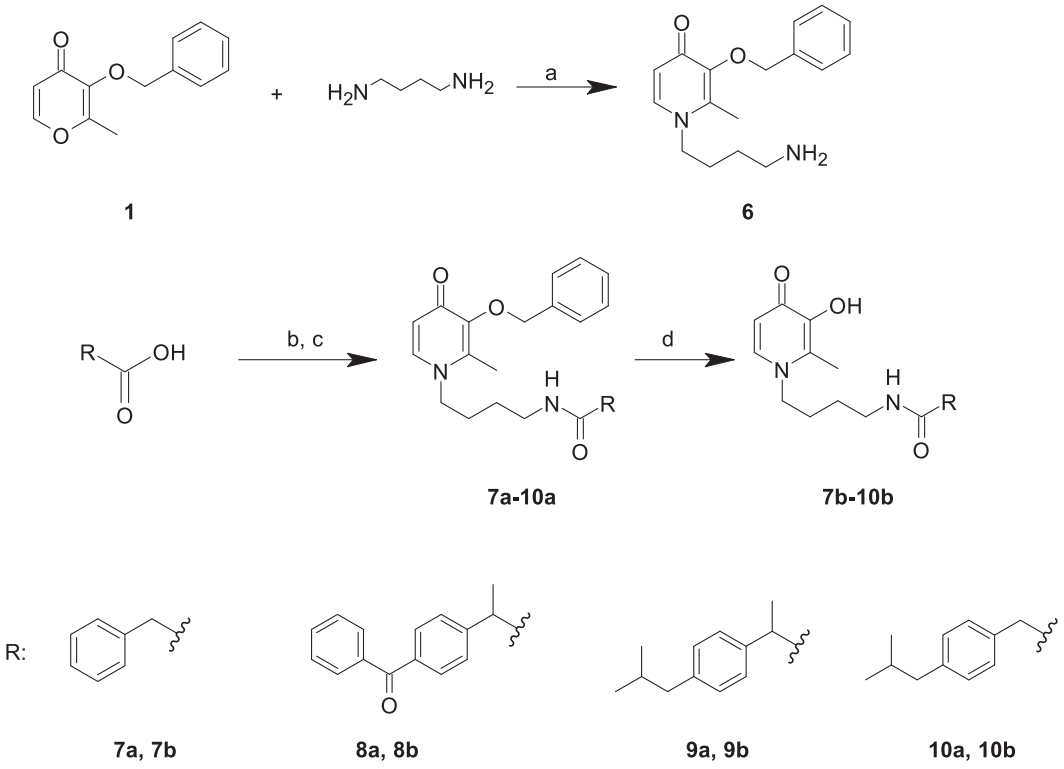


Chart 1. DFP derivatives studied in this work.



Scheme 1. *Reagents and conditions:* a) benzyl bromide, EtOH, NaOH, reflux, 6 h; b) H_2N-R, EtOH/H_2O, NaOH (pH = 13), reflux, 18 h; c) 6 N HCl, reflux, 2 h.



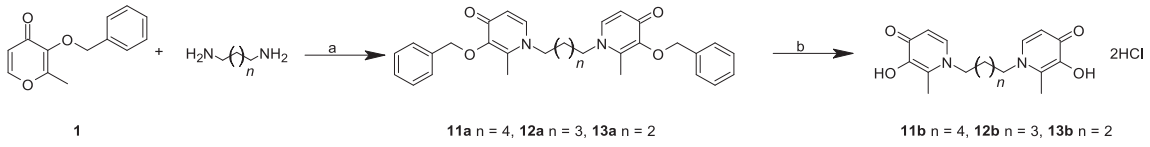
Scheme 2. *Reagents and conditions:* a) EtOH/H_2O, NaOH (pH = 13), reflux, 18 h; b) CDI, AcOEt, reflux, 5 h; c) **6**, reflux, 12 h; d) 10% Pd/C, Zn, MeOH, H_2SO_4.

Michael addition product **6**, which was furtherly coupled with different carboxylic acid, via carbonyldiimidazole (CDI) intermediates, to obtain the amide derivatives **7a-10a**. The subsequent removal of the benzyl group by catalytic hydrogenation in presence of 10% Pd/C gave the amides **7b-10b**.

