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Bioorganic & Medicinal Chemistry 12 (2004) 5465–5483
Bioorganic & Medicinal Chemistry

Synthesis, antimicrobial activity and molecular modeling studies of halogenated 4-[1*H*-imidazol-1-yl(phenyl)methyl]-1,5-diphenyl-1*H*-pyrazoles

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Received 19 March 2004; accepted 13 July 2004
Available online 1 September 2004

Abstract—During the course of our studies in the azole antifungals area, we synthesized a number of 1,5-disubstituted 4-[1*H*-imidazol-1-yl(phenyl)methyl]-1*H*-pyrazoles, analogues of bifonazole. 1,5-Diphenyl-1*H*-pyrazole **3** showed weak antimycotic and antibacterial activities in vitro against *Candida albicans*, *Cryptococcus neoformans* and *Staphylococcus aureus*. In order to increase these properties, given that the halo substitution was found to be capable of enhancing antifungal effects, we prepared a series of fluoro and chloro derivatives of **3**. The microbiological evaluation carried out on newly synthesized compounds included in vitro assays for antifungal, antibacterial and antimycobacterial activities. Among the tested compounds, some dichloro and trichloro-derivatives showed interesting antimicrobial properties. In particular, compounds **10j**,**k**,**l** produced inhibitory effects against pathogen representatives of yeast (*C. albicans*, *C. neoformans*) and Gram positive bacteria (*S. aureus*) similar or superior to those of bifonazole. In addition, their activity against *Mycobacterium tuberculosis* was superior to that of clotrimazole and econazole, which were used as reference drugs. The replacement, in these compounds, of chlorine with fluorine atoms led to inactive derivatives. Docking studies were carried out on the most active compounds, in order to rationalize the pharmacological results.

1. Introduction

Fungi usually cause infections on body surfaces, although occasionally they may cause systemic infections. The latter generally occur in immunosuppressed subjects (AIDS patients or patients receiving anticancer therapy) who are especially susceptible because their immune system is not functioning normally.

In recent years the widespread use of antifungal agents has resulted in the development of resistance to these drugs by pathogenic microorganisms, causing an increase in morbidity and mortality. Therefore, new therapeutic options are required.

At the same time, *Mycobacterium tuberculosis* is a re-emerging pathogen both in developed and developing countries. In some areas, the human immunodeficiency virus (HIV) infection is primarily responsible for the global resurgence of tuberculosis. In fact, 50% of people living with HIV/AIDS in Africa are affected by tuberculosis and about one third of the 40 million HIV-infected people worldwide are co-infected with *M. tuberculosis*.[1] This increased risk of developing the infection and the emergence of multi-drug resistant mutants are dictating new approaches in the treatment of mycobacterial disease.

The potential benefit of drugs endowed with both antimycobacterial and antifungal properties represents a valuable goal, since the association between

Keywords: Azoles; Bifonazole analogues; Antifungals; Antimycobacterials.
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doi:10.1016/j.bmc.2004.07.029

mycobacterial and mycotic infections often occurs in immunocompromised patients.

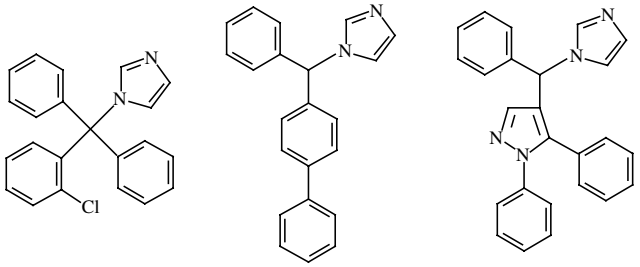
In recent years the class of azoles (imidazoles and triazoles) has supplied many effective antifungal agents, which are currently in clinical use; drugs such as fluconazole and voriconazole can be used to treat generalized systemic mycoses. The mechanism of their antifungal action includes the inhibition of cytochrome P450 51 (CYP51), which is essential for ergosterol biosynthesis at the step of lanosterol-14-demethylation.[2]

Recent studies have demonstrated that selected azole drugs are very potent antimycobacterial agents, which show inhibitory concentrations in the nanomolar range. A soluble protein from *M. tuberculosis* (MT-CYP51), similar in sequence to CYP51 isozymes, has been cloned, expressed and purified. Several azole compounds with known antifungal activity proved to bind MT-CYP51 with high affinity and to impair the growth of *Mycobacterium bovis* and *Mycobacterium smegmatis*, two mycobacterial species which closely resemble *M. tuberculosis*.[2] Clotrimazole and econazole, in particular, were reported to be more effective than rifampin and isoniazid against *M. smegmatis*.[3]

More recently, the expression, characterization and crystallization of a novel cytochrome P450 (CYP121) from *M. tuberculosis* has been reported.[4,5] MT-CYP121 binds commercially available azole drugs with a higher affinity than MT-CYP51. P450 drug-binding titrations and gene-knockout studies suggested that MT-CYP121 may be the true target for the antimycobacterial activity of the azoles in vivo, even though its physiological role remains unclear.[6]

These studies may lead to the development of a new generation of azole antimycotic compounds that may prove effective against multi-drug resistant strains of the tuberculosis pathogen.[3,5]

The high affinity of the azole antifungals as clotrimazole (Fig. 1: **1**) for MT-CYP121 appears to be determined primarily by their bulky, polycyclic structure, giving favourable interactions with the hydrophobic residues in the largely apolar active site of the enzyme.[3]



1 **2** **3**

Figure 1. Structures of chlotrimazole (**1**), bifonazole (**2**) and 1,5-diphenylpyrazole analogue of bifonazole (**3**).

Bifonazole (**2**) derives from the structure of clotrimazole but, unlike the parent compound, it contains no halogen atoms. It is a broad-spectrum antimycotic, employed in the therapy of various dermatomycoses. Recent studies performed on some imidazole antifungal drugs displayed other biological properties of bifonazole, such as antiproliferative action on melanoma cells[7] and an inhibitory effect on prostanoid biosynthesis in various cell systems.[8]

Since the discovery of bifonazole, many modifications on its structure were carried out, so as to increase its antifungal potency and selectivity and to improve its chemical physical properties. Among other changes, several bioisosteric replacements of either or both benzene rings, constituting the biphenyl moiety of the molecule, have been investigated.[9]

Several pyrazole,[10] isoxazole and pyrimidine[11] heteroanalogues of bifonazole were synthesized and tested for their antifungal and antibacterial activities, in previous research carried out in our laboratory; among these, the 1,5-diphenylpyrazole **3** (Fig. 1) showed weak antifungal and antibacterial properties.[10]

Given that in many studies the halo substitution was found to be capable of enhancing both antifungal and antimycobacterial activities,[12–14] we decided to synthesize a number of fluoro and chloro derivatives of compound **3**, extending the biological investigation to the antimycobacterial activity too. Fluorine or chlorine atoms are present as common substituents on aromatic rings, in the most potent antifungal azoles, as fluconazole, voriconazole, ketoconazole, itraconazole and others.

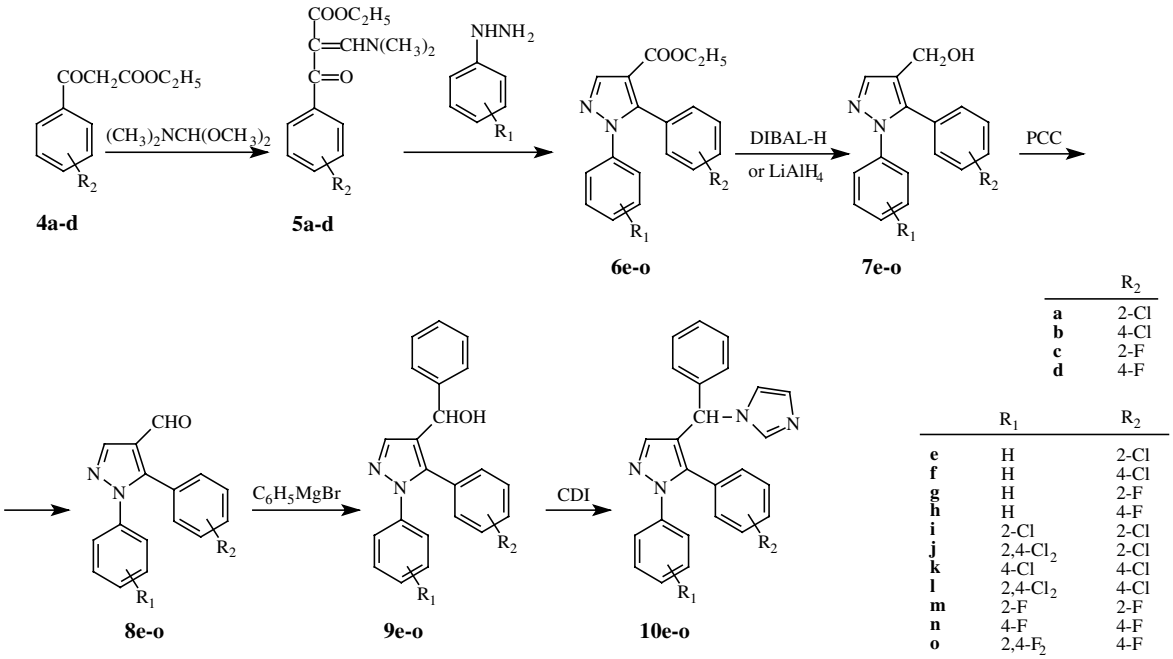
Thus, in this paper we are presenting the synthesis, preliminary microbiological evaluation (antimycotic, antibacterial and antimycobacterial activities) and molecular modeling studies (docking into the active site of cytochrome P450 from *M. tuberculosis* MT-CYP121) of halogenated pyrazole analogues of bifonazole **10e–u**.

