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Design, synthesis and evaluation of biphenyl imidazole analogues as potent antifungal agents

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ABSTRACT:

To further explore the structure activity relationships (SARs) of our previously discovered antifungal lead compound (**1**), a series of biphenyl imidazole analogues were designed, synthesized and evaluated for their *in vitro* antifungal activity. Many of the synthesized compounds showed excellent activity against *Candida albicans* and *Candida tropicalis*. Among these compounds, 2-F substituted analogue **12m** displayed the most remarkable *in vitro* activity against *C. albicans*, *C. neoformans*, *A. fumigatus* and fluconazole-resistant *C.alb.* strains, which is superior or comparable to the activity of the reference drugs fluconazole and itraconazole. Notably, the compound **12m** exhibited low inhibition profiles for various human cytochrome P450 isoforms and showed low toxicity to mammalian A549 cells and U87 cells. The SARs and binding mode established in this study will be useful for further lead optimization.

KEYWORDS:

Antifungal activity, CYP51, Structure-activity relationship, Azole antifungals

Over the past decades, the incidence of fungal infections has been steadily increasing and

presents a serious threat to human health[1]. Especially, the invasive fungal infections (IFIs) has greatly increased, leading to high morbidity and mortality in immunocompromised individuals including patients with AIDS and those receiving immunomodulatory therapies and organ transplants[2-5]. There are three major species of human fungal pathogens responsible for the IFIs including *Candida albicans*(mortality rate 20-40%), *Cryptococcus neoformans*(mortality rate 20-70%), and *Aspergillus fumigatus*(mortality rate 50-90%)[6, 7].

The therapeutic antifungal drugs available for systemic clinical use can be divided into four different classes based on their mode of action: including polyenes (e.g. amphotericin B and Nystatin), nucleoside analogues (e.g. 5-fluorocytosine), echinocandins (e.g. caspofungi and micafungin) and azoles(e.g. fluconazole and itraconazole)[8, 9]. Amphotericin B is a broad-spectrum antifungal agent with good therapeutic effects, but nephrotoxicity and other serious side effects restict its use[10, 11]. Echinocandins have potent antifungal activities but low oral absorption[12]. Among these agents, azoles antifungal drugs, such as fluconazole, itraconazole and voriconazole, are widely used as first-line antifungal therapy (Figure 1). However, several factors have limited their clinical applications, such as drug resistance, narrow antifungal spectrum, and low bioavailability[13, 14]. Therefore, discovering novel, safer and more effective azole antifungal agents has great value in study and application.

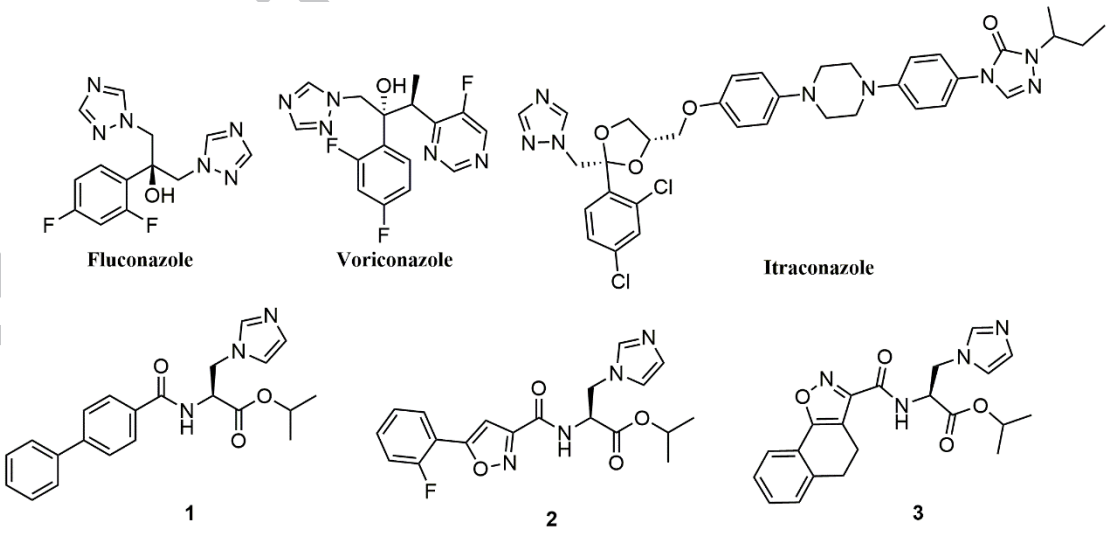


Figure 1. Chemical structures azole antifungal agents and lead compounds.