Compounds **11a,b-13a,b** were synthesized as previously reported (Scheme 3).[42]

The detailed synthetic procedures, the analytical and spectroscopic data of the synthesized compounds are reported in the supplementary material and are in agreement with the proposed structures.

In order to verify the chelating capability of synthesized DFP derivatives, chelation studies of Fe^{3+} and Cu^{2+} were performed using an UV–visible spectrophotometer on four selected compounds, two amine derivatives (**3a**, **3b**) and two amide derivatives conjugated with ibuprofen (**9a**, **9b**). These molecules were chosen to evaluate whether the presence of benzyl group linked to oxygen of DFP may influence the chelating abilities of the studied compounds. Since the molecular fragment responsible for the chelating activity is DFP, it is quite reasonable to assume that the chelating behaviour of these selected compounds can be extended to the other benzylated and debenzylated derivatives. The UV spectra of the pure ligands were recorded and compared with the spectra obtained by adding an excess of metal ion, maintaining the same



Scheme 3. *Reagents and conditions:* a) EtOH/H_2O, NaOH (pH = 13), reflux, 18 h; b) 6 N HCl, reflux, 2 h.

concentration of ligand. The variation of the UV spectra of the ligand in presence of metal ions is indicative of the complex formation. These spectra showed that benzylated derivatives **3a** and **9a** can chelate only Fe^{3+}, while debenzylated compounds **3b** and **9b** chelate both Fe^{3+} and Cu^{2+} (Fig. S1–S8 supporting information).

The synthesized compounds **2a,b-13a,b** have been screened to evaluate the activity against *C. albicans* planktonic cells and biofilm, both in formation and mature (48 h), using *C. albicans* ATCC 10231, a strain sensitive to fluconazole on planktonic cells (0.5 μg/mL) and resistant in the different phases of biofilm formation ($BMIC_{50}$ 128 μg/mL on biofilm formation and > 128 μg/mL on mature biofilm).[45] The results are reported in Table 1.

Among the amine derivatives **2a,b-5a,b**, the compounds **2b** and **3b** are the most interesting, both for their activity against biofilm formation ($BMIC_{50}$ 32 μg/mL and 16 μg/mL respectively), comparable or superior to that of the parent compound DFP ($BMIC_{50}$ 32 μg/mL), and for their activity against planktonic cells (MIC_{50} 32 μg/mL and 16 μg/mL respectively), much higher than that of DFP (MIC_{50} 128 μg/mL). These compounds contain a phenyl alkyl moiety connected with DFP; it can be noted that passing from three (compound **2b**) to four (compound **3b**) methylene units the biofilm inhibition activity probably increases due to the higher lipophilicity of **3b** (MLogP = 1.83) compared to **2b** (MLogP = 1.58).[46,47]

It is noteworthy that the corresponding benzylated molecules **2a** and **3a** are not active up to a concentration of 128 μg/mL, indicating that the free hydroxyl function in position 3 is of great importance for obtaining antibiofilm activity and this could be related to the depletion of metals from the cellular environment by a chelation mechanism; indeed, the presence of the benzyl protecting group on the oxygen atom in position 3 of DFP makes the latter less available for the chelation of metals.

Regarding the amide derivatives **7a,b-10a,b**, the most potent inhibitors are compounds **9a,b** and **10a,b** which derive from the connection between DFP with NSAIDs, in particular ibuprofen and ibufenac respectively. In particular, the benzylated DFP derivatives **9a** and **10a** are active against biofilm formation, with $BMIC_{50}$ values of 32 μg/mL and 64 μg/mL respectively, but not towards planktonic cells. The corresponding deprotected compounds **9b** and **10b** show a lower or a similar potency against biofilm formation, with $BMIC_{50}$ values of 128 μg/mL and 64 μg/mL respectively. These data suggest that their activity on biofilm could be due not only to chelation proprieties, but also to other inhibition mechanisms, probably related to the NSAIDs moiety. On the contrary, the debenzylated derivatives **9b** and **10b** show a very interesting activity against *C. albicans* planktonic cells, with MIC_{50} of 4 μg/mL and 8 μg/mL respectively.

