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Article

1,5-Diarylidene-4-piperidones as Promising Antifungal Candidates Against *Cryptococcus neoformans*

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Abstract

The study investigates the antifungal potential of 1,5-diarylidene-4-piperidones. These compounds were synthesized via Claisen-Schmidt condensation, yielding 26 derivatives with varied electronic and hydrophobic properties. Their antifungal efficacy against *Cryptococcus neoformans* was evaluated using minimum inhibitory concentration (MIC) assay, revealing that piperidone with tetrabutoxy groups exhibited potent activity (MIC of 7.8 μ M), surpassing standard antifungals like fluconazole. The selectivity index (SI) values confirmed their safety profile across various human cell lines. Mechanistic insights via docking studies suggest that these compounds inhibit ergosterol biosynthesis by targeting sterol 14-demethylase, with E binding score of 8.2 close to that of fluconazole. The compounds also demonstrated broad-spectrum activity against other pathogenic yeasts and fungi, including *Candida* and *Aspergillus* species, with some efficacy against fluconazole-resistant strains. These findings underscore the potential of 1,5-diarylidene-4-piperidones as promising antifungal candidates with a favorable safety profile.

Keywords: antifungal; cryptococcus neoformans; piperidones; curcumins

1. Introduction

The infectious agent *Cryptococcus neoformans* is a capsulated yeast present in soils and organic debris transmitted by air. This invasive fungal infection with *C. neoformans* is a serious cause of morbidity in HIV/AIDS immunocompromised patients. The most clinical form of infection is meningoencephalitis or pneumonia which is fatal when it is not treated even in immunocompetent patients.[1] In these cases, the standard treatment is amphotericin B combined with flucytosine.[2] However, both agents are toxic and laboratory monitoring of the patients is needed. Moreover, intravenous administration of amphotericin B is required limiting therefore the access of patients from resource-constrained countries. [3,4] In infections with no meningoencephalitis, fluconazole has been a primary antifungal agent in managing cryptococcosis, however, the excess use of azole drugs has caused the drug-resistance. Indeed, the ability of *Cryptococcus* to alter its genomic architecture in the face of antifungal stress primarily occurs through a phenomenon referred to as heteroresistance, necessitating the search and identification of novel antifungal drugs directed toward *C. neoformans*.

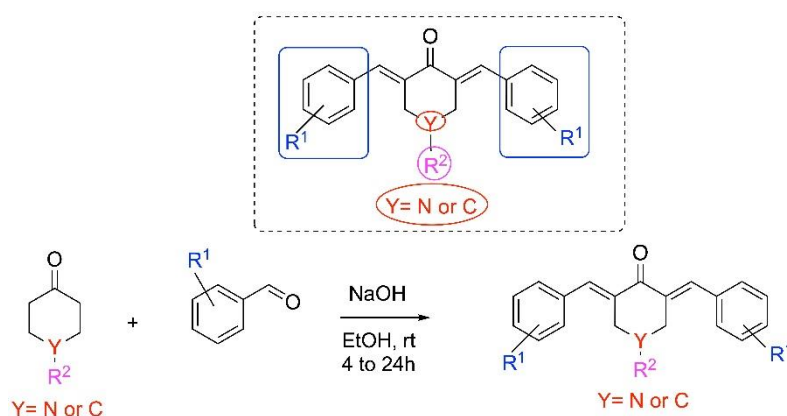
Numerous reports have demonstrated that curcumins derived from piperidones have a wide range of biological activities such as anti-cancer,[5] anti-bacterial anti-inflammatory and anti-fungal.[6–8] α,β -Unsaturated carbonyl compounds are known to be Michael acceptors particularly to thiol nucleophiles,[9] this faculty has been nicely exploited to design curcuma derivatives as potential anti-fungal drugs including 1,5-diarylidene-4-piperidones.[10–12] This assumption prompted us to design new piperidone library to investigate their potential anti-fungal activities against *C. neoformans*. The synthesis of curcumin derived drug is simple and based on Claisen-Schmidt reaction

in which ketone reacts with two molecules of benzaldehyde analogues in the presence of sodium hydroxide to reach benzylidene structures. Motivated by the simplicity and the potential of such molecules, we aimed to target series of benzylidene compounds since there is a need for the development of novel hits to face the drug-resistance.[13]

2. Results

2.1. Synthesis of Piperidones

To target structure-activity relationship (SAR), preparation of several analogues of 1,5-diarylidene-4-piperidones (**Scheme 1**) was planned by varying their electronic and/or hydrophobic properties (**Figure 1**). The Claisen-Schmidt condensation of piperidones and aldehyde derivatives gave rise to twenty-six compounds in one-step procedure with, in most cases, no need for column chromatography since a precipitation in ethanol is conclusive to obtain pure products with yields ranging from 48 to 86%.[14] We introduced a structural diversity through the modification of alkoxy groups, N-alkyl functions and the lateral moieties. Except **3h** and **3iso-h** (**Figure 1**), all compounds were isolated as intensely colored solids and characterized by ^1H NMR, ^{13}C NMR and Mass spectroscopy.



Scheme 1. Chemical syntheses of 1,5-diarylidene-4-piperidinones.