2. Results and discussion

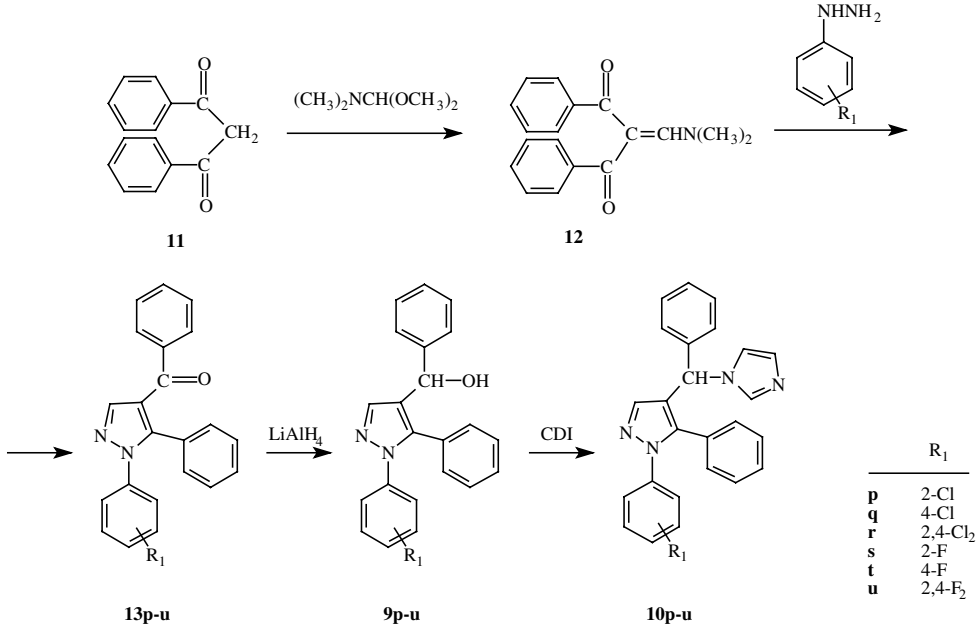
2.1. Chemistry

Compounds **10e–u** were synthesized as depicted in Schemes 1 and 2.

1,5-Diarylpyrazoles **10e–o** were obtained following the same synthetic pathway described for the compound **3**[10] (Scheme 1): treatment of ethyl aroylacetates **4a–d**[15] with *N*,*N*-dimethylformamide dimethylacetal provided α-dimethylaminomethylene β-ketoesters **5a–d**,[16] which were condensed with appropriated arylhydrazines to afford pyrazole esters **6e–o**. Following reduction by lithium aluminium hydride in diethyl ether (**6k**,**n**) or by diisobutylaluminium hydride (DIBAL-H) in toluene (**6e–j**,**l**,**m**,**o**) gave alcohols **7e–o**, which were oxidized to the corresponding aldehydes **8e–o** by pyridinium chlorochromate (PCC) in dichloromethane. Grignard's reac-



Scheme 1. Synthesis of compounds **10e**–**o**.



Scheme 2. Synthesis of compounds **10p**–**u**.

tion of **8e**–**o** with phenylmagnesium bromide led to carbinols **9e**–**o**, which, by reaction with *N*,*N*′-carbonyldiimidazole (CDI), yielded final imidazole compounds **10e**–**o**.

1-Aryl-5-phenylpyrazole derivatives **10p**–**u** were prepared by a shorter pathway (Scheme 2), from 2-dimethylaminomethylene-1,3-diphenylpropane-1,3-dione **12**, an intermediate already used by us to synthesize other pyrazoles.[17] By condensation of **12** with suitable substituted phenylhydrazines, ketones **13p**–**u** were obtained, which, by lithium aluminium hydride reduction, led to carbinols **9p**–**u**. Finally, the reaction of **9p**–**u** with CDI afforded imidazole derivatives **10p**–**u**.

The aim of treating esters **6e**–**j**,**l**,**m**,**o** with DIBAL-H was to obtain directly corresponding aldehydes **8**,[18] but the reduction, even if carried out at a temperature of −78 °C and under nitrogen atmosphere, led to alcohols **7e**–**j**,**l**,**m**,**o**, which were easily isolated from the reaction mixture with high yields. On the contrary, the treatment of the same esters with lithium aluminium hydride produced unsatisfactory results.

It is interesting to emphasize that the ^{1}H NMR spectra of the ethyl esters **6e**,**i**,**j** and of the carbinols **7e**,**i**,**j**, having an *o*-chlorophenyl group at C5 of the pyrazole ring, show complex signals in the δ ranges of 4.05–4.30 and 4.42–4.54 ppm, respectively (Tables 1, 2). These signals correspond to methylene protons belonging to ethoxycarbonyl (esters **6e**,**i**,**j**) or hydroxymethyl (carbinols **7e**,**i**,**j**) group at C4 of the pyrazole ring, respectively. On the contrary, ethyl CH_2 protons of the other esters **6** resonate as a quartet, while the hydroxymethyl CH_2 protons of the other carbinols **7** as a singlet or a doublet (if coupled with OH), as expected (Tables 1, 2).

Moreover, ^{1}H NMR spectra of alcohols **9e**,**i**,**j** (Table 5) and of imidazole derivatives **10e**,**i**,**j** (Table 6) show double signals corresponding to:

- pyrazole H-3, methyne proton and hydroxyl proton of **9e**,**i**,**j**;
- imidazole H-2 and methyne proton of **10e**,**i**,**j**.

These data are in agreement with the existence, in all these cases, of stable conformers, rotationally constrained, due to the presence of both *o*-chlorine atom on the C5 phenyl substituent and bulky groups at pyrazole C4. In fact, ^{1}H NMR spectra of analogues 2-fluorophenyl derivatives (**6g**,**m**, **7g**,**m**, **9g**,**m**, **10g**,**m**) (Tables 1, 2, 5, 6) show regular signals, as well as those of aldehydes **8e**,**i**,**j** (Table 3), in which is evident that the formyl group at pyrazole C4 does not hinder the free rotation of the *o*-chlorophenyl ring at pyrazole C5.

Currently, crystallographic studies are in progress on some significant 2-chlorophenyl derivatives so as to define their spatial arrangement, while preliminary 'in silico' conformational studies on compounds **6e**,**i**,**j**, **7e**,**i**,**j** confirm the hypothesis of two stable conformers, rotationally constrained (Fig. 6).

2.2. Biological properties

The activities of the title compounds were evaluated in vitro against:

(a) Representative human pathogenic fungi (*Candida albicans*, *Cryptococcus neoformans*, *Aspergillus fumigatus*);
(b) Gram positive (*Staphylococcus aureus*) and Gram negative (*Salmonella* spp.) bacteria;
(c) Several strains of *Mycobacteria*: *M. smegmatis* ATCC 19420, *M. fortuitum* (clinical isolate), *M. tuberculosis* ATCC 27294 (wild type), *M. tuberculosis* ATCC 35820 (Str.^{R}), *M. tuberculosis* ATCC 35822 (Ison.^{R}), *M. tuberculosis* ATCC 35828 (Pyr.^{R}), *M. tuberculosis* ATCC 35837 (Eth.^{R}).

Only antimycobacterial activity of the parent compound **3** was investigated here; its antifungal and antibacterial properties had formerly been evaluated.[10]

All title compounds were also evaluated at random for antiretroviral activity in a lymphoid cell line (MT-4) infected with HIV-1.

In vitro cytotoxicity in MT-4 cells, carried out in parallel with anti-HIV-1 activity, was performed in order to determine whether the compounds were endowed with selective antimicrobial/antiviral activity.

Miconazole, bifonazole and amphotericin B were used as reference compounds in antimycotic assays. Streptomycin and AZT were used in antibacterial and antiretroviral assays, respectively.

Clotrimazole and econazole were used as reference drugs in antimycobacterial assays.

The results of biological tests are summarized in Tables 7 and 8; compounds completely devoid of antimicrobial properties are not reported.

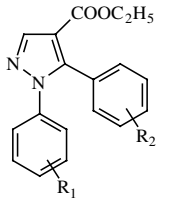
None of the tested compounds was capable of protecting MT-4 cells from the cytopathic effect induced by HIV-1, at least in concentrations, which were lower than the cytotoxic ones.

When tested against yeast and mold representatives, chloro derivatives **10j–l** showed an appreciable activity against both *C. albicans* (MIC = MFC values, ranging between 11.1 and 66.6 μM) and *C. neoformans* (ratio MIC/MFC = 11.1/33.3 μM for all three compounds) (Table 7). Derivative **10l** displayed the best activity, three times superior to that of bifonazole and twice that of the parent compound **3**, with a ratio MIC/MFC = 11.1/11.1 μM towards *C. albicans* and with a ratio MIC/MFC = 11.1/33.3 μM towards *C. neoformans*. Compound **10r** was active only against *C. neoformans* with a ratio MIC/MFC = 33.3/33.3. None of the tested compounds showed significant inhibitory activity against *A. fumigatus*, as well as bifonazole.

With regard to antibacterial activity, compounds **10j**,**k**,**l** turned out to be weakly efficacious against representative Gram positive bacteria (*S. aureus*) whereas other tested derivatives showed no activity; all compounds were inactive towards representative Gram negative bacteria (*Salmonella* spp).

The results of the in vitro antimycobacterial activity are reported in Table 8 (derivatives **10i**,**p** were not cited, because lacking in any antimycobacterial effect). In particular, **10j** emerged as the most potent derivative, also against *M. tuberculosis* strains resistant to drugs commonly employed in the antitubercular therapy, showing MIC values ranging between 6.7 and 57.7 μM. All compounds, except **10j**, were inactive against *M. fortuitum*, as well as clotrimazole and econazole, used as reference azole drugs. Among *M. tuberculosis* strains used for biological assays, ATCC 27294 (wt) was generally the most susceptible to the majority of tested compounds: the lowest values of MIC towards this strain were shown by the three derivatives **10j–l** (MIC = 6.7, 6.1, 6.7 μM, respectively) (Table 8), which showed the highest activity in antimycotic assays too. Compounds **3**, **10e**,**r** exhibited moderate activity against all *M. tuberculosis* strains.

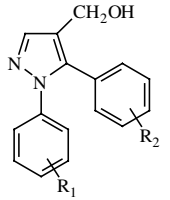
Table 1. Chemical, physical, IR and ^{1}H NMR spectral data of compounds **6e–o**