Finally, none of the compounds obtained by molecular duplication of DFP group **11a,b-13a,b** were found active against biofilm and planktonic cells up to a concentration of 128 μg/mL.

Table 1
Antifungal activity of the DFP derivatives **2a,b-13a,b** against *C. albicans* ATCC 10,231 biofilms and planktonic cells.

	$BMIC_{50}$ (μg/mL)		MIC_{50} (μg/mL)
Compound	Mature biofilm	Biofilm formation	Planktonic cells
2a	>128	>128	>128
2b	>128	32	32
3a	>128	>128	>128
3b	>128	16	16
4a	>128	>128	>128
4b	>128	>128	>128
5a	>128	>128	>128
5b	>128	>128	>128
7a	>128	>128	>128
7b	>128	>128	>128
8a	>128	>128	>128
8b	>128	128	>128
9a	>128	32	>128
9b	>128	128	4
10a	>128	64	>128
10b	128	64	8
11a	>128	>128	>128
11b	>128	>128	>128
12a	>128	>128	>128
12b	>128	128	>128
13a	>128	128	>128
13b	>128	>128	>128
DFP	128	32	128

$BMIC_{50}$: the lowest drug concentration producing a 50% decrease of biofilm relative to the untreated growth control. MIC_{50}: the lowest drug concentration producing 50% growth inhibition. The antifungal activities are the result of three independent experiments performed in triplicate. The data were presented as median.

Finally, none of the compounds obtained by molecular duplication of DFP group **11a,b-13a,b** were found active against biofilm and planktonic cells up to a concentration of 128 μg/mL.

In order to support the hypothesis that a chelation mechanism could be involved in the activity of the best inhibitor of biofilm formation **3b**, other experiments were carried out using culture medium enriched with increasing concentration of Fe^{3+} and Cu^{2+}. The obtained data are reported in the Fig. 1. As expected, a dose/related decrease of the activity against biofilm formation was observed in presence of increasing concentration of the metallic ions. Recently, the relationship between the inhibition of biofilm formation and the reduction of available iron in *C. albicans* has also been demonstrated by Hsu *et al.* These authors showed the antifungal activity of a compound, which inhibits yeast-to-hyphal transition and biofilm formation of *C. albicans* by interfering with iron ion homeostasis.[22] Moreover, Sumant Puri *et al.* have demonstrated that *C. albicans* cells treated with the iron chelator deferasirox have a significantly reduced adhesion ability.[19] Adhesion is the first step in the formation of the biofilm. This could explain the activity of **3b** in the formation of the biofilm and not in the dispersion of the preformed biofilm.

The stoichiometry of **3b** complexed with Fe^{3+} and Cu^{2+} was determined by the method of continuous variations of Job,[48] which is described in detailed in supporting information. In Fig. 2 the UV titration spectra of ligand **3b** with Fe^{3+} and the related Job's plot obtained at 302 nm are depicted. Both for Fe^{3+} and Cu^{2+} complexes a 1:1 stoichiometry among the ligand and the metal ion was observed. Known the stoichiometry, it was possible to calculate the stability constants (K_{stab}) of these complexes using UV–Vis spectroscopy method, as described in the supporting information.[49] The corresponding $logK_{stab}$ of **3b**-Fe^{3+} and **3b**-Cu^{2+} complexes were 5.16 ± 0.27 and 4.63 ± 0.09 respectively.

Finally, we evaluated the *in vivo* toxicity of the most active antibiofilm compound **3b** on larvae of *Galleria mellonella*. This is a simple and low-cost validated model widely used, because its results correlate with those observed in mammals.[50] The detailed procedure is reported in the supplementary materials. The lethal dose that reduces the number of *G. mellonella* larvae by 50% (LD_{50}) of **3b** has not been found even when testing a dose more of 10 times higher (512 μg/mL) than the active against *C. albicans* biofilm ($BMIC_{50}$ 16 μg/mL). The results, reported in Table 2, showed that *G. mellonella* larvae treated with **3b** at the concentration of 512 μg/mL displayed a 100%, 96% and 86% of survival rate respectively at 24 h, 48 h and 72 h; no toxic effects were observed at lower concentration of **3b** (Table 2).