In our previous studies, a series of biphenyl imidazole derivatives showed excellent antifungal activity against C*andida albicans* and *Cryptococcus neoformans*, with MIC values in the range of 0.25 μg/mL to 2 μg/mL.[15] However, almost all of the target compounds were inactive

against *Aspergillus fumigatus*, which prompted us to continue studying the structural modification of our potent original compounds to improved anti-*Aspergillus fumigatus* efficacy. Our previous study also identified that compounds aromatic isoxazole (**2**) and 4,5-dihydronaphtho[2,1-*d*] isoxazole (**3**) displayed the most remarkable *in vitro* activity against *A. fumigatus*[16, 17]. Molecular docking suggests that the reason may be the formation of the hydrogen bonds with between isoxazole ring of compounds and *A. fumigatus* CYP51 Tyr122 (Figure 2).

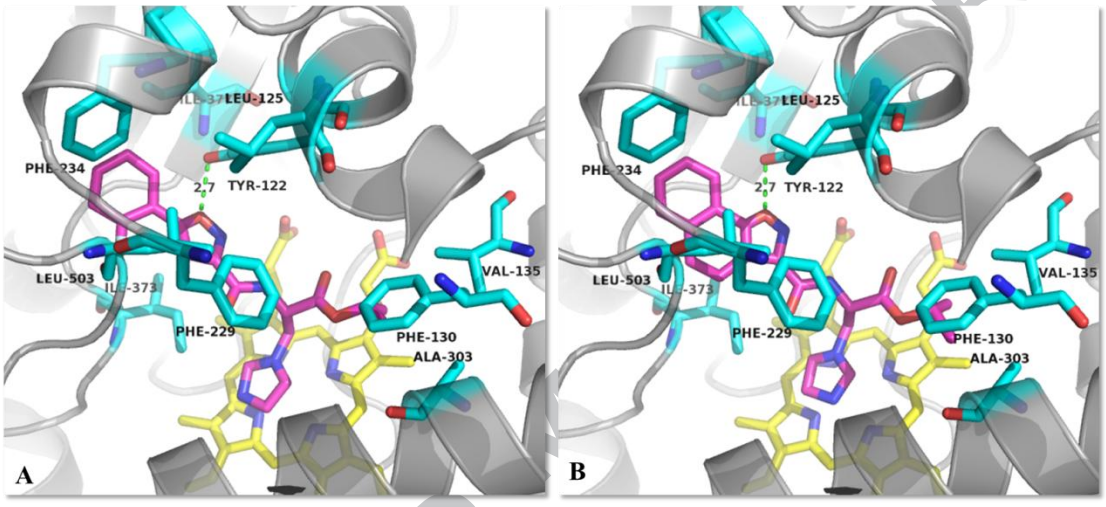
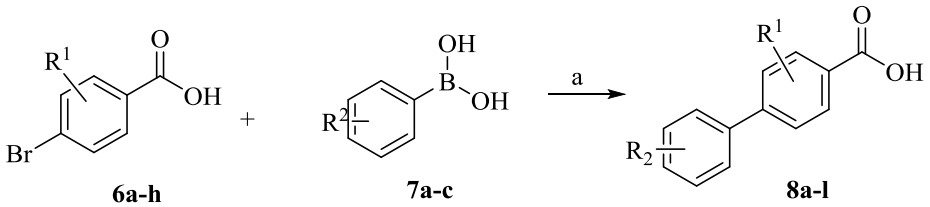


Figure 2. The binding mode of compound **2** and compound **3** in the active site of *A. fumigatus* CYP51B(PDB:4YUM).

To further explore the potent and broad antifungal spectrum of biphenyl derivatives, compound **1** was docked into the active site of *A. fumigatus* CYP51B (PDB ID:4UYM)[16]. Based on the interactions between compound **1** and *A. fumigatus* CYP51, the middle benzene ring of compound **1** was investigated with various substituents to get insights into the structure−activity relationships (SARs) and improved anti-*Aspergillus* efficacy.