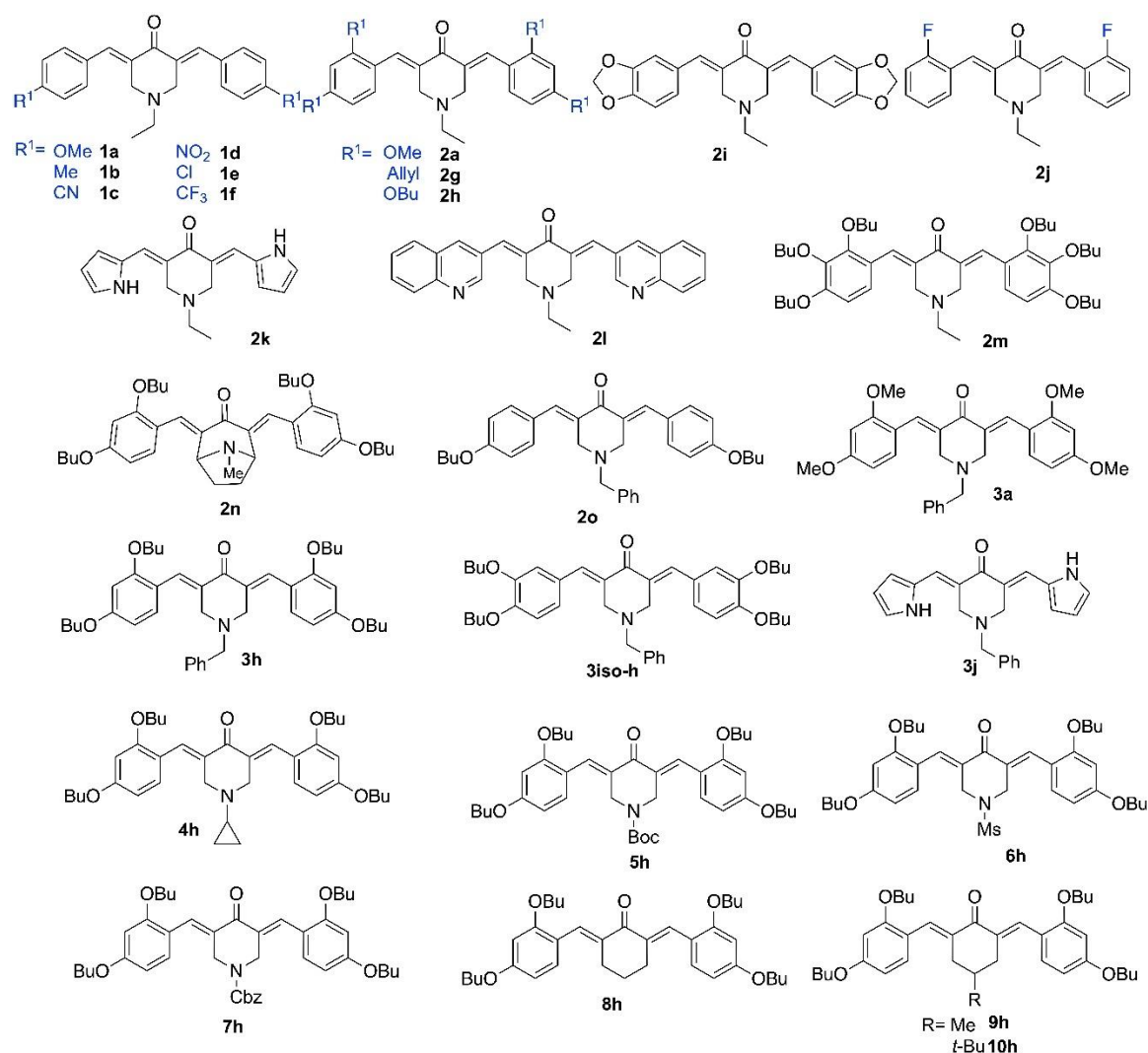


Figure 1. Chemical structures of tested 1,5-diarylidene-4-piperidinones.

2.1. Antifungal Activities and Toxicity of Synthesized Piperidones

The antifungal activities of the twenty-three piperidone compounds were first determined against *C. neoformans* using minimum inhibitory concentration (MIC) assay and are reported in Table 1. The antifungal potency was found to be modest for the monosubstituted aryl series **1a-1f** as well as for compound **2j**, with MIC ranging from 125 to 250 μ M, regardless of the substitution ($R^1 =$ OMe, Me, CN, NO₂, Cl, F, CF₃). Disubstituted methoxy and allyloxy groups (**2a** and **2g**) afforded even worse results. However, the dibutoxy compound **2h** exhibited a high ability to inhibit the growth of *C. neoformans* (MIC of 31.2 μ M). Compound **2i**, derived from piperonal, showed weak activity with a MIC of 250 μ M. Piperidone **2l**, possessing a quinoline scaffold analogous to those reported by Shingate,[15] was tested but showed lower efficiency (MIC = 250 μ M). Since butoxy groups appeared to exhibit the best activity against *C. neoformans*, we decided to synthesize and evaluate the compound **2m** which contains six butoxy groups enhancing therefore the hydrophobic ability of the piperidone. Compound **2m** displayed a good MIC of 62.5 μ M, but it was less efficient than **2h**. Bicyclic piperidone **2n** was found to be inactive against *C. neoformans*. We then varied the nature of N-alkyl substitution. The results indicated that cyclopropyl, Boc, mesyl and Cbz groups were detrimental to the activity. Benzyl amines (e.g. butenafine) are known to be antifungal agents [16] with activity on the cell membrane through squalene epoxidase bounding.[17] We decided therefore to synthesize compound **3h** that significantly improved activity, presenting a MIC as low as 7.8 μ M. To confirm this effect, we compared the activity of **2k** and **3j** by switching from ethyl alkyl to benzyl group on the nitrogen atom. Again, the results showed that benzyl substitution (compound **3j**) was benefit presenting a