In conclusion, in this work we describe the synthesis of a new series of DFP derivatives with the aim of obtaining compounds able to chelate the metal cations Fe^{3+} and Cu^{2+} and endowed with antibiofilm activity. Two representative compounds of the benzylated series on the oxygen 3 of DFP (**3a**, **9a**) showed the ability to chelate exclusively the Fe^{3+} ion, while the corresponding debenzylated compounds with free hydroxyl group (**3b**, **9b**) resulted chelators of both Fe^{3+} and Cu^{2+}.

The data obtained by antifungal and antibiofilm activity tests in cultured ATCC 10,231*C. albicans* indicate that none of the synthesized compound resulted active on mature biofilm. Otherwise, among the amine derivatives **2a,b-5a,b**, only **2b** and **3b** resulted moderately active both on biofilm formation and on planktonic cells, with **3b** being the most active antibiofilm compound identified ($BMIC_{50}$ = 16 μg/mL). The reduction in the inhibitory effect of compound **3b** on biofilm formation in the presence of increasing amounts of Fe^{3+} and Cu^{2+} suggests that this

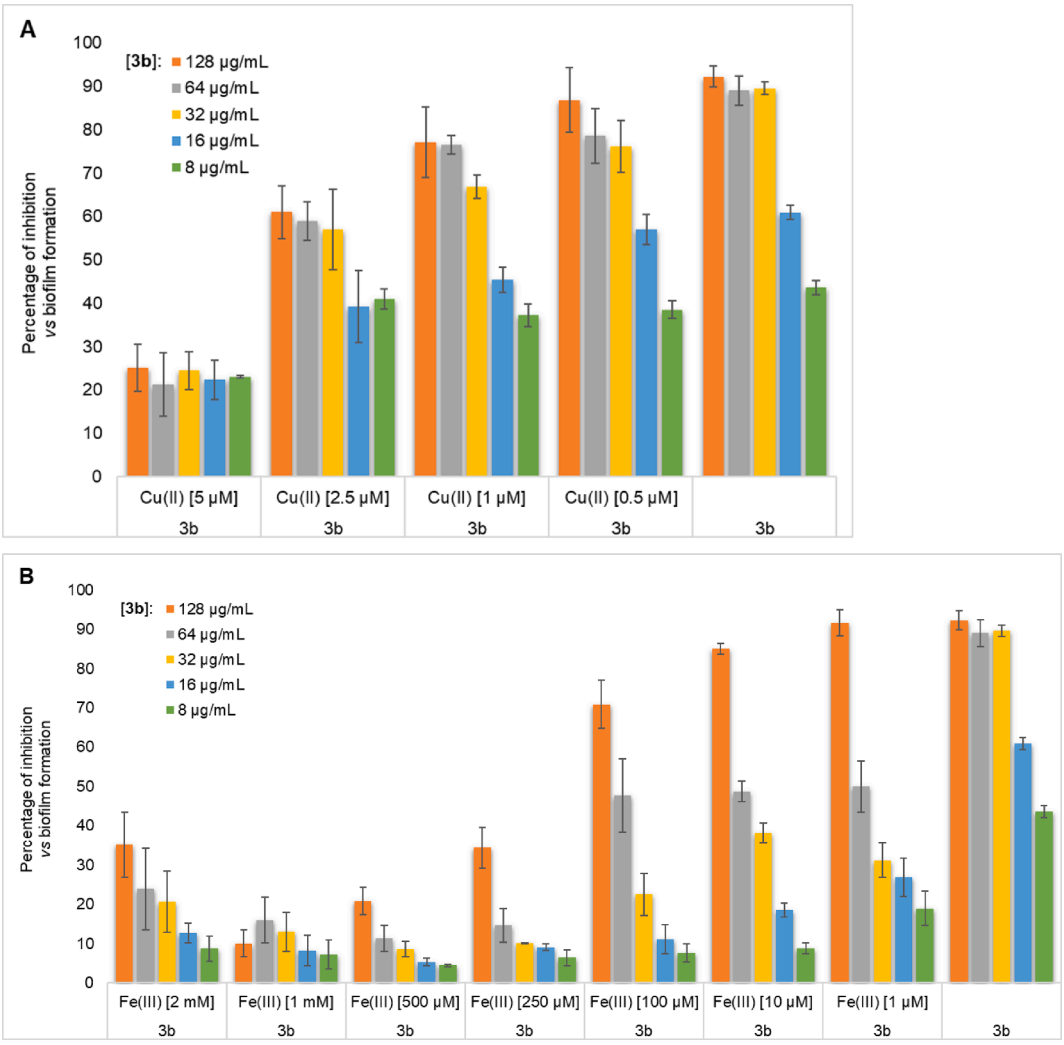


Fig. 1. Inhibition of biofilm formation obtained by compound **3b** in presence of increasing concentration of Cu^{2+} (Panel A) and Fe^{3+} (Panel B). Data are reported as percentage of inhibition ± standard deviation.

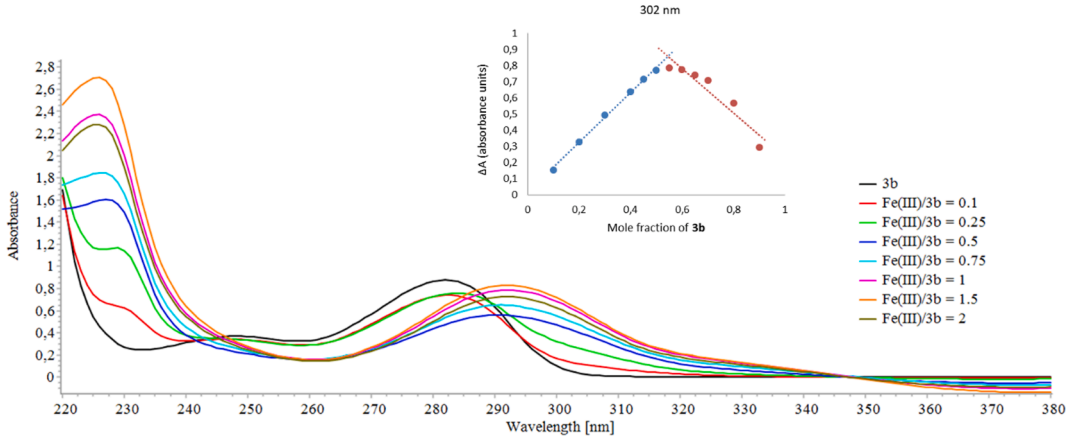


Fig. 2. The UV titration spectra of ligand **3b** with Fe^{3+} and the related Job's plot obtained at 302 nm.