The target compounds **12a-v** were synthesized according to our previously reported procedure which were outlined in Scheme 1 and Scheme 2[15, 18]. Firstly the key intermediates **8a-l** were synthesized by Suzuki coupling. Commercially available the substituted 4-bromobenzoic acid 6**a-h** and the substituted phenylboronic acids **7a-c** were subjected to a Suzuki coupling in the presence of $Pd(PPh_3)_4$ to afford the key intermediates **8a-l**.



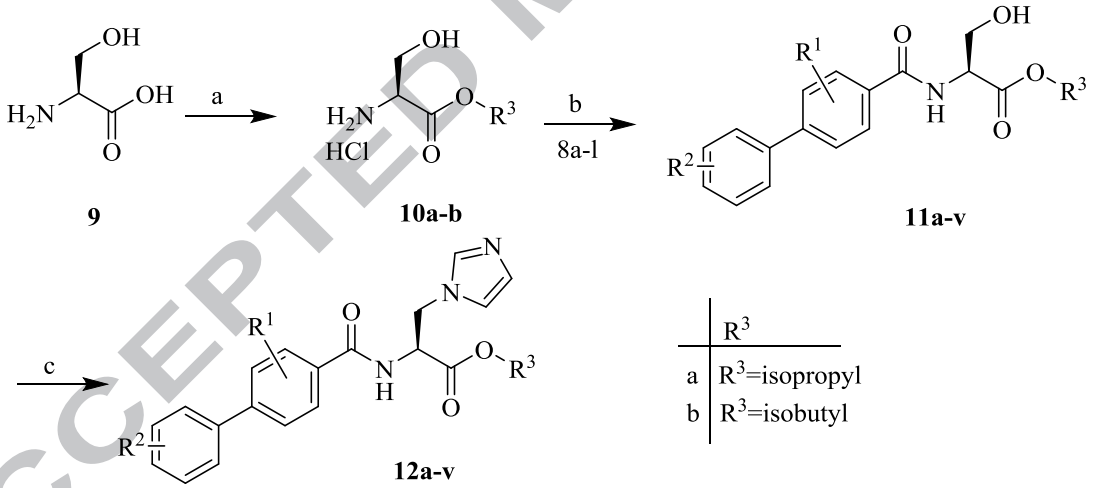
	R^1
a	R^1=2-F
b	R^1=2-Cl
c	R^1=2-CH_3
d	R^1=2-OCF_3

	R^1
e	R^1=3-F
f	R^1=3-Cl
g	R^1=3-CH3
h	R^1=2,6-F

	R^2
a	R^2=H
b	R^2=2-F
c	R^2=4-CN

Scheme 1. Synthesis of intermediates 8a-l. Reagents and conditions: (a) $Pd(PPh_3)_4$, K_2CO_3, reflux, 5h.

Secondly, the L-serine esters **10a-b** were synthesised by L-serine and alcohols (isopropanol, isobutanol) in the presence of $SOCl_2$. The L-serine esters **10a-b** were then treated with intermediates **8a-l** in the presence of a condensing agent to give intermediates **11a-v**. Finally, the target compounds **12a-v** were successfully obtained by introducing the imidazole group of intermediates 11a-z using CDI/imidazole.



	R^3
a	R^3=isopropyl
b	R^3=isobutyl

Scheme 2. Synthesis of target compounds **12a-v**. Reagents and conditions: (a) alcohol reagent, $SOCl_2$, reflux, 1–2 h; (b) EDCI, HOBt, DIEA, r.t., 7 h; (c) CDI, imidazole, CH_3CN, reflux, 7 h.

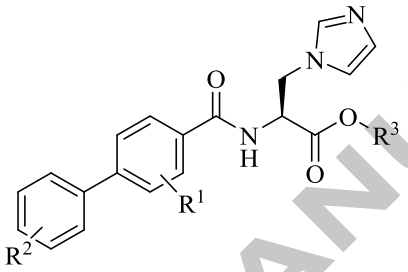
The *in vitro* antifungal activities of target compounds were determined against five important fungal pathogens according to the protocols from the NCCLS[18, 19]. The broth microdilution method was used to determine the minimum inhibitory concentration (MIC) of the target compounds in 96-well microtest plates. Fluconazole (FLC) and Itraconazole (ITR) were used as reference drugs.