lower MIC (62.5 μ M) compared to the ethyl-substituted piperidone **2k** (MIC = 250 μ M). Testing compound **2o**, which contains one butoxy group on each aromatic moiety, again proved detrimental, with a MIC of 125 μ M (vs 7.8 μ M for **3h**) highlighting the importance of the presence two butoxy groups on each aromatic moiety. A similar trend was observed when changing the position of the alkoxy group from ortho to meta (**3iso-h** being less active than **3h**, with a MIC of 62.5 and 7.8 μ M, respectively). The comparison between compounds **3a** (MIC >250 μ M) with methoxy instead of butoxy groups and **3h** strongly suggests that the **hydrophobic nature and the length of the alkyl chains** are crucial for the activity of these piperidone derivatives. The longer butyl chains in **3h** likely facilitate stronger hydrophobic interactions with a specific region (hydrophobic pocket) on the target protein, leading to enhanced biological activity. In addition, to underscore the significance of the piperidone core, we synthesized and evaluated 1,5-diarylidene cyclohexanone analogues **8-10h** which demonstrated no activity with MIC > 250 μ M. These results suggest that the amphiphilic character of piperidones- due to the presence of four alkyl chains and nitrogen atom- plays an important role. Interestingly, a control test revealed that compound **3h** was more active than fluconazole (MIC of 25 μ M), an antifungal recommended to treat *C. neoformans* infection,[18] particularly in low-income and middle-income countries.[19]

To better evaluate the therapeutic potential of the active compounds, their toxicity was then measured using various human cells: i.e. A498 (kidney), BEAS-2B (lung), Caco-2 (intestine), HaCaT (skin) and HepG2 (liver) cells (Figure 2). For comparison, the toxicity of fluconazole was also determined (Figure 3). The CC₅₀ values, i.e. the concentrations causing 50% reduction of the cell viability, were determined from the graphs and are given in Table 1.

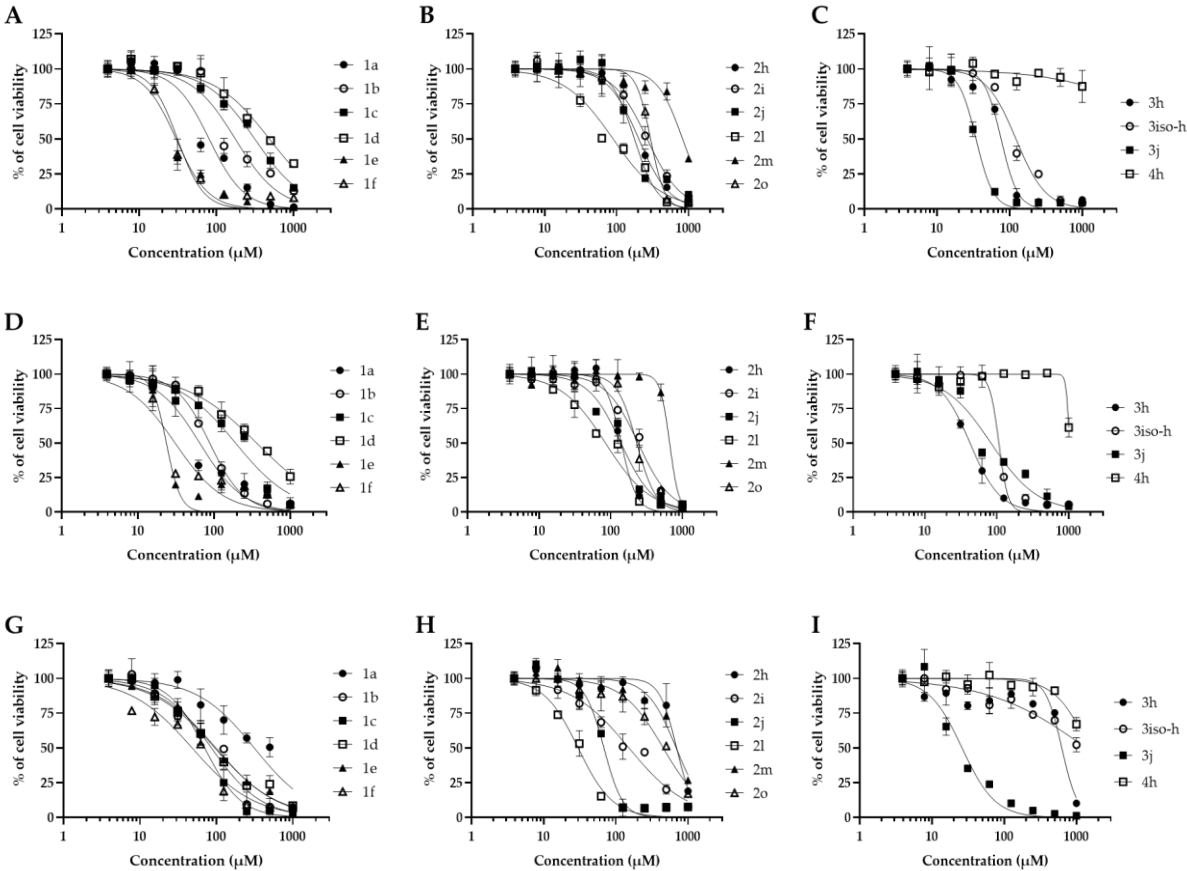
The CC₅₀ values of compounds range from 18.3 to > 1000 μ M depending on the compound and of the cell model tested. Compounds possessing mono-substituted aryl groups in para-position **1b**, **1c**, **1e**, **1f**, and **2j** afforded the highest toxicity (CC₅₀ of 18.3 to 313.9 μ M). Introducing substituted alkoxy group, like in **1a** and **2o**, was beneficial giving lower toxicity (CC₅₀ of 64.9 to >1000 μ M). In addition to higher anti-fungal activity, disubstituted butoxy groups **2h** revealed low toxicity (CC₅₀ of 140 to 696.6 μ M). When the aryl groups were connected to acetal, a detrimental effect was observed on toxicity of compound **2l** (CC₅₀ of 30.0 to 89.5 μ M). However, incorporating a third butoxy group in **2m** drug, afforded even a lower toxicity (CC₅₀ of 663 to >1000 μ M). We then evaluated the effect of the substitution on the nitrogen atom by introducing alkyl groups. Benzyl group **3h** revealed a small elevation of toxicity when it is compared to **2h** (CC₅₀ of 42.4 to 628.6 μ M) whereas compound with cyclopropyl group **4h** showed the lowest toxicity regarding all the cell lines tested (>1000 μ M).