Compound	R_1	R_2	Yield (%)	Mp (°C)	Molecular formula	Analyses% calcd/found			IR (cm^{-1}) ($CHCl_3$)	^{1}H NMR, δ ($CDCl_3$)
						C	H	N		
6e	H	2-Cl	83	80–81[a]	$C_{18}H_{15}ClN_2O_2$	66.16	4.63	8.57	1708	1.15 (t, J = 7.1, 3H, CH_3), 4.05–4.3 (m, 2H, CH_2), 7.2–7.45
						66.18	4.52	8.76		(m, 9H, ar), 8.21 (s, 1H, H-3 pyr)
6f	H	4-Cl	83	102–103[a]	$C_{18}H_{15}ClN_2O_2$	66.16	4.63	8.57	1715	1.26 (t, J = 7.1, 3H, CH_3), 4.23 (q, J = 7.1, 2H, CH_2),
						66.13	4.84	8.78		7.15–7.4 (m, 9H, ar), 8.18 (s, 1H, H-3 pyr)
6g	H	2-F	93	79–80[b]	$C_{18}H_{15}FN_2O_2$	69.67	4.87	9.02	1712	1.20 (t, J = 7.1, 3H, CH_3), 4.20 (q, J = 7.1, 2H, CH_2),
						69.61	4.71	9.00		7.0–7.5 (m, 9H, ar), 8.22 (s, 1H, H-3 pyr)
6h	H	4-F	89	114–115[b]	$C_{18}H_{15}FN_2O_2$	69.67	4.87	9.02	1712	1.25 (t, J = 7.1, 3H, CH_3), 4.22 (q, J = 7.1, 2H, CH_2),
						69.41	4.81	8.95		6.95–7.1 (m, 2H, ar), 7.15–7.35 (m, 7H, ar), 8.19 (s, 1H, H-3 pyr)
6i	2-Cl	2-Cl	91	83–84[b]	$C_{18}H_{14}Cl_2N_2O_2$	59.85	3.91	7.75	1710	1.14 (t, J = 7.1, 3H, CH_3), 4.05–4.3 (m, 2H, CH_2),
						60.00	3.98	7.77		7.1–7.45 (m, 8H, ar), 8.25 (s, 1H, H-3 pyr)
6j	2,4-Cl_2	2-Cl	91	102–103[a]	$C_{18}H_{13}Cl_3N_2O_2$	54.64	3.31	7.08	1705	1.14 (t, J = 7.1, 3H, CH_3), 4.05–4.3 (m, 2H, CH_2),
						54.79	3.20	7.18		7.15–7.45 (m, 7H, ar), 8.25 (s, 1H, H-3 pyr)
6k	4-Cl	4-Cl	87	130–131[c]	$C_{18}H_{14}Cl_2N_2O_2$	59.85	3.91	7.75	1708	1.25 (t, J = 7.1, 3H, CH_3), 4.22 (q, J = 7.1, 2H, CH_2),
						59.68	3.92	7.76		7.1–7.4 (m, 8H, ar), 8.17 (s, 1H, H-3 pyr)
6l	2,4-Cl_2	4-Cl	89	103–104[d]	$C_{18}H_{13}Cl_3N_2O_2$	54.64	3.31	7.08	1708	1.26 (t, J = 7.1, 3H, CH_3), 4.24 (q, J = 7.1, 2H, CH_2),
						54.90	3.01	7.17		7.15–7.35 (m, 6H, ar), 7.43 (s, 1H, ar), 8.21 (s, 1H, H-3 pyr)
6m	2-F	2-F	88	85–86[b]	$C_{18}H_{14}F_2N_2O_2$	65.85	4.30	8.53	1708	1.20 (t, J = 7.1, 3H, CH_3), 4.21 (q, J = 7.1, 2H, CH_2), 6.95–7.45 (m, 8H, ar),
						65.70	4.11	8.55		8.26 (s, 1H, H-3 pyr)
6n	4-F	4-F	84	110–111[d]	$C_{18}H_{14}F_2N_2O_2$	65.85	4.30	8.53	1705	1.25 (t, J = 7,1, 3H, CH_3), 4.22 (q, J = 7,1, 2H, CH_2), 6.95–7.1 (m, 4H, ar),
						65.71	4.25	8.69		7.15–7.3 (m, 4H, ar), 8.17 (s, 1H, H-3 pyr)
6o	2,4-F_2	4-F	88	78–79[b]	$C_{18}H_{13}F_3N_2O_2$	62.43	3.78	8.09	1707	1.24 (t, J = 7,1, 3H, CH_3), 4.23 (q, J = 7,1, 2H, CH_2), 6.75–7.05 (m, 4H, ar),
						62.68	ND[e]	8.13		7.2–7.45 (m, 3H, ar), 8.20 (s, 1H, H-3 pyr)

[a] From anhydrous diethyl ether.
[b] From anhydrous diethyl ether/petroleum ether (bp 40–60 °C).
[c] From 95% ethanol.
[d] From petroleum ether (bp 40–60 °C).
[e] ND = not determined.

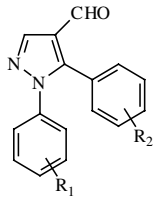
Table 2. Chemical, physical, IR and ^{1}H NMR spectral data of compounds **7e–o**



Compound	R_1	R_2	Yield (%)	Bp (°C/mmHg) or mp (°C)	Molecular formula	Analyses% calcd/found			IR (cm^{-1}) ($CHCl_3$)	^{1}H NMR, δ ($CDCl_3$)
						C	H	N		
7e	H	2-Cl	96	165–170/0.25	$C_{16}H_{13}ClN_2O$	67.49 67.31	4.60 4.92	9.84 9.50	3605, 3380	1.82 (s, 1H, OH, ex), 4.42 and 4.52 (2d, J = 12.5, 2H, CH_2), 7.3–7.5 (m, 9H, ar), 7.85 (s, 1H, H-3 pyr)
7f	H	4-Cl	98	140–141[a]	$C_{16}H_{13}ClN_2O$	67.49 67.20	4.60 4.79	9.84 9.82	3605, 3350	~1.9 (very br s, 1H, OH, ex), 4.53 (s, 2H, CH_2), 7.15–7.35 (m, 9H, ar), 7.79 (s, 1H, H-3 pyr)
7g	H	2-F	90	77–78[a]	$C_{16}H_{13}FN_2O$	71.63 71.54	4.88 5.02	10.44 10.56	3610, 3380	1.73 (t, J = 5.6, 1H, OH, ex), 4.52 (d, J = 5.6, 2H, CH_2, became s with D_2O), 7.0–7.45 (m, 9H, ar), 7.86 (s, 1H, H-3 pyr)
7h	H	4-F	71	99–100[a]	$C_{16}H_{13}FN_2O$	71.63 71.40	4.88 5.10	10.44 10.53	3610, 3370	1.73 (t, J = 5.1, 1H, OH, ex), 4.54 (d, J = 5.1, 2H, CH_2, became s with D_2O), 6.95–7.1 (m, 2H, ar), 7.15–7.4 (m, 7H, ar), 7.81 (s, 1H, H-3 pyr)
7i	2-Cl	2-Cl	83	84–86[b]	$C_{16}H_{12}Cl_2N_2O$	60.20 60.34	3.79 4.00	8.78 8.69	3600, 3370	1.92 (s, 1H, OH, ex), 4.45 and 4.54 (2d, J = 12.5, 2H, CH_2), 7.1–7.4 (m, 8H, ar), 7.88 (s, 1H, H-3 pyr)
7j	2,4-Cl_2	2-Cl	88	85–87[b]	$C_{16}H_{11}Cl_3N_2O$	54.34 54.24	3.13 3.19	7.92 7.90	3595, 3360	1.73 (br s, 1H, OH, ex), 4.46 and 4.53 (2d, J = 12.6, 2H, CH_2), 7.15–7.45 (m, 7H, ar), 7.90 (s, 1H, H-3 pyr)
7k	4-Cl	4-Cl	92	135–136[b]	$C_{16}H_{12}Cl_2N_2O$	60.20 60.24	3.79 3.77	7.78 7.84	3610, 3370	~1.9 (very br s, 1H, OH, ex), 4.52 (s, 2H, CH_2), 7.1–7.4 (m, 8H, ar), 7.79 (s, 1H, H-3 pyr)
7l	2,4-Cl_2	4-Cl	92	118–119[a]	$C_{16}H_{11}Cl_3N_2O$	54.34 54.25	3.13 3.15	7.92 8.07	3595, 3330	1.82 (br s, 1H, OH, ex), 4.56 (s, 2H, CH_2), 7.16 (d, J = 8.4, 2H, ar), 7.27 (s, 2H, ar), 7.30 (d, J = 8.4, 2H, ar), 7.43 (s, 1H, ar), 7.84 (s, 1H, H-3 pyr)
7m	2-F	2-F	74	83–85[b]	$C_{16}H_{12}F_2N_2O$	67.13 66.92	4.23 4.01	9.78 9.83	3605, 3380	1.58 (t, J = 5.3, 1H, OH, ex), 4.55 (d, J = 5.3, 2H, CH_2, became s with D_2O), 6.5–6.8 (m, 8H, ar), 7.91 (s, 1H, H-3 pyr)
7n	4-F	4-F	92	112–113[a]	$C_{16}H_{12}F_2N_2O$	67.13 67.20	4.22 4.40	9.78 9.64	3600, 3360	1.89 (t, J = 4.7, 1H, OH, ex), 4.52 (d, J = 4.7, 2H, CH_2), 6.9–7.1 (m, 4H, ar), 7.15–7.3 (m, 4H, ar), 7.78 (s, 1H, H-3 pyr)
7o	2,4-F_2	4-F	92	82–83[a]	$C_{16}H_{11}F_3N_2O$	63.16 62.85	3.64 ND[c]	9.21 9.14	3600, 3370	1.73 (br s, 1H, OH, ex), 4.56 (s, 2H, CH_2), 6.75–7.1 (m, 4H, ar), 7.15–7.25 (m, 2H, ar), 7.3–7.45 (m, 1H, ar), 7.84 (s, 1H, H-3 pyr)

[a] From anhydrous diethyl ether/petroleum ether (bp 40–60 °C).
[b] From anhydrous diethyl ether.
[c] ND = not determined.

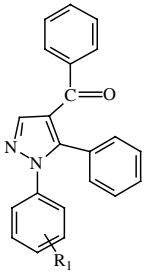
Table 3. Chemical, physical, IR and ^{1}H NMR spectral data of compounds **8e–o**