The *in vitro* antifungal activities of the target compounds are summarized in Table 1. The

results showed that many of these compounds exhibited moderate to good antifungal activity with broad spectrums of activity. Compounds **12i-j, 12m-p** and **12s-v** showed the most potent activity against *C. albicans* and *C. tropicalis* with MIC values in the range of 0.03125 to 2 μg/mL. Among these compounds, 2-F substituted analogue **12m** displayed the most remarkable *in vitro* activity against *all of the tested strains*, with the exception of *Aspergillus fumigatus*, and was superior or comparable to those of the reference drugs FLC and ITR.

Table 1

In vitro antifungal activities of the target compounds (MIC, μg/mL)[a].



Compd.	R^1	R^2	R^3	*C. alb.*(I)	*C. alb*(II).	*C. neo.*	*C.tro.*	*A. fum.*
12a	3-CH_3	H	-$CH(CH_3)_2$	>16	2	>16	0.5	>16
12b	3-CH_3	H	-$CH_2CH(CH_3)_2$	>16	2	>16	0.5	>16
12c	3-OCH_3	H	-$CH(CH_3)_2$	8	1	>16	1	>16
12d	3-OCH_3	H	-$CH_2CH(CH_3)_2$	>16	4	>16	2	>16
12e	2-Cl	H	$CH(CH_3)_2$	8	8	8	1	>16
12f	2-Cl	H	-$CH_2CH_2CH_3$	4	8	8	0.25	>16
12g	2-CH_3	H	$CH(CH_3)_2$	>16	>16	>16	8	>16
12h	2-CH_3	H	-$CH_2CH(CH_3)_2$	>16	>16	>16	4	>16
12i	3,5-F_2	H	-$CH(CH_3)_2$	0.5	2	>16	0.5	>16
12j	3,5-F_2	H	-$CH_2CH(CH_3)_2$	0.5	2	>16	2	>16
12k	3-Cl	H	-$CH(CH_3)_2$	4	8	8	0.5	>16
12l	3-Cl	H	-$CH_2CH(CH_3)_2$	4	8	8	0.25	>16
12m	2-F	H	-$CH(CH_3)_2$	0.5	0.03125	8	0.03125	2
12n	2-F	H	-$CH_2CH(CH_3)_2$	0.25	0.03125	>16	0.03125	4
12o	3-F	H	-$CH(CH_3)_2$	0.25	0.03125	>16	0.03125	4
12p	3-F	H	-$CH_2CH(CH_3)_2$	0.25	0.03125	>16	0.03125	8

12q	3-F	4-CN	-$CH(CH_3)_2$	8	4	>16	2	>16
12r	3-F	4-CN	-$CH_2CH(CH_3)_2$	>16	>16	>16	0.5	>16
12s	3-F	2-F	-$CH(CH_3)_2$	0.5	0.125	>16	0.25	>16
12t	3-F	2-F	-$CH_2CH(CH_3)_2$	0.5	0.125	>16	0.25	>16
12u	2-F	2-F	$CH(CH_3)_2$	0.0625	0.03125	8	0.03125	>16
12v	2-F	2-F	-$CH_2CH(CH_3)_2$	0.25	0.0625	>16	0.0625	>16
FCZ	-		-	0.5	1	4	1	>16
ITZ	-		-	0.0625	0.25	1	0.5	4

[a]Abbreviations: *C.alb.*(I), *Candida albicans* (ATCC SC5314); *C.alb.*(II), *Candida albicans* (CPCC400523); *C. neo.*, *Cryptococcus neoformans* (cgmcc 2.3161); *A.fum.*, *Aspergillus fumigatus* (cgmcc 3.7795); *C.tro.*, *Candida tropicalis* (cgmcc 2.3739); FCZ: Fluconazole; ITZ: Itraconazole.

The fluconazole is the most widely used today and can seriously increase the problem of azole resistance for treating fungal infections[20]. Based on the results of *in vitro* antifungal activity assays, the most potent compounds **12m**, **12o** and **12u-v** were further evaluated against fluconazole-resistant strains of *C. alb*. (*strain 100* and *strain 103*). As shown in Table 2, compounds **12m** and **12v** showed moderate antifungal activities against fluconazole-resistant strains of *C. alb*. with MIC values in the range of 2 to 8 μg/mL.

Table 2

In vitro antifungal activities of the target compounds (MIC, μg/mL) [a].