For comparison, Fluconazole gave CC₅₀ ranging from 841.2 to >1000 μ M, respectively (Figure 3 and Table 1). In order to further identify the most promising candidates, the Selectivity Indexes (SI) of each compound (i.e. the ratio of CC₅₀ to MIC values) were calculated (Table 1).

Table 1. Anti-*C. neoformans* activity and cytotoxicity of piperidone compounds. The MIC values (in μ M) of piperidone compounds and fluconazole were measured on *C. neoformans* as explained in Materials and Methods section. Their Cytotoxic Concentration 50 (CC₅₀) (in μ M) were calculated from Figures 1 and 2 after 48 h incubation of human cells with increasing concentrations of molecules (n=3). Selectivity Indexes (SI) were calculated by dividing CC₅₀ by MIC values. Molecules with MIC superior or equal to 250 μ M were not tested in toxicity assay and are indicated as “NT”.

Compound	MIC (μ M)	CC ₅₀ (μ M)					SI
		A498	BEAS-2B	Caco-2	HaCaT	HepG2	
1a	125	80.4	64.9	313.7	368.6	471.9	0.5-3.7
1b	250	175.6	87.9	86.2	60.0	113.5	0.2-0.7
1c	250	313.9	191.8	70.3	122.7	49.5	0.1-1.2
1d	250	486.2	363.5	95.4	162.9	313.6	0.3-1.9
1e	250	31.3	23.7	98.6	34.9	68.5	0.09-0.4
1f	250	30.0	30.9	47.0	18.3	82.1	0.07-0.3
2a	>250	NT	NT	NT	NT	NT	NT

2g	>250	NT	NT	NT	NT	NT	NT
2h	31.2	203.7	140.2	696.6	680.3	595.6	4.5-22.3
2i	250	266.6	241.2	152.0	140.0	169.6	0.5-1.0
2j	250	177.3	139.0	68.3	44.2	136.4	0.1-0.7
2k	>250	NT	NT	NT	NT	NT	ND
2l	250	89.5	84.5	30.0	32.6	45.3	0.1-0.3
2m	62.5	829.4	663.7	686.9	764.3	>1000	10.6->16
2n	>250	NT	NT	NT	NT	NT	NT
2o	125	297.4	230.5	454.7	979.3	>1000	1.8->8
3a	>250	NT	NT	NT	NT	NT	NT
3h	7.8	76.4	42.4	628.6	377.3	178.3	5.4-80.5
3iso-h	62.5	122.4	106.9	>1000	753.8	>1000	1.7->16
3j	62.5	34.5	82.9	26.1	28.2	85.6	0.4-1.3
4h	250	>1000	>1000	>1000	>1000	>1000	>4
5h	>250	NT	NT	NT	NT	NT	NT
6h	>250	NT	NT	NT	NT	NT	NT
7h	>250	NT	NT	NT	NT	NT	NT
8h	>250	NT	NT	NT	NT	NT	NT
9h	>250	NT	NT	NT	NT	NT	NT
10h	>250	NT	NT	NT	NT	NT	NT
Fluconazole	25	993.6	942.5	977.0	841.2	>1000	33.6->40