Compound	R_1	R_2	Yield (%)	Mp (°C)	Molecular formula	Analyses% calcd/found			IR (cm^{-1}) (KBr)	^{1}H NMR, δ ($CDCl_3$)
						C	H	N		
8e	H	2-Cl	88	107–108[a]	$C_{16}H_{11}ClN_2O$	67.97 67.74	3.92 3.92	9.90 9.85	1680	7.2–7.5 (m, 9H, ar), 8.26 (s, 1H, H-3 pyr), 9.65 (s, 1H, CHO)
8f	H	4-Cl	88	124–125[b]	$C_{16}H_{11}ClN_2O$	67.97 67.86	3.92 4.01	9.90 10.03	1675	7.2–7.45 (m, 9H, ar), 8.24 (s, 1H, H-3 pyr), 9.77 (s, 1H, CHO)
8g	H	2-F	95	104–105[b]	$C_{16}H_{11}FN_2O$	72.17 72.40	4.16 4.31	10.52 10.38	1672	7.05–7.55 (m, 9H, ar), 8.27 (s, 1H, H-3 pyr), 9.73 (s, 1H, CHO)
8h	H	4-F	93	149–150[b]	$C_{16}H_{11}FN_2O$	72.17 72.01	4.16 3.92	10.52 10.63	1678	7.05–7.45 (m, 9H, ar), 8.24 (s, 1H, H-3 pyr), 9.76 (s, 1H, CHO)
8i	2-Cl	2-Cl	97	116–117[b]	$C_{16}H_{10}Cl_2N_2O$	60.59 60.90	3.18 3.20	8.83 9.04	1680	7.2–7.5 (m, 8H, ar), 8.29 (s, 1H, H-3 pyr), 9.68 (s, 1H, CHO)
8j	2,4-Cl_2	2-Cl	91	163–164[a]	$C_{16}H_9Cl_3N_2O$	54.65 54.46	2.58 2.56	7.97 7.99	1682	7.2–7.5 (m, 7H, ar), 8.29 (s, 1H, H-3 pyr), 9.67 (s, 1H, CHO)
8k	4-Cl	4-Cl	95	134–135[c]	$C_{16}H_{10}Cl_2N_2O$	60.59 60.53	3.18 3.27	8.83 8.99	1676	7.15–7.5 (m, 8H, ar), 8.22 (s, 1H, H-3 pyr), 9.75 (s, 1H, CHO)
8l	2,4-Cl_2	4-Cl	92	137–138[b]	$C_{16}H_9Cl_3N_2O$	54.65 54.72	2.58 2.44	7.97 7.93	1678	7.15–7.5 (m, 7H, ar), 8.27 (s, 1H, H-3 pyr), 9.79 (s, 1H, CHO)
8m	2-F	2-F	91	141–142[a]	$C_{16}H_{10}F_2N_2O$	67.60 67.67	3.54 3.30	9.85 9.89	1683	7.0–7.55 (m, 8H, ar), 8.30 (s, 1H, H-3 pyr), 9.76 (s, 1H, CHO)
8n	4-F	4-F	98	117–118[c]	$C_{16}H_{10}F_2N_2O$	67.60 67.41	3.54 3.60	9.85 9.82	1677	7.0–7.35 (m, 8H, ar), 8.22 (s, 1H, H-3 pyr), 9.75 (s, 1H, CHO)
8o	2,4-F_2	4-F	85	98–99[a]	$C_{16}H_9F_3N_2O$	63.58 63.55	3.00 ND[d]	9.27 9.29	1680	6.8–7.55 (m, 7H, ar), 8.26 (s, 1H, H-3 pyr), 9.77 (s, 1H, CHO)

[a] From anhydrous diethyl ether.
[b] From anhydrous diethyl ether/petroleum ether (bp 40–60 °C).
[c] From 95% ethanol.
[d] ND = not determined.

Table 4. Chemical, physical, IR and ^{1}H NMR spectral data of compounds **13p–u**



Compound	R_1	Yield (%)	Mp (°C)	Molecular formula	Analyses% calcd/found			IR (cm^{-1}) ($CHCl_3$)	^{1}H NMR, δ ($CDCl_3$)
					C	H	N		
13p	2-Cl	62	115–116[a]	$C_{22}H_{15}ClN_2O$	73.64 73.76	4.21 4.39	7.81 7.88	1643	7.15–7.55 (m, 12H, ar), 7.83 (d, J = 6.8, 2H, ar), 8.11 (s, 1H, H-3 pyr)
13q	4-Cl	79	155–156[b]	$C_{22}H_{15}ClN_2O$	73.64 73.45	4.21 4.34	7.81 7.78	1645	7.15–7.55 (m, 12H, ar), 7.80 (d, J = 6.8, 2H, ar), 8.05 (s, 1H, H-3 pyr)
13r	2,4-Cl_2	70	157–158[b]	$C_{22}H_{14}Cl_2NO$	67.19 67.03	3.59 3.57	7.12 7.23	1640	7.15–7.55 (m, 11H, ar), 7.82 (d, J = 6.8, 2H, ar), 8.10 (s, 1H, H-3 pyr)
13s	2-F	68	145–146[b]	$C_{22}H_{15}FN_2O$	77.18 77.17	4.41 4.48	8.18 8.14	1642	6.95–7.1 (m, 2H, ar), 7.2–7.6 (m, 10H, ar), 7.81 (d, J = 6.8, 2H, ar), 8.04 (s, 1H, H-3 pyr)
13t	4-F	81	144–145[a]	$C_{22}H_{15}FN_2O$	77.18 76.81	4.41 4.21	8.18 8.13	1640	6.95–7.1 (m, 2H, ar), 7.2–7.6 (m, 10H, ar), 7.81 (d, J = 6.8, 2H, ar), 8.02 (s, 1H, H-3 pyr)
13u	2,4-F_2	68	130–131[b]	$C_{22}H_{14}F_2N_2O$	73.33 73.66	3.92 3.63	7.77 7.89	1643	6.8–7.0 (m, 2H, ar), 7.2–7.6 (m, 9H, ar), 7.81 (d, J = 6.8, 2H, ar), 8.09 (s, 1H, H-3 pyr)

[a] From anhydrous diethyl ether/petroleum ether (bp 40–60 °C).
[b] From anhydrous diethyl ether.

As reported in Table 8, MIC values of tested compounds appear higher than those related to the common antitubercular agents, used as reference drugs, but lower or comparable to values of the azole antifungal drug econazole. Clotrimazole, furthermore, was active only towards the strain *M. tuberculosis* ATCC 27294 (wt), moreover with a MIC value higher than those of **10j–l**,**q**,**r**.

With regard to in vitro cytotoxycity assays, all chloro derivatives were cytotoxic for MT-4 cells, at concentrations near the active doses.

All fluoro derivatives, on the contrary, showed very low cytotoxicity (CC50 > 100 μM), but produced poor biological effects. As already observed in other similar cases,[22] replacing chlorine with fluorine atoms led to a dramatic decrease in activity. All fluoro derivatives were inactive against the fungi or bacteria tested, but **10m–o** and **10s–u** produced a moderate inhibition of the growth of *M. smegmatis* ATCC 19420 and *M. tuberculosis* ATCC 27294 (wt) (MIC values ranging from 48.9 to 88.2 μM and from 21.1 to 65.3 μM, respectively).

The results of the preliminary biological tests thus pointed out that the introduction of halo substituents on the phenyl rings linked to the pyrazole nucleus of compound **3**, yielded modifications in the microbiological profile. In particular, derivatives bearing 2 or 3 chlorine atoms emerged as the most interesting for antifungal, antibacterial and antimycobacterial activities, in accordance with literature.[12–14] Moreover, as already observed,[12] the number of chloro substituents rather than their position on phenyl rings is the major determinant for antimicrobial activities. Monochloro and all fluoro derivatives are inactive as antimycotics, whereas a number of them showed some antimycobacterial activity. In particular, the monochloro derivative **10e** exhibited appreciable inhibitory effects against all *Mycobacteria* strains tested, except *M. fortuitum*, but it also proved to be highly toxic in the assay on the MT-4 cells.

2.3. Molecular modeling studies

In order to further rationalize the biological results, a molecular docking study was performed on compound **10j**, the most active antimycobacterial agent, endowed with a significant antifungal activity. The compound was docked into the active site of cytochrome P450 from *M. tuberculosis*,[19] MT-CYP121, using FlexX.[20] FlexX is a widely used docking algorithm in drug design whose ability in predicting a conformation of the ligand very close to its X-ray structure has been extensively reviewed in literature.[21]

According to the results of our docking experiments, compound **10j** could bind to the cytochrome catalytic site orienting the nitrogen in position 2 of pyrazole ring towards the iron atom of the haem group. Previous docking studies reported by Munro et al. for the binding of miconazole and clotrimazole with MT-CYP121, put in evidence a steric clash that could be present between the ligand and the Arg386 side chain[6] if the azole nucleus was positioned perpendicularly to the iron atom. On the contrary, FlexX proposes a binding model where **10j** is shifted so as to avoid any negative interaction with Arg386, while a favourable π–π interaction is present between the phenyl ring of substituent on position 4 and Phe280 (Fig. 2A). Since Munro described a positive interaction between clotrimazole and side chain of Phe280, this residue seems to play an important role in the binding of azole drugs. Only site-directed mutagenesis experiments, however, will be able to confirm this hypothesis.

Interestingly, in the case of docking studies performed with **10j** and MT-CYP51,[23] which shares a significative structure similarity with *C. albicans* CYP51 (CA-CYP51),[24] the binding model obtained was fully comparable to the bioactive conformation of fluconazole into the crystallized complex, thus demonstrating the reliability of the selected computational methodology.

With the aim of investigating which molecular characteristics could affect the differences in biological responses of chlorinated versus fluorinated derivatives, a docking study was also performed on **10o**, one of the less active fluorinated compounds. Figure 2B shows the different pose of **10o** into the catalytic pocket, in comparison with **10j**. A superimposition of FlexX solutions for **10j** and **10o** underlined that conformers of **10j** occupy in the catalytic area different regions than conformers

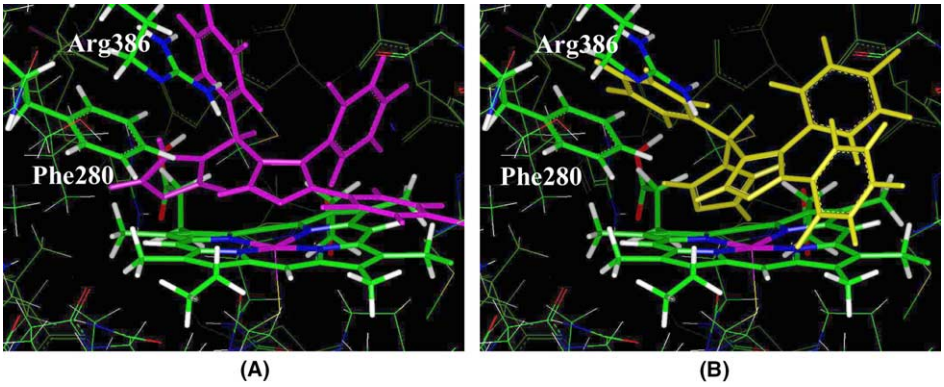


Figure 2. Binding mode proposed for **10j** (A) and **10o** (B) into the catalytic site of MT-CYP121. For the sake of clarity, only few amino acids of the macromolecule are displayed as sticks.

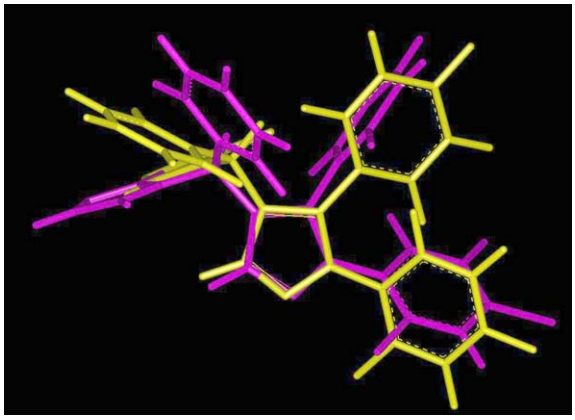


Figure 3. A superimposition of the best FlexX docking solution for **10j** (magenta) and for **10o** (yellow).