Compd.	**R^1**	**R^2**	***C. alb.***	
			Strain100	***Strain103***
12m	2-F	H	8	2
12o	3-F	H	16	4
12u	3-F	2-F	16	2
12v	3-F	2-F	8	4
FCZ	-	-	>64	>64

[a]Abbreviations: *C.alb.*, *Candida albicans*; *strain 100*, fluconazole-resistant strains of *Candida albicans*; *strain 103*, fluconazole-resistant strains of *Candida albicans*; FCZ: Fluconazole.*Strain 100* and *strain 103* were provided by The Second Military Medical University.

Cytochrome P450 (CYP) enzymes play an important role in phase I of xenobiotic biotransformation. Drug-drug interactions (DDIs) caused by inhibition of cytochrome P450 enzymes can result in dangerous side effects, therefore it is important to evaluate the CYP

inhibition of our compounds. The most potent compounds **12m** and **12o** were tested against the five major human CYP isoforms (CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4-M). As shown in Table 3, compound **12m** showed no inhibitory activity against CYP1A2, CYP2C9, CYP2C19, and CYP2D6, whereas showed weak activity against CYP3A4 with IC_{50} values 4.05 μM. However, compound **12o** showed strong inhibition against CYP3A4 with IC_{50} values 0.0429 μM.

Table 3.

In vitro CYP inhibition assessment of compounds.

Compd.	IC50 (μM)				
	CYP1A2	**CYP2C9**	**CYP2C19**	**CYP2D6**	**CYP3A4-M**
12m	>50	>50	43.4	>50	4.05
12o	>50	4.49	7.44	19.1	0.0429

The compounds designed to target fungal cells may cause unwanted toxicity to mammalian cells. Therefore, it is important to evaluate the toxicity of the compounds to mammalian cells by in WTT assays. We tested the most potent compounds **12m** and **12o** against A549 cells and U87 cells. As shown in Table 4, compounds **12m** and **12o** exhibited low toxicity in A549 cells and U87 cells (IC_{50}>50μM).

Table 4

In vitro cytotoxicity of compounds on A549 cells and U87 cells.

Compd.	IC50 (μM)	
	A549	**U87**
12m	>50	>50
12o	>50	>50

The structure-activity relationship between various substituents on the middle aromatic ring was investigated (compounds **12a-p**). Compounds with electron-donating groups at the 2-position or 3-position, such as $-CH_3$ (**12a-b** and **12g-h**) and $-OCH_3$ (**12c-d)** moieties, resulted in a significant decrease in antifungal activity. Compounds with halogens such as -F and –Cl (**12e-f** and **12k-p**) at the 2-position or 3-position, exhibited higher antifungal activity than those with electron-donating groups. Among these, compounds **12m-p** with 2-F or 3-F substituents showed the most potent activity against *all of the tested strains*, with the exception of *Aspergillus*

fumigatus. Moreover, Compounds **12i-j** with multi-halogens substituents showed no advantage.

Based on the results above, we selected 2-F or 3-F substituents compounds as our starting point for further modification to expand the SAR studies. *In vitro* antifungal activity tests have shown that compounds with substituent (2-F and 4-CN) on the terminal benzene ring led to only a slight decrease in antifungal activity.

To better understand the binding mode of compound **12m**, a highly potent compound, docking simulations were carried out using the CDOCKER program in the Discovery Studio 3.0 software. The published X-ray structure of *A. fumigatus* CYP51(PDB ID:4UYM, Figure 3) served as a useful template for generating binding modes. Images depicting the proposed binding modes were generated using PyMOL. As shown in Figure 6, the imidazole ring of compound **12m** coordinated the iron in the heme group, and the alkyl ester formed a hydrophobic interaction with Val135, Ala303and Ala307. The biphenyl side chain extended into CYP51 channel could form favourable van der Waals and hydrophobic interactions with the surrounding residues Ile373, Leu503 and Phe504. Interestingly, the 2-F substituent of compound **12m** could form a hydrogen bond with the –OH of *A. fumigatus* Tyr122. Binding studies may provide a good explanation for the excellent *in vitro* antifungal activity against *A. fumigatus* of compound **12m**.