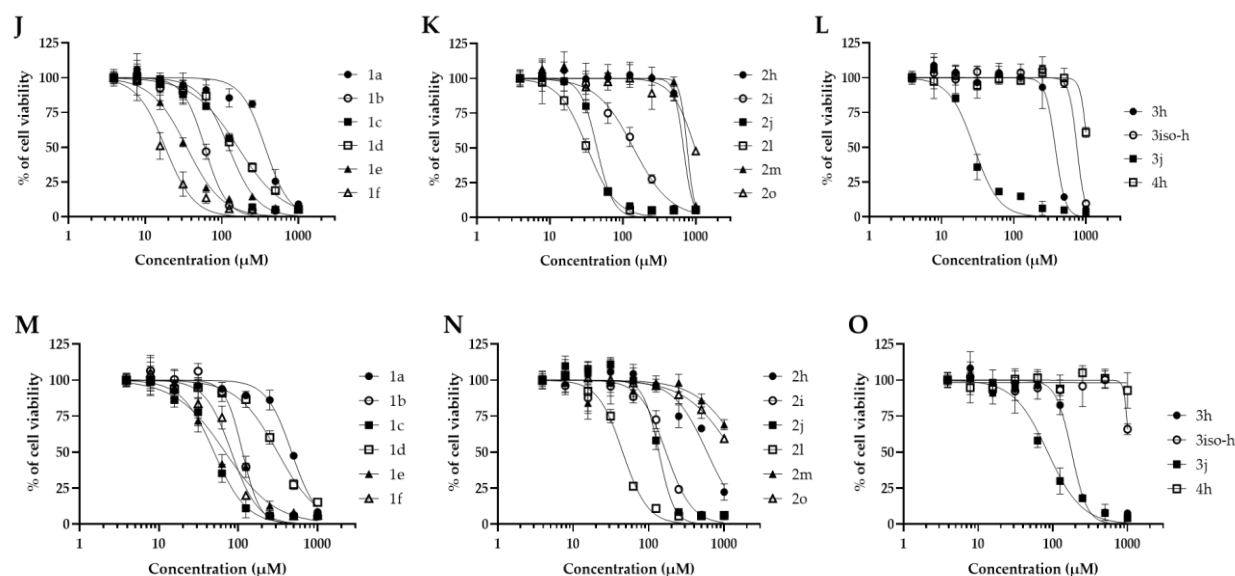


Figure 2. Toxicity of piperidone compounds against human cells. Human cells were exposed to increasing concentrations of piperidone compounds active against *C. neoformans* (1a, 1b, 1c, 1d, 1e, 1f, 2h, 2i, 2j, 2m, 2o, 3h, 3iso-h, 3j, and 4h) for 48h before measurement of the cell viability using resazurin assay. A-C: A498 cells (kidney), D-F: BEAS-2B cells (lung), G-I: Caco-2 cells (intestine), J-L: HaCaT cells (skin), and M-O: HepG2 cell (liver). Curves were fitted using GraphPad® Prism 8 software (means \pm SD, n=3).

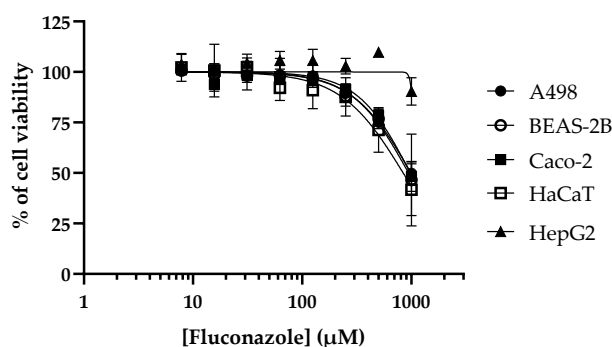


Figure 3. Toxicity of Fluconazole against human cells. Human cells were exposed to increasing concentrations of Fluconazole for 48h before measurement of the cell viability using resazurin assay. Curves were fitted using GraphPad® Prism 8 software (means \pm SD, n=3).

These investigations revealed that **2h**, **2m**, **3iso-h**, and **3h** have the best safety/activity profile with very interesting SI up to 80.5. Importantly, **3h** gave the highest SI values ranging from 5.4 to 80.5 depending on the human cell model considered. Although less active than **3h** (with MIC values on *C. neoformans* of 7.8 and 62.5 μ M, respectively), **2m** showed to be the less toxic among the active compounds, with CC₅₀ ranging from 663.7 to > 1000 μ M and SI ranging from 10.6 to > 16. For comparison, Fluconazole gave SI ranging from 33.6 to >40, close to the ones obtained with the safest compounds of this study.

The spectrum of antifungal activity of the more active compounds (i.e. **2h**, **2m**, **3iso-h**, and **3h**) was further screened using various fungal strains (yeasts and filamentous species) infecting humans or plants (Tables 2 and 3). Regarding human pathogenic yeasts (Table 2), **2h**, **2m**, **3iso-h** and **3h** gave MIC values similar to the ones obtained on *C. neoformans* when tested against *Candida auris* and *C. glabrata* (MIC of 7.8 to 31.2 μ M) but were found less active against *C. albicans* and *C. tropicalis* (MIC of 62.5 to 250 μ M). Regarding filamentous fungi (Table 3), for the human pathogen *Aspergillus fumigatus* and the plant pathogen *A. flavus*, **3h** was the only active compound (MIC of 31.2 μ M), **2h**, **2m**, and

3iso-h being found less or not active (MIC of 125 to > 250 μ M). **2h**, **2m**, **3iso-h** and **3h** were also found less or not active against the plant pathogens *Colletotrichum graminicola*, *Fusarium graminearum*, and *Penicillium verrucosum* (MIC of 125 to > 250 μ M) except for **3h** giving an MIC of 62.5 μ M on *P. verrucosum*. Regarding the plant pathogens *Magnaporthe oryzae* and *Microdochium bolleyi* and the human pathogen *Trichophyton rubrum*, **2h**, **2m**, **3iso-h** and **3h** displayed good activities (MIC of 7.8 to 62.5 μ M) except for **2m** on *M. bolleyi* (MIC >250 μ M) and **3iso-h** on *T. rubrum* (MIC of 125 μ M), **3h** giving the lowest MIC on those strains (i.e. 7.8 μ M). Interestingly, it must be noted that yeasts and filamentous fungi found resistant to Fluconazole (MIC of 250 to >1000 μ M) were still sensitive to **2h**, **2m**, **3iso-h** and **3h**. Thus, again, **3h** gave the best antifungal activity with the lowest MIC values for each strain.

Table 2. Activity of piperidone compounds against pathogenic yeasts. The MIC values (in μ M) of piperidone compounds **2h**, **2m**, **3h**, and **3iso-h** were measured using various yeast models as explained in Materials and Methods section. Fluconazole was used as antifungal control.