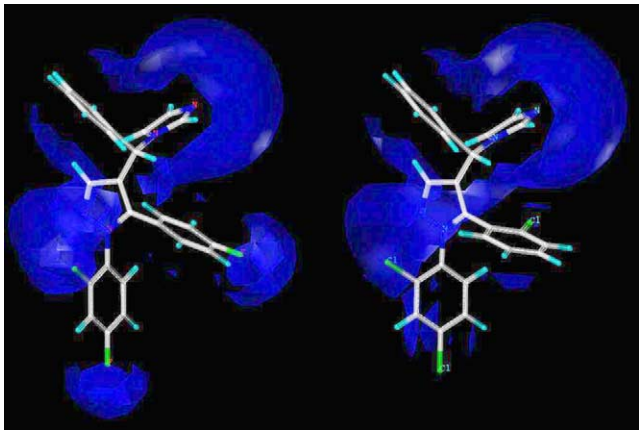


Figure 4. Isopotential contour maps for **10o** (left) and **10j** (right). Only negative potentials have been represented, coloured in blue. The cut has been done at −4.00 level.

of **10o**, due to their different conformational freedom. In Figure 3 the best FlexX solutions for **10j** and **10o** are depicted. This finding could suggest that the rotationally constrained **10j** could have some specific interactions with MT-CYP121, and thus a different degree of activity. In addition, isopotential contour maps evaluation (Fig. 4), highlights higher values of negative charge for fluorine atom, in comparison with chlorine one. This marked negative potential could be responsible of unfavourable interactions with the macromolecule and, consequently, of the reduced activity of fluorinated compounds.

Furthermore, so as to support the presence of two stable conformers for compounds **6e**,**i**,**j**, **7e**,**i**,**j**, **9e**,**i**,**j** and **10e**,**i**,**j** such as proposed by the ^{1}H NMR data, a conformational analysis based on a systematic molecular mechanics search was carried out on these structures (Fig. 5).

The energy profiles for the rotation of the angles $\tau 1$ and $\tau 2$ were calculated and the absolute minima plus local minima were found for all mentioned compounds. $\tau 1$, $\tau 2$ and corresponding energy values are reported in Table 9.

Ramachandran plots for compounds **6e**,**i**,**j** identify two couples of conformers, **I** and **II**, separated by an energy barrier of about 95 kJ/mol (around 25 kcal/mol), which do not convert one into the other. Conformers **I** (namely **Ia** and **Ib**) and conformers **II** (namely **IIa** and **IIb**) could instead easily convert one into the other. In the case of compounds **7e**,**i**,**j**, **9e**,**i**,**j** and **10e**,**i**,**j**, the absolute minima, located in a large isoenergetical area of the plot, are separated from a specular large isoenergetical area, the relative minima, by an energy barrier of about 95 kJ/mol. In Figure 6 are reported, as an example, the relevant conformers and the energy profiles for the rotation of $\tau 1$ and $\tau 2$ in **6j** and **7j**.

These calculations support the hypothesis of two stable and distinct conformations at room temperature for compounds **6e**,**i**,**j**, **7e**,**i**,**j**, **9e**,**i**,**j** and **10e**,**i**,**j**. However, only experimental data from crystallographic studies (in progress) will fully elucidate what are the conformers ratio at room temperature and the structure of the crystal of the above mentioned compounds.

3. Conclusion

In conclusion, we have synthesized and evaluated in vitro the antimicrobial activity of chloro and fluoro derivatives **10e**–**u**, in order to improve the antimycotic and antibacterial activities of 4-[1*H*-imidazol-1-yl(phenyl)methyl]-1,5-diphenyl-1*H*-pyrazole **3**, heteroanalogue of bifonazole.

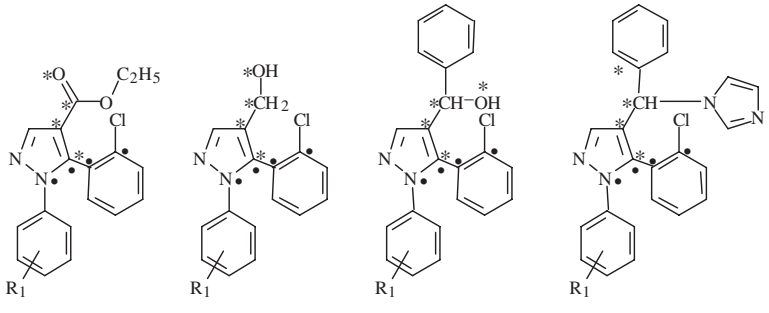
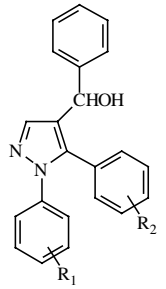


Figure 5. Torsional angles $\tau 1$ (∗) and $\tau 2$ (●) selected for the conformational analysis.

Table 5. Chemical, physical, IR and ^{1}H NMR spectral data of compounds **9e–u**



Compound	R_1	R_2	Yield (%)	Mp (°C)	Molecular formula	Analyses% calcd/found			IR (cm^{-1}) (KBr)	^{1}H NMR, δ ($CDCl_3$)
						C	H	N		
9e	H	2-Cl	71	153–154[a]	$C_{22}H_{17}ClN_2O$	73.23 73.25	4.75 4.62	7.76 7.80	3295	~2.5 (very br s, 1H, OH, ex), 5.63 and 5.65 (2s, 1H, CH-Ph), 7.15–7.4 (m, 14H, ar), 7.59 and 7.77 (2s, 1H, H-3 pyr)
9f	H	4-Cl	84	216–218[a]	$C_{22}H_{17}ClN_2O$	73.23 73.26	4.75 4.76	7.76 7.86	3260	~2.3 (very br s, 1H, OH, ex), 5.70 (s, 1H, CH-Ph), 7.15–7.45 (m, 14H, ar), 7.64 (s, 1H, H-3 pyr)
9g	H	2-F	95	177–178[a]	$C_{22}H_{17}FN_2O$	76.73 76.48	4.97 5.02	8.13 8.24	3275	~2.2 (very br s, 1H, OH, ex), 5.71 (s, 1H, CH-Ph), 7.0–7.45 (m, 14H, ar), 7.70 (s, 1H, H-3 pyr)
9h	H	4-F	88	197–198[a]	$C_{22}H_{17}FN_2O$	76.73 76.72	4.97 4.88	8.13 8.04	3265	~2.1 (very br s, 1H, OH, ex), 5.69 (s, 1H, CH-Ph), 6.95–7.1 (m, 2H, ar), 7.15–7.45 (m, 12H, ar), 7.63 (s, 1H, H-3 pyr)
9i	2-Cl	2-Cl	89	158–159[a]	$C_{22}H_{16}Cl_2N_2O$	66.85 66.69	4.08 3.94	7.09 7.05	3310	2.19 and 2.32 (2d, J = 3, 1H, OH, ex), 5.65–5.75 (m, 1H, CH-Ph, became s with D_2O), 7.1–7.45 (m, 13H, ar), 7.65 and 7.82 (2s, 1H, H-3 pyr)
9j	2,4-Cl_2	2-Cl	67	146–147[b]	$C_{22}H_{15}Cl_3N_2O$	61.49 61.75	3.52 3.62	6.52 6.52	3270	2.32 and 2.39 (2d, J = 3, 1H, OH, ex), 5.6–5.7 (m, 1H, CH-Ph became s with D_2O), 7.05–7.4 (m, 12H, ar), 7.65 and 7.81 (2s, 1H, H-3 pyr)
9k	4-Cl	4-Cl	70	158–159[c]	$C_{22}H_{16}Cl_2N_2O$	66.85 66.70	4.08 4.08	7.09 7.04	3285	2.29 (d, J = 4, 1H, OH, ex), 5.68 (d, J = 4, 1H, CH-Ph, became s with D_2O), 7.1–7.2 (m, 4H, ar), 7.25–7.45 (m, 9H, ar), 7.64 (s, 1H, H-3 pyr)
9l	2,4-Cl_2	4-Cl	76	197–198[b]	$C_{22}H_{15}Cl_3N_2O$	61.49 61.57	3.52 3.33	6.52 6.61	3280	~1.6 (very br s, 1H, OH, ex), 5.74 (s, 1H, CH-Ph), 7.1–7.2 (m, 2H, ar), 7.25–7.45 (m, 10H, ar), 7.70 (s, 1H, H-3 pyr)
9m	2-F	2-F	51	174–175[d]	$C_{22}H_{16}F_2N_2O$	72.92 73.05	4.45 4.77	7.73 7.80	3265	~2.2 (br s, 1H, OH, ex), 5.74 (s, 1H, CH-Ph), 6.95–7.4 (m, 13H, ar), 7.73 (s, 1H, H-3 pyr)
9n	4-F	4-F	69	144–145[c]	$C_{22}H_{16}F_2N_2O$	72.92 72.73	4.45 ND[e]	7.73 7.69	3295	2.25–2.35 (m, 1H, OH, ex), 5.69 (d, J = 3, 1H, CH-Ph, became s with D_2O), 6.9–7.4 (m, 13H, ar), 7.62 (s, 1H, H-3 pyr)
9o	2,4-F_2	4-F	68	145–146[b]	$C_{22}H_{15}F_3N_2O$	69.47 69.66	3.97 ND[e]	7.36 7.45	3305	2.29 (d, J = 4, 1H, OH, ex), 5.73 (d, J = 4, 1H, CH-Ph, became s with D_2O), 7.1–7.2 (m, 2H, ar) 7.25–7.45 (m, 10H, ar), 7.69 (s, 1H, H-3 pyr)
9p	2-Cl	H	91	170–171[a]	$C_{22}H_{17}ClN_2O$	73.23 72.97	4.75 5.05	7.76 7.67	3255	2.22 (d, J = 4, 1H, OH, ex), 5.79 (d, J = 4, 1H, CH-Ph, became s with con D_2O), 7.2–7.5 (m, 14H, ar), 7.70 (s, 1H, H-3 pyr)
9q	4-Cl	H	55	200–201[a]	$C_{22}H_{17}ClN_2O$	73.23 73.58	4.75 4.84	7.76 7.89	3310	2.24 (d, J = 4, 1H, OH, ex), 5.72 (d, J = 4, 1H, CH-Ph, became s with con D_2O), 7.1–7.45 (m, 14H, ar), 7.65 (s, 1H, H-3 pyr)
9r	2,4-Cl_2	H	93	160–161[d]	$C_{22}H_{16}Cl_2NO$	66.85 67.13	4.08 3.92	7.09 7.20	3310	2.35 (d, J = 4, 1H, OH, ex), 5.77 (d, J = 4, 1H, CH-Ph, became s with con D_2O), 7.15–7.45 (m, 13H, ar), 7.69 (s, 1H, H-3 pyr)

(*continued on next page*)

Table 5 (*continued*)