activity may be related to the chelating properties of **3b** observed in UV spectrophotometric tests. Among the amide derivatives **7a,b**-**10a,b**, compounds **9a** and **10a** resulted moderately active against biofilm formation, whereas **9b** and **10b** showed an interesting activity on planktonic cells of *C. albicans*; in this case, the mechanism of action could be related to the combination of the chelating effects of DFP moiety with the antifungal activity reported in the literature for some NSAIDs.

These data, together with the very low toxicity shown *in vivo* by **3b**, suggest that this compound can be considered as an interesting hit compound for the development of new metal chelating agents with

Table 2
In vivo toxicity of compound **3b** observed on *Galleria mellonella* larvae.

3b (μg/mL) [a]	*G. mellonella* survival (%) [b]		
	24 h	48 h	72 h
512	100	96	86
256	100	100	100
128	100	100	100
64	100	100	100
32	100	100	100

[a] Compound was dissolved in sterile water (containing less than 1% v/v of DMSO) and 20 μL was injected into the last, left pro-leg, of the *G. mellonella* larvae. [b] Results represent the mean percentage of survival of *G. mellonella* larvae, of three independent experiments, as a function of the administered dosage.

antibiofilm properties.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2021.128087. These data include MOL files and InChiKeys of the most important compounds described in this article.

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