Compound	<i>C. albicans</i>	<i>C. auris</i>	<i>C. glabrata</i>	<i>C. tropicalis</i>	<i>C. neoformans</i>
2h	250	31.2	31.2	250	31.2
2m	125	31.2	15.6	250	62.5
3h	62.5	7.8	7.8	125	7.8
3iso-h	250	31.2	62.5	125	62.5
Fluconazole	6.25	500	250	1000	25

Table 3. Activity of piperidone compounds against pathogenic filamentous fungi. The MIC values (in μ M) of piperidone compounds **2h**, **2m**, **3h**, and **3iso-h** were measured using various filamentous fungi models (human pathogens or plant pathogens) as explained in Materials and Methods section. Fluconazole was used as antifungal control.

Compound	<i>A. flavus</i>	<i>A. fumigatus</i>	<i>C. graminicola</i>	<i>F. graminearum</i>	<i>M. oryzae</i>	<i>M. bolleyi</i>	<i>P. verrucosum</i>	<i>T. rubrum</i>
2h	>250	125	>250	>250	15.6	62.5	125	62.5
2m	>250	250	>250	>250	62.5	>250	>250	31.2
3h	31.2	31.2	250	125	7.8	31.2	62.5	7.8
3iso-h	>250	125	125	>250	15.6	62.5	125	125
Fluconazole	>1000	>1000	50	>1000	12.5	>1000	>1000	>1000

Importantly, the twenty-three piperidone compounds were tested against Gram-positive and Gram-negative bacteria belonging to the ESKAPE group and were found inactive with MIC > 250 μ M for all compounds, except **2k** giving an MIC of 250 μ M on the Gram-negative bacteria *A. baumannii* and *E. cloacae* and **2l** and **3j** giving an MIC of 250 μ M on the Gram-positive bacteria *S. aureus*. This demonstrates that the compounds possess an antimicrobial activity selectively directed against yeasts and fungi that can be explained by the specific targeting of fungal enzyme(s).

2.3. Molecular Modeling

It has been demonstrated that similar compounds are able to interact and inhibit cytochrome P450 14 α -sterol 14-demethylase (CYP51), an enzyme involved in the ergosterol biosynthesis in fungal.[15] Docking analysis was therefore performed to evaluate the affinity of best piperidones molecules with sterol 14-demethylase using its crystal structure (PDB code: 3KHM). Except for piperidone **2m** which contains three butoxy groups, the results indicated that compounds **2h**, **3h**, **3iso-h** effectively interacted in the active site of CYP51 complex. The docking binding energy for **3h** score was found -8,2 kcal/mol similar to that found with Fluconazole (binding energy = -8,1 kcal/mol) which supports the fact that **3h** may act through inhibition of ergosterol synthesis as previously shown for other piperidone compounds described in the literature.[11,15] In this molecular docking

study, all docked compounds (**2m**, **2h**, **3h**, **3iso-h**) formed interactions with Hem300 and hydrophobic interactions with numerous amino-acids residues (Table 4 and Figure 5-8). These hydrophobic interactions with butoxy chains may explain the difference in activity between **3h** (MIC=7.8 μ M) and its methyl analogue **3a** (MIC > 250 μ M). In addition, piperidones **2h** and **3h** revealed T-shaped interactions with Tyr103 whereas **3iso-h** showed T-shaped interactions with His294 and Phe290.

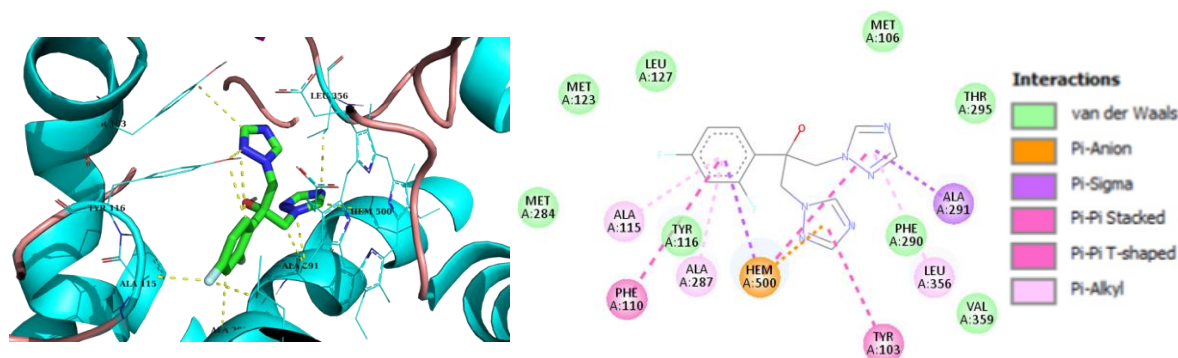


Figure 4. Best binding mode of Fluconazole into the active site of CYP51. Docking score: -8.1 kcal/mol.