Compound	R_1	R_2	Yield (%)	Mp (°C)	Molecular formula	Analyses% calcd/found			IR (cm^{-1}) (KBr)	^1H NMR, δ ($CDCl_3$)
						C	H	N		
9s	2-F	H	70	162–163[b]	$C_{22}H_{17}FN_2O$	76.73	4.97	8.13	3305	2.25–2.35 (m, 1H, OH, ex), 5.72 (d, $J = 4$, 1H, CH-Ph, became s with D_2O), 6.9–7.05 (m, 2H, ar), 7.15–7.45 (m, 12H, ar), 7.63 (s, 1H, H-3 pyr)
						77.01	5.09	8.22		
9t	4-F	H	79	162–163[b]	$C_{22}H_{17}FN_2O$	76.73	4.97	8.13	3300	2.4–2.6 (m, 1H, OH, ex), 5.7–5.75 (m, 1H, CH-Ph, became s with D_2O), 6.9–7.05 (m, 2H, ar), 7.15–7.45 (m, 12H, ar), 7.61 (s, 1H, H-3 pyr)
						76.58	5.12	8.09		
9u	2,4-F_2	H	59	152–153[c]	$C_{22}H_{16}F_2N_2O$	72.92	4.45	7.73	3285	2.34 (d, $J = 4$, 1H, OH, ex), 5.76 (d, $J = 4$, 1H, CH-Ph, became s with D_2O), 6.7–6.95 (m, 2H, ar), 7.2–7.5 (m, 11H, ar), 7.68 (s, 1H, H-3 pyr)
						73.01	4.43	7.68		

[a] From ethyl acetate.
[b] From anhydrous diethyl ether.
[c] From anhydrous diethyl ether/petroleum ether (bp 40–60 °C).
[d] From ethyl acetate/petroleum ether (bp 40–60 °C).
[e] ND = not determined.

Dichloro and trichloro derivatives **10j**,**k**,**l** emerged as the most interesting compounds, confirming the effectiveness of chloro substituents to enhance antifungal activity.[10–12] These compounds produced inhibitory effects against pathogen representatives of yeast (*C. albicans*, *C. neoformans*) and Gram positive bacteria (*S. aureus*) similar or superior to those of bifonazole. In addition, they showed an antimycobacterial activity against *M. tuberculosis* superior to that of clotrimazole and econazole, which were used as reference drugs.

Computational studies suggest an inhibition of CA-CYP51 and MT-CYP121 as possible mechanism of action for these compounds.

4. Experimental

4.1. Chemistry

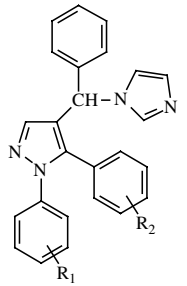
Melting points were determined with a Fisher–Johns apparatus and are uncorrected. IR spectra were registered on a Perkin–Elmer 398 spectrophotometer and are expressed in cm^{-1}. ^1H NMR spectra were registered on a Varian Gemini 200 (200 MHz) spectrometer; chemical shifts are reported as δ values (ppm) relative to TMS as internal standard; coupling constants (J) are expressed in Hertz (Hz). The following NMR abbreviations are used: br (broad), s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), ex (exchangeable with D_2O), ar (aromatic proton), pyr (pyrazole), imid (imidazole). Microanalyses for C, H, N were performed using a Carlo Erba Elemental Analyzer Model EA 1110 and results agree within ±0.4% with calculated values.

4.1.1. General procedure for the synthesis of compounds 6e–o and 13p–u. The appropriate arylhydrazine hydrochloride (11 mmol) was added to a stirred solution of **5a–d** or **12** (10 mmol) in absolute ethanol (50 mL). The mixture was heated to reflux for 2 h and evaporated under reduced pressure. The residue was taken up in chloroform and the organic solution was washed with water, dried (magnesium sulfate), filtered and evaporated in vacuo. The solid residue was purified by recrystallization from suitable solvent.

Yields, mp values, recrystallization solvents, IR and ^1H NMR spectral data are reported in Table 1 (**6e–o**) and in Table 4 (**13p–u**).

4.1.2. General procedure for the synthesis of compounds 7k,n. A solution of **6k**,**n** (20 mmol) in anhydrous diethyl ether (150 mL) was slowly added to a stirred solution of lithium aluminium hydride (1.52 g, 40 mmol) in the same solvent (100 mL). The mixture was refluxed with stirring for 7 h, cooled at 0 °C and treated in succession with water (2 mL), 10% sodium hydroxide solution (2.5 mL) and water (8 mL). The supernatant organic solution was decanted, the insoluble residue was treated with 3 N hydrochloric acid (pH ~ 1) and the mixture was then thoroughly extracted with diethyl ether. The combined organic extracts were washed with water, dried

Table 6. Chemical, physical and ^{1}H NMR spectral data of compounds **10e–u**



Compound	R_1	R_2	Yield (%)	Mp (°C) or bp (°C/mm Hg)	Molecular formula	Analyses% calcd/found			^{1}H NMR, δ ($CDCl_3$)
						C	H	N	
10e	H	2-Cl	65	—[a]	$C_{25}H_{19}ClN_4$	73.07 73.17	4.66 4.91	13.63 13.46	6.16 and 6.21 (2s, 1H, CH-Ph), 6.85–7.4 (m, 16H, ar and imid), 7.44 and 7.61 (2s, 1H, H-2 imid), 7.53 (s, 1H, H-3 pyr)
10f	H	4-Cl	42	156–157[b]	$C_{25}H_{19}ClN_4$	73.07 72.97	4.66 4.78	13.63 13.59	6.22 (s, 1H, CH-Ph), 6.85–7.4 (m, 16H, ar and imid), 7.49 (s, 2H, H-2 imid and H-3 pyr)
10g	H	2-F	47	113–114[b]	$C_{25}H_{19}FN_4$	76.12 75.99	4.85 5.07	14.20 14.10	6.26 (s, 1H, CH-Ph), 6.85–7.4 (m, 16H, ar and imid), 7.46 (s, 1H, H-2 imid), 7.53 (s, 1H, H-3 pyr)
10h	H	4-F	64	156–157[b]	$C_{25}H_{19}FN_4$	76.12 75.79	4.85 5.03	14.20 14.10	6.24 (s, 1H, CH-Ph), 6.9–7.4 (m, 16H, ar and imid) 7.51 (s, 1H, H-3 pyr), 7.57 (s, 1H, H-2 imid)
10i	2-Cl	2-Cl	55	128–129[b]	$C_{25}H_{18}Cl_2N_4$	67.42 67.52	4.07 4.23	12.58 12.62	6.20 and 6.23 (2s, 1H, CH-Ph), 6.85–7.45 (m, 15H, ar and imid), 7.49 and 7.64 (2s, 1H, H-2 imid), 7.54 (s, 1H, H-3 pyr)
10j	2,4-Cl_2	2-Cl	67	205–215/0.38	$C_{25}H_{17}Cl_3N_4$	62.58 62.28	3.17 3.43	11.68 11.33	6.18 and 6.21 (2s, 1H, CH-Ph), 6.85–7.4 (m, 14H, ar and imid), 7.46 and 7.64 (2s, 1H, H-2 imid), 7.54 (s, 1H, H-3 pyr)
10k	4-Cl	4-Cl	68	134–135[b]	$C_{25}H_{18}Cl_2N_4$	67.42 67.41	4.07 4.02	12.58 12.64	6.21 (s, 1H, CH-Ph), 6.85–7.4 (m, 15H, ar and imid), 7.49 (s, 2H, H-2 imid and H-3 pyr)
10l	2,4-Cl_2	4-Cl	39	166–167[c]	$C_{25}H_{17}Cl_3N_4$	62.58 62.37	3.57 3.32	11.68 11.46	6.25 (s, 1H, CH-Ph), 6.8–7.45 (m, 14H, ar and imid), 7.50 (s, 1H, H-2 imid), 7.53 (s, 1H, H-3 pyr)
10m	2-F	2-F	53	140–141[b]	$C_{25}H_{18}F_2N_4$	72.80 72.70	4.40 ND[d]	13.58 13.59	6.30 (s, 1H, CH-Ph), 6.9–7.4 (m, 15H, ar and imid), 7.46 (s, 1H, H-2 imid), 7.58 (s, 1H, H-3 pyr)
10n	4-F	4-F	80	141–142[b]	$C_{25}H_{18}F_2N_4$	72.80 73.05	4.40 4.26	13.58 13.59	6.21 (s, 1H, CH-Ph), 6.9–7.4 (m, 15H, ar and imid), 7.47 (s, 2H, H-2 imid and H-3 pyr)
10o	2,4-F_2	4-F	60	121–122[e]	$C_{25}H_{17}F_3N_4$	69.76 69.50	3.98 3.71	13.01 12.79	6.24 (s, 1H, CH-Ph), 6.75–7.45 (m, 14H, ar and imid), 7.49 (s, 1H, H-2 imid), 7.53 (s, 1H, H-3 pyr)
10p	2-Cl	H	74	131–132[e]	$C_{25}H_{19}ClN_4$	73.07 72.92	6.66 4.51	13.63 13.80	6.30 (s, 1H, CH-Ph), 6.9–7.45 (m, 16H, ar), 7.52 (s, 1H, H-2 imid), 7.55 (s, 1H, H-3 pyr)
10q	4-Cl	H	70	122–123[b]	$C_{25}H_{19}ClN_4$	73.07 73.10	4.66 4.34	13.63 13.68	6.24 (s, 1H, CH-Ph), 6.9–7.4 (m, 16H, ar and imid), 7.49 (s, 1H, H-2 imid), 7.51 (s, 1H, H-3 pyr)
10r	2,4-Cl_2	H	72	152–153[b]	$C_{25}H_{18}Cl_2N_4$	67.42 67.39	4.07 3.92	12.58 12.64	6.29 (s, 1H, CH-Ph), 6.9–7.45 (m, 15H, ar and imid), 7.50 (s, 1H, H-2 imid), 7.55 (s, 1H, H-3 pyr)
10s	2-F	H	75	126–127[b]	$C_{25}H_{19}FN_4$	76.12 76.24	4.85 4.80	14.20 14.30	6.25 (s, 1H, CH-Ph), 6.9–7.4 (m, 16H, ar and imid), 7.50 (s, 2H, H-2 imid and H-3 pyr)

(*continued on next page*)

Table 6 (*continued*)

Compound	R_1	R_2	Yield (%)	Mp (°C) or bp (°C/mm Hg)	Molecular formula	Analyses% calcd/found			^{1}H NMR, δ ($CDCl_3$)
						C	H	N	
10t	4-F	H	77	126–127[b]	$C_{25}H_{19}FN_4$	76.12 76.07	4.85 5.03	14.20 14.31	6.24 (s, 1H, CH-Ph), 6.9–7.4 (m, 16H, ar and imid), 7.50 (s, 2H, H-2 imid and H-3 pyr)
10u	2,4-F_2	H	62	112–113[e]	$C_{25}H_{18}F_2N_4$	72.80 72.96	4.40 4.45	13.58 13.69	6.28 (s, 1H, CH-Ph), 6.75–7.45 (m, 15H, ar and imid), 7.50 (s, 1H, H-2 imid), 7.55 (s, 1H, H-3 pyr)

[a] Not crystallizable, vitreous oil.
[b] From anhydrous diethyl ether.
[c] From ethyl acetate/petroleum ether (bp 40–60 °C).
[d] ND = not determined.
[e] From anhydrous diethyl ether/petroleum ether (bp 40–60 °C).