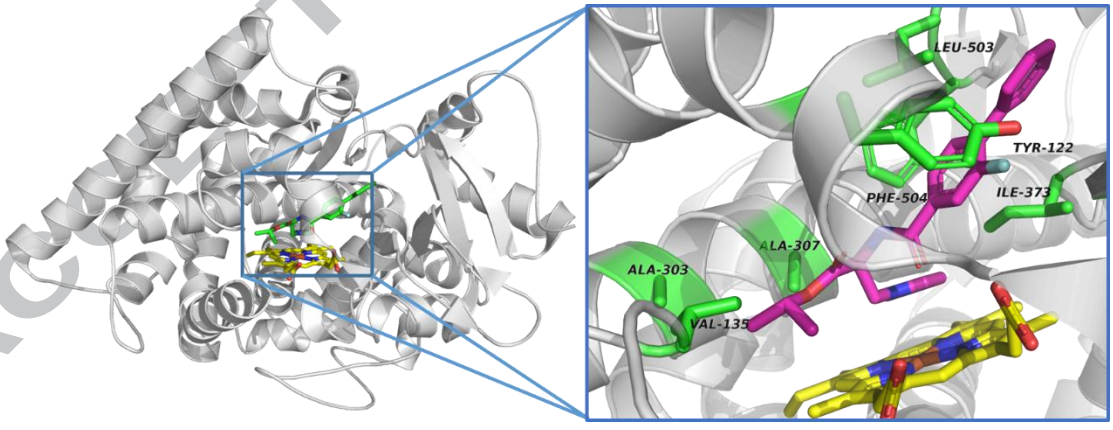


Figure 3. The binding mode of compounds **12m** in the active site of *A. fumigatus* CYP51 (PDB ID: 4UYM).

Based on our laboratory´s work we designed and synthesed a series of biphenyl imidazole derivatives and their *in vitro* antifungal activity were evaluated. Many of these compounds exhibited moderate to good antifungal activity against *C. albicans* and *C. tropicalis* with MIC values in the range of 0.03125 to 2 μg/mL. Among these, 2-F or 3-F substituted analogues **12m-p** displayed the most remarkable *in vitro* activity against *all of the tested strains*, with the exception

of *Aspergillus fumigatus*, and was superior or comparable to those of the reference drugs FLC and ITR. Notably, the compound **12m** had weak inhibition for various human cytochrome P450 isoforms, which indicated they had a low potential to cause DDIs. Compounds **12m** and **12o** were almost nontoxic to mammalian A549 cells and U87 cells. The SARs and binding mode established in this study will be useful for further lead optimization.

Acknowledgements

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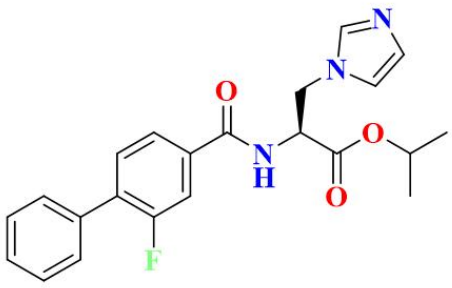
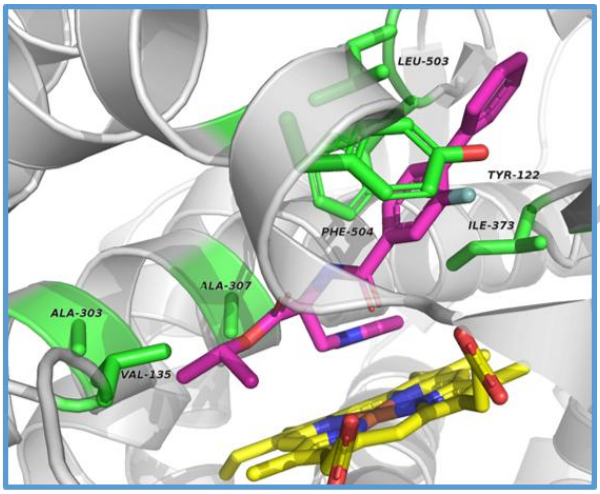


Compound 12m

C.albicans MIC=0.5 μg/mL
A. fumigatus MIC=2 μg/mL



Highlights

- 22 new compounds with biphenyl imidazole scaffolds were designed and synthesized.
- Compound **12m** showed better antifungal activity than fluconazole.
- The structure-activity relationships of compounds were discussed.
- Compound **12m** exhibited low inhibition profiles for human cytochrome P450 isoforms.