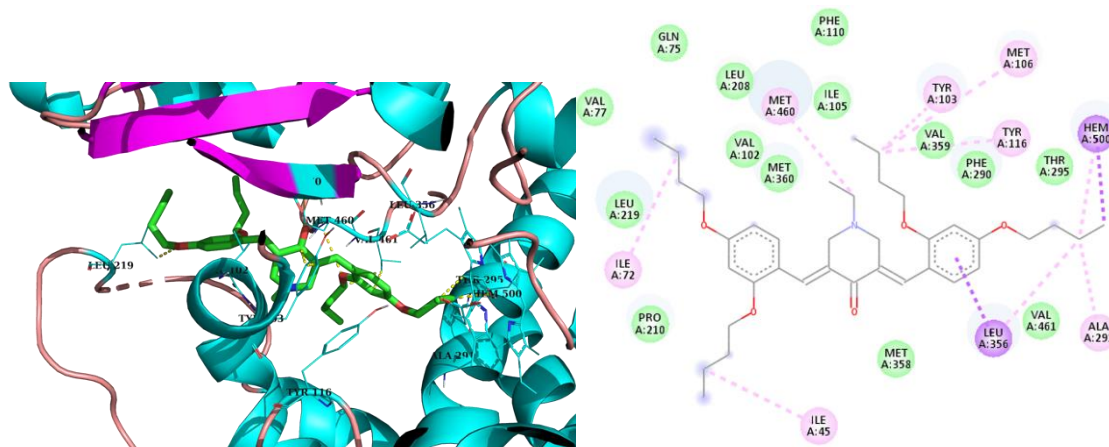


Figure 5. Best binding mode of **2h** into the active site of CYP51. Docking score: -7.8 kcal/mol.

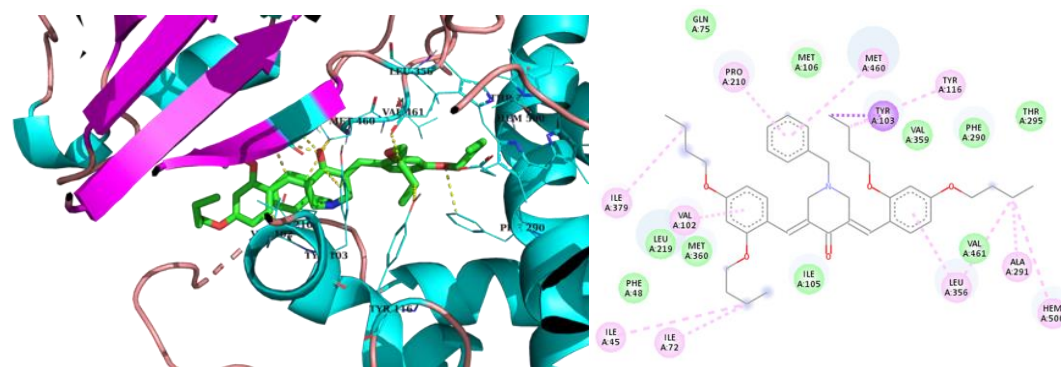


Figure 6. Best binding mode of **3h** into the active site of CYP51. Docking score: -8.2 kcal/mol.

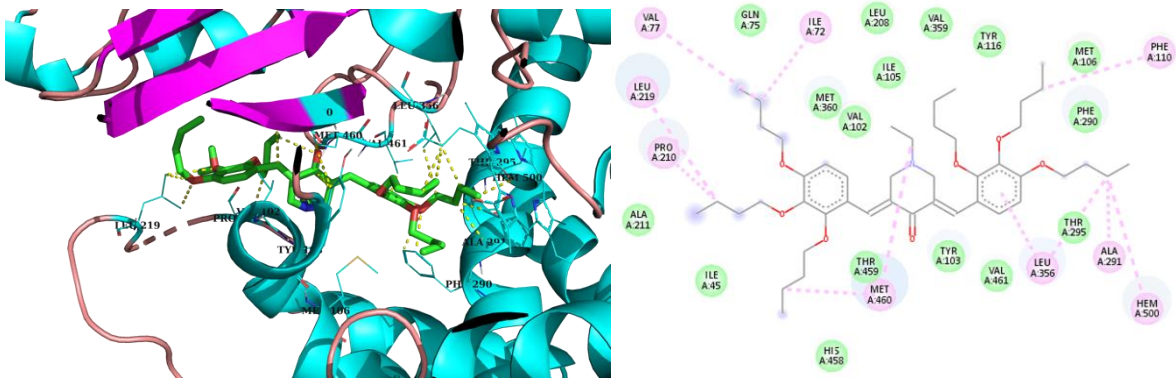


Figure 7. Best binding mode of 2m into the active site of CYP5. Docking score: -7.2 kcal/mol.

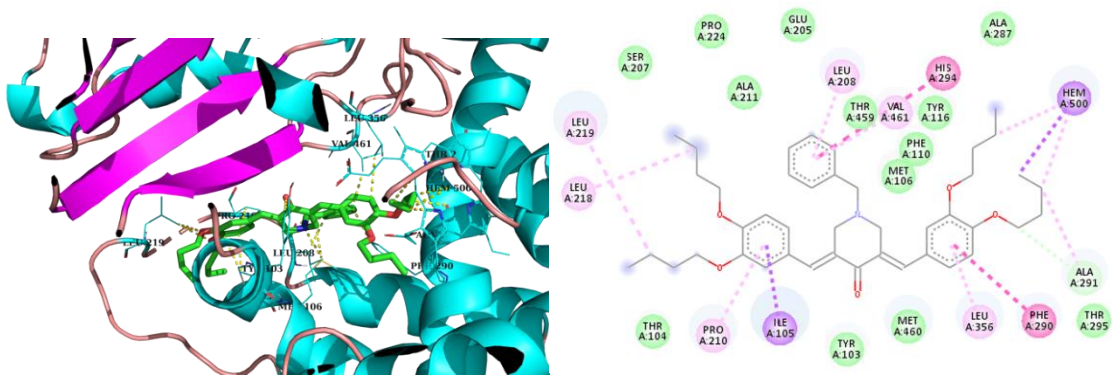


Figure 8. Best binding mode of 3iso-h into the active site of CYP51. Docking score: -8.8 kcal/mol.