(magnesium sulfate), filtered and evaporated. The crude product was then recrystallized from suitable solvent.

Yields, mp values, recrystallization solvents, IR and ^{1}H NMR spectral data are reported in Table 2.

4.1.3. General procedure for the synthesis of compounds 7e–j,l,m,o. Diisobutylaluminium hydride (DIBAL-H) (1.5 mol dm^{-3} in toluene, 13 mL, 20 mmol) was added dropwise, under dry nitrogen, to a solution of **6e–j**,**l**,**m**,**o** (10 mmol) in anhydrous toluene, cooled to −78 °C. After 45 min of stirring at −78 °C, the mixture was quenched by cautious addition of 2 N hydrochloric acid (70 mL) and thoroughly extracted with ethyl acetate. The combined organic extracts were washed with water, dried (magnesium sulfate), filtered and evaporated under reduced pressure. The crude product was then recrystallized from suitable solvent. Crude compounds **7e** and **7i** were obtained as liquid residues, which were purified by silica gel chromatography, using ethyl acetate–petroleum ether (bp 40–60 °C) 1:1 as eluant, followed by bulb-to-bulb distillation in vacuo (**7e**) or by recrystallization from anhydrous diethyl ether (**7i**).

Yields, bp and mp values, recrystallization solvents, IR and ^{1}H NMR spectral data are reported in Table 2.

4.1.4. General procedure for the synthesis of compounds 8e–o. Compounds **7e–o** (20 mmol) dissolved in anhydrous dichloromethane (100 mL) were added to a magnetically stirred suspension of pyridinium chlorochromate (6.46 g, 30 mmol) in the same solvent (50 mL). The resulting mixture was stirred at room temperature for 24 h and, therefore, diluted with anhydrous diethyl ether (50 mL). The supernatant organic solution was decanted from black gum and the insoluble residue was washed with anhydrous diethyl ether (3 × 30 mL). The combined organic solution was filtered on florisil and the solvent was removed by distillation. The crude residues were purified by recrystallization from suitable solvent.

Yields, mp values, recrystallization solvents, IR and ^{1}H NMR spectral data are reported in Table 3.

4.1.5. General procedure for the synthesis of compounds 9e–o. Compounds **8e–o** (10 mmol) dissolved in anhydrous diethyl ether (**8e**,**g–i**,**k**,**l**,**n**,**o**) or tetrahydrofuran (**8f**,**j**,**m**) (50 mL) were slowly added with stirring to an ice-cooled solution of phenylmagnesium bromide, prepared from magnesium turnings (0.73 g, 30 mmol) and bromobenzene (3.60 g, 43 mmol) in the same solvent (20 mL). The mixture was refluxed for 1 h, cooled at 0 °C and treated with 15% sulfuric acid (pH ∼ 1). The organic layer was separated from the acid phase, which was extracted thoroughly with diethyl ether. The combined organic solution was washed with saturated sodium hydrogen carbonate solution and with water, dried (magnesium sulfate), filtered and evaporated to give a white solid residue, which was purified by recrystallization from a suitable solvent.

Yields, mp values, recrystallization solvents, IR and ^{1}H NMR spectral data are reported in Table 5.

Table 7. In vitro antifungal and antibacterial activity of compounds **3**, **10j–l,r**

Compound	CC50[a]	EC50[b]	MIC[c]/MFC (MBC)[d]				
	MT-4	HIV-1	*C. albicans*	*C. neoformans*	*A. fumigatus*	*S. aureus*	*Salmonella* spp.
3[e]	22.2	>22.2	22/>100	22/22	In[f]	66/>100	In[f]
10j	33	>33	33.3/33.3	⩾11.1/33.3	In[f]	33.3/33.3	In[f]
10k	34	>34	66.6/66.6	11.1/33.3	In[f]	25/100	In[f]
10l	12	>12	11.1/11.1	11.1/33.3	In[f]	33.3/33.3	In[f]
10r	32	>32	In[f]	33.3/33.3	In[f]	In[f]	In[f]
AZT	>100	0.008	—	—	—	—	—
Streptomycin	>100	—	—	—	—	3.1/3.1	10/23.6
Bifonazole	>100	—	33.3/33.3	33.3/100	In[f]	—	—
Miconazole	18	—	6.0/10.6	3.7/3.7	27.6/44.4	—	—
Amphotericin B	>100	—	3.7/3.7	1.2/3.7	11.1/11.1	—	—

[a] Compound concentration (μM) required to reduce the viability of mock-infected MT-4 cells by 50%, as determined by the MTT method.
[b] Compound concentration (μM) required to achieve 50% protection of MT4 cells from the HIV-1 induced cytopathogenicity, as determined by the MTT method.
[c] Minimum inhibitory concentration (μM).
[d] Minimum fungicidal (bactericidal) concentration (μM).
[e] Ref. 10.
[f] Inactive up to MIC ⩾ 100 μM.

4.1.6. General procedure for the synthesis of compounds 9p–u. A solution of **13p–u** (10 mmol) in anhydrous diethyl ether (50 mL) was slowly added with stirring to a solution of lithium aluminium hydride (0.38 g, 10 mmol) in the same solvent (20 mL). The mixture was refluxed with stirring for 4 h, cooled at 0 °C and treated in succession with water (0.5 mL), 10% sodium hydroxide solution (0.5 mL) and water (2 mL). The supernatant organic solution was decanted and the insoluble residue was treated twice with diethyl ether. The combined organic extracts were washed with water, dried (magnesium sulfate), filtered and evaporated. The crude product was then recrystallized from suitable solvent.

Yields, mp values, recrystallization solvents, IR and ^{1}H NMR spectral data are reported in Table 5.

4.1.7. General procedure for the synthesis of compounds 10e–u. *N*,*N*′-Carbonyldiimidazole (1.78 g, 11 mmol) was added to a solution of **9e–u** (10 mmol) in dry toluene (60 mL) and the mixture was refluxed for 4 h. The solvent was removed under reduced pressure and the residue was dissolved in diethyl ether (200 mL). The organic solution was washed with water, dried (magnesium sulfate), filtered and evaporated. The crude residue was then purified by chromatography on florisil, using diethyl ether for **10k**,**l**,**q**, or diethyl ether–petroleum ether (bp 40–60 °C) 1:1 for **10g**,**j**,**m–p**,**r**,**t**,**u**, or ethyl acetate–petroleum ether (bp 40–60 °C) 1:1 for **10i**,**f** as eluent, and recrystallization from a suitable solvent.

Yields, bp and mp values, recrystallization solvents, ^{1}H NMR spectral data are reported in Table 6. IR spectral data are consistent with the proposed structures and are not reported, because lacking of characteristic absorption values.

4.2. Biological assays

Test compounds were dissolved in DMSO at an initial concentration of 100 μM and then were serially diluted in culture medium. Cell lines were from American Type Culture Collection (ATCC). H9/III$_B$, MT-4 and C8166 cells [grown in RPMI 1640 containing 10% foetal calf serum (FCS), 100 UI/mL penicillin G and 100 μg/mL streptomycin] were used for anti-HIV-1 assays. Cell cultures were checked periodically for the absence of mycoplasma contamination with a MycoTect Kit (Gibco). Human immunodeficiency virus type-1 (HIV-1, III$_B$ strain) was obtained from supernatants of persistently infected H9/III$_B$ cells. HIV-1 stock solutions had a titre of 5×10^7 cell culture infectious dose 50 (CCID$_{50}$)/mL. Bacterial and fungal strains were either clinical isolates (obtained from Clinica Dermosifilopatica, University of Cagliari) or collection strains from ATCC. *M. smegmatis* 19420 and *M. tuberculosis* 27294, 35820, 35822, 35828, 35837 strains were purchased from American Type Culture Collection (ATCC), whereas *M. fortuitum* and all bacterial strains were clinical isolates provided by University Hospitals of Cagliari, Italy.

4.2.1. Antiviral assays. Activity against the HIV-1 multiplication in acutely infected cells was based on inhibition of virus-induced cytopathogenicity in MT-4 cells.[25] Briefly, 50 μL of RPMI/10% FCS containing 1×10^4 cells were added to each well flat-bottomed microtitre trays containing 50 μL of medium and serial dilutions of test compounds. HIV-1 suspension (20 μL) containing 100 CCID$_{50}$ were then added. After a 4 days incubation at 37 °C, the number of viable cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) method.[26,27] Cytotoxicity of compounds, based on the viability of mock-infected cells as monitored by the MTT method, was evaluated in parallel with their antiviral activity.

4.2.2. Antibacterial assays. *S. aureus* and *Salmonella* spp. were recent clinical isolates. Assays were carried out in nutrient broth (DIFCO), pH 7.2, with an inoculum of 10^3 bacterial cells/tube. Minimum inhibitory concentrations (MICs) were determined after incubation at 37 °C

Table 8. In vitro antimycobacterial activity of compounds **3**, **10e–h,j–o,q–u**