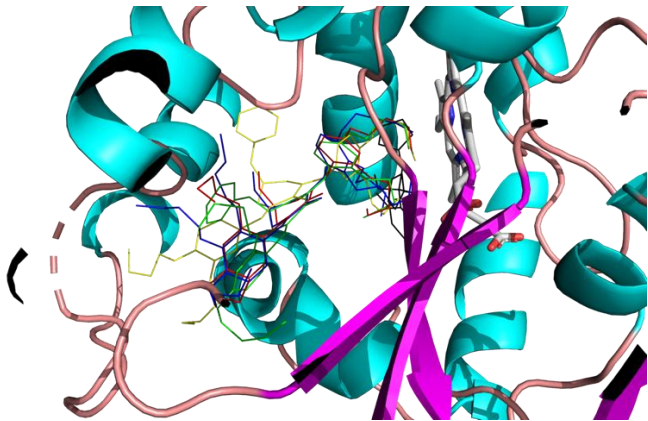


Figure 9. Best binding mode of 2h(red), 3h(green), 2m(blue), 3iso-h(yellow) and Fluconazole (black) into the active site of CYP51.

Table 4. Interaction parameters. The interaction analysis of the sterol 14 α -demethylase protein on the basis of molecular docking studies with piperidones **2h**, **3h**, **2m**, **3iso-h**.

Compound	Binding energy kcal/mol	Hydrophobic interactions with amino acids	Aromatic interactions
Fluconazole	-8.1	Phe110, Tyr116, Leu127, Ala287, Ala291, Leu356	T-Shaped (Tyr103), Hem500
2h	-7.8	Tyr103, Met106, Tyr116, Leu219, Phe290, Ala291, Leu356, Met360, Met460	Hem500 T-Shaped (Tyr103),
3h	-8.2	Val102, Tyr103, Ile105, Pro210, Phe290, Ala291, Leu356, Met360, Met460	Hem500 T-Shaped (Tyr103),
2m	-7.2	Ile72, Tyr103, Phe110, Pro210, Leu219, Phe290, Thr295, Leu356, Met360, Met460, Val461	Hem500
3iso-h	-8.8	Ile105, Met106, Leu208, Pro210, Leu219, Phe290, Ala291, Leu356, Thr459, Val461	Hem500 T-Shaped (His294, Phe290),

3. Discussion

C. neoformans is an important human pathogen but, other fungi should be considered when developing antifungal drugs. *Candida* sp infect mainly immunocompromised patients but also immunocompetent ones leading every year to more than 1.5 millions bloodstream infection or invasive candidiasis and around 900,000 deaths.[20] In addition to *C. albicans*, among the various *Candida* sp infecting humans, *C. auris*, *C. glabrata*, and *C. tropicalis* have attracted more attention due to their increasing prevalence and their ability to develop resistance to antifungal drugs used in medicine, including fluconazole.[21–25] Similarly, *Aspergillus fumigatus* is responsible for infection mainly in immunocompromised patients and is estimated to be responsible for over 600,000 deaths annually.[26] Although not infecting humans, fungi infecting plants and crops are also important to consider when developing antifungal since they cause massive losses (for example, *Fusarium graminearum* only causes every year losses of around 28 million metric tons of wheat grain valued at \$5.6 billion[27] and/or produce mycotoxins harmful for humans and animals).[28] When testing the spectrum of antifungal activity of the best piperidone compounds, data showed that in addition to possess good activity against *C. neoformans*, **2h**, **2m**, **3iso-h**, and **3h** demonstrated also antifungal activity against important humans and plants pathogens, such as *C. albicans*, *C. auris*, *C. glabrata*, *A. fumigatus*, and *T. rubrum* but also *A. flavus*, *M. oryzae*, *M. bolleyi*, and *P. verrucosum*. In all case, **3h** was identified as the most active piperidone compound with MIC ranging from 7.8 to 62.5 μ M. Importantly, fungi resistant to Fluconazole were found still sensitive to **2h**, **2m**, **3h**, and/or **3iso-h**. This observation is not in contradiction with the docking data indicating that these molecules target ergosterol biosynthesis like Fluconazole. Indeed, the resistance to Fluconazole is not due to mutation in its target but classically involves a reduction of its entry and/or an increase in its efflux in resistant strains.[29] The fact that strains resistant to Fluconazole remain sensitive to **2h**, **2m**, **3h**, and/or **3iso-h** is of major importance due to the growing incidence of Fluconazole resistance in fungal strains infecting humans including *Candida* species and *A. fumigatus*.[30,31]

4. Materials and Methods

4.1. Chemistry

All reagents and solvents were purchased from Aldrich, FluoroChem or TCI and used as received without further purification. Mass spectroscopy was performed by the Spectropole of Aix-Marseille University. ESI mass spectral analyses were recorded with a 3200 QTRAP (Applied Biosystems SCIEX) mass spectrometer. The HRMS mass spectral analysis was performed with a QStar Elite (Applied Biosystems SCIEX) mass spectrometer. Elemental analyses were recorded with a Thermo Finnigan EA 1112 elemental analysis apparatus driven by the Eager 300 software. ^1H and ^{13}C NMR spectra were determined at room temperature in 5 mm o.d. tubes on a Bruker Avance 400 spectrometer or a Bruker Avance 300 spectrometer of the Spectropole: ^1H (400 MHz), ^1H (300 MHz), ^{13}C (100 MHz) and ^{13}C (75 MHz). The ^1H chemical shifts were referenced to the solvent peak CDCl_3 (7.26 ppm) or DMSO_d6 (2.50 ppm) and the ^{13}C chemical shifts were referenced to