Compound	CC50[a]	MIC50[b]/MIC90[c]						
	MT-4	*M. smegmatis* ATCC 19420	*M. fortuitum* (Cl. is.)	*M. tuberculosis* ATCC 27294 (wt)	*M. tuberculosis* ATCC 35820 (Str.^{R})	*M. tuberculosis* ATCC 35822 (Ison.^{R})	*M. tuberculosis* ATCC 35828 (Pyr.^{R})	*M. tuberculosis* ATCC 35837 (Eth.^{R})
3	22.2	In[d]	In[d]	69.9/>100	43.5/>100	43.3/86.6	30.0/>100	33.0/>100
10e	11	90.0/>100	In[d]	56.3/>100	41.1/>100	44.1/87.4	27.3/89.2	41.1/>100
10f	26	In[d]	In[d]	25.3/90.0	In[d]	In[d]	In[d]	In[d]
10g	>100	50.4/>100	In[d]	In[d]	In[d]	In[d]	In[d]	In[d]
10h	>100	55.3/>100	In[d]	In[d]	In[d]	In[d]	In[d]	In[d]
10j	33	29.8/>100	57.7/>100	6.7/26.2	22.4/100	23.9/>100	15.6/60.2	36.2/>100
10k	34	16.2/67.3	In[d]	6.1/27.3	In[d]	In[d]	In[d]	In[d]
10l	12	In[d]	In[d]	6.7/25.3	In[d]	91.7/>100	73.1/>100	In[d]
10m	>100	88.2/>100	In[d]	47.2/>100	In[d]	In[d]	In[d]	In[d]
10n	>100	50.0/⩾ 100	In[d]	In[d]	In[d]	In[d]	In[d]	In[d]
10o	>100	59.0/>100	In[d]	21.1/>100	In[d]	In[d]	In[d]	In[d]
10q	⩾ 100	In[d]	In[d]	10.7/84.0	In[d]	In[d]	In[d]	In[d]
10r	32	In[d]	In[d]	11.7/29.3	60.8/98.3	61.2/99.6	48.9/88.5	64.2/>100
10s	>100	51.6/>100	In[d]	47.0/>100	In[d]	In[d]	In[d]	In[d]
10t	>100	50.0/⩾ 100	In[d]	65.3/>100	In[d]	In[d]	In[d]	In[d]
10u	>100	48.9/⩾ 100	In[d]	57.5/>100	In[d]	In[d]	In[d]	In[d]
Ofloxacin	>100	2.0/7.6	1.1/7.2	0.7/3.0	0.6/2.1	1.6/3.4	0.9/2.8	1.1/2.9
Ciprofloxacin	60	1.0/4.0	2.7/15.2	0.4/3.0	0.4/1.1	1.3/3.2	0.7/2.4	0.9/2.4
Isoniazid	>100	2.3/8.1	In[d]	0.2/1.1	0.07/1.1	In[d]	0.1/2.4	0.1/0.4
Streptomycin	>100	0.2/0.36	15.7/>100	0.01/0.06	In[d]	0.08/0.3	0.1/0.3	0.1/0.3
Rifampin	>100	3.2/11.7	0.7/5.9	0.1/0.3	0.2/1.1	0.6/1.0	0.6/1.1	0.4/1.0
Ethambutol	>100	0.96/2.6	In[d]	2.7/6.6	2.3/9.7	5.0/>100	4.3/24.2	In[d]
Clotrimazole	>100	42.2/>100	In[d]	20.4/61.2	In[d]	In[d]	In[d]	In[d]
Econazole	>100	19.6/62.3	In[d]	12.5/52.1	13.1/30.9	18.2/30.7	13.7/28.3	20.0/32.3

[a] Compound concentration (μM) required to reduce the viability of mock-infected MT-4 cells by 50%, as determined by the MTT method.
[b] Minimum inhibitory concentration (μM) required to reduce the number of viable mycobacteria by 50%, as determined by the MTT method.
[c] Minimum inhibitory concentration (μM) required to reduce the number of viable mycobacteria by 90%, as determined by the MTT method.
[d] Inactive up to MIC50 ⩾ 100 μM.

Table 9. Absolute and local minima calculated for **6e**,**i**,**j**, **7e**,**i**,**j**, **9e**,**i**,**j**, **10e**,**i**,**j**

Conformer	Angle $\tau 1$ (°)	Angle $\tau 2$ (°)	E (kJ/mol)
6e_Ia	0	260	154.7
6e_Ib	190	259	159.1
6e_IIa	0	100	155.0
6e_IIb	172	110	158.2
6i_Ia	5	241	176.6
6i_Ib	194	240	179.0
6i_IIa	0	98	177.4
6i_IIb	168	104	180.6
6j_Ia	0	260	144.6
6j_Ib	180	258	146.9
6j_IIa	0	90	149.3
6j_IIb	159	118	150.0
7e_Ia	142	245	196.2
7e_IIa	217	115	196.2
7i_Ia	264	247	220.0
7i_IIa	240	69	215.4
7j_Ia	90	290	186.0
7j_IIa	260	60	188.0
9e_a	100	180	194.3
9e_b	269	171	196.1
9i_a	79	169	213.3
9i_b	259	171	220.5
9j_a	81	168	183.7
9j_b	249	171	189.0
10e_a	160	279	197.0
10e_b	73	80	199.1
10i_a	90	90	216.5
10i_b	162	276	222.9
10j_a	60	80	186.2
10j_b	158	289	191.6

for 18 h in the presence of serial dilutions of test compounds.

4.2.3. Antimycobacterial assays. Mycobacterial strains were grown in Middlebrook 7H9 medium with 0.5% glycerol and 10% albumine-dextrosecatalase (ADC) enrichment (Microbiol diagnostici, Italy). MICs were assessed in microtitre plates by adding 20 μL aliquots of a culture suspension [whose turbidity was equal to that of a No 1 McFarland standard containing 10^8 colony forming units (CFU)/mL] to 80 μL of the same medium containing various concentrations of test compounds. Plates were then incubated for 1 day (*M. smegmatis* and *M. fortuitum*) or 9 days *M. tuberculosis*) at 37 °C. At the end of incubation, the number of viable mycobacteria was determined by the MTT method, as already reported.[25]

4.2.4. Antimycotic assays. Yeast inocula were obtained by properly diluting cultures incubated at 37 °C for 30 h in Sabouraud dextrose broth to obtain 5×10^3 cells/mL. On the contrary, dermatophyte inocula were obtained from cultures grown at 37 °C for 5 days in Sabouraud dextrose broth by finely dispersing clumps with a glass homogenizer and then diluting to 0.05 OD_{590}/mL. Then, 20 μL of the above suspensions were added to each well of flat-bottomed microtitre trays containing 80 μL of medium with serial dilutions of test compounds, and were incubated at 37 °C. Growth controls were visually determined after 2 days (yeast) or 3 days (dermatophytes). MIC was defined as the compound concentration at which no macroscopic sign of fungal growth was detected. The minimal germicidal concentrations (MBC or MFC) were determined by subcultivating in Sabouraud dextrose agar samples from cultures with no apparent growth.

4.2.5. Linear regression analysis. Viral and cell growth at each drug concentration was expressed as percentage of untreated controls and the concentrations resulting in 50% (EC_{50}, CC_{50}) growth inhibition were determined by linear regression analysis.

4.3. Molecular modeling

Molecular structures of ligands were built and energy minimized within MacroModel.[28] Conformational analysis was carried out using the AMBER* force field, as included in MacroModel. The initially minimized structure was used as a starting point to perform a systematic search using the DRIVE option implemented in MacroModel. Torsional angles $\tau 1$ and $\tau 2$ (Fig. 5) were increased by 5° increments in the range 0°–360° and the obtained geometries were fully relaxed. Since a third torsional angle should be taken into account for a complete conformational analysis of these compounds, a more accurate exploration of the conformational space was done by random search, using the Monte Carlo option implemented in MacroModel. Starting from one of the lowest energy conformations retrieved with the previous strategy, several Monte Carlo cycles were run. For each cycle the following parameters were used: automatic setup, maximum number of search interactions 1000, energy cut-off set differently to 5, 10, 20, 30 kcal/mol. Only in the case of an energy cut-off set to 30 kcal/mol, the high energy barrier could be transpassed and from conformation 'a', namely the absolute minimum, conformation 'b' could be found among the possible results for all compounds examined.

For all compounds, the resulting geometries of all the lower energy conformers were re-optimized with semi-empirical quantum mechanic calculations, using the Hamiltonian AM1 as implemented in Spartan.[29] In the case of compounds **10j** and **10o** atomic charges calculated with AM1 were used to evaluate the corresponding isopotential contour surfaces.

The crystallographic coordinates of CYP121 and CYP51 from *M. tuberculosis* were obtained from the Brookhaven Protein Databank, entries 1N4O[19] and 1EA1.[24]

The structural models of selected compounds, were docked into the active site of the cytochrome using FlexX.[20] Preparation of the protein for FlexX requires definition of the binding pocket in terms of 'interaction points'. In this work the active sites were defined as all atoms within a distance of 10 Å from the iron atom of the haem. The specific distance was determined in order to ensure a significant portion of the active site for the docking experiments. The results were evaluated in terms of total estimated binding energy (FlexX total

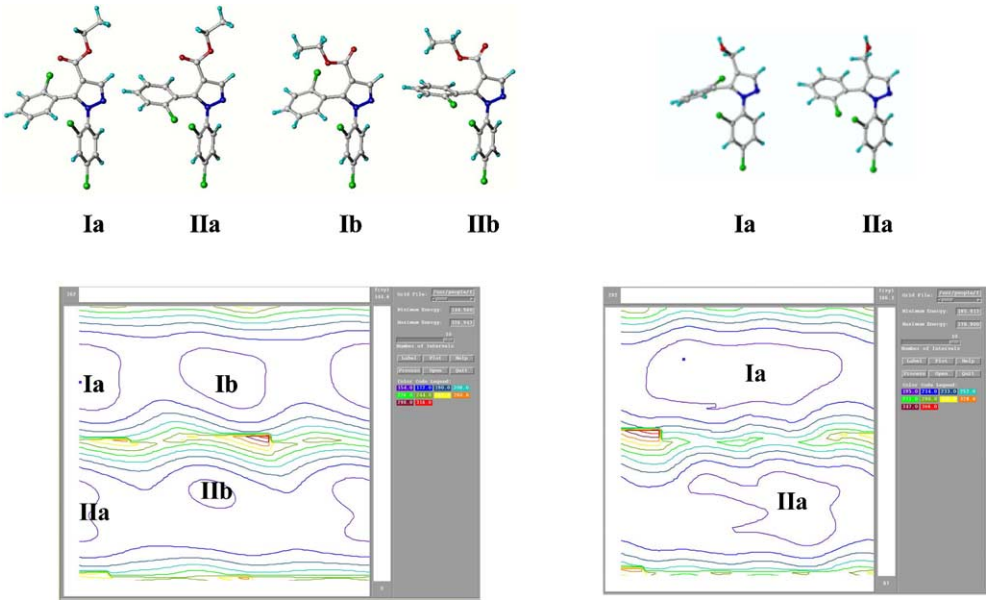


Figure 6. Ramachandran plots of compound **6j**, **7j** and their identified conformers. Different colours define different energetical areas. Colour code (kJ/mol): violet 154.0, dark-blue 172.0, green-blue 190, turquoise 208.0, light-green 226.0, dark-green 244.0, yellow 262.0, orange 280.0, brown 298.0, red 316.0.

score) and lipophilicity interactions (FlexX lipophilicity score). Since the macromolecule has a high degree of hydrophobic residues into the catalytic core,[3] this latest parameter, decoding for hydrophobic interactions, has been taken into particular account. All selected complexes underwent to a final energy minimization, before the model would be achieved within Charmm as implemented in INSIGHT-II.[30]

All calculations were carried out on SGI O2 workstations and on a standard personal computer running under Linux.

Acknowledgements

We would like to thank the 'Ministero dell'Istruzione, dell'Università e della Ricerca' for its financial support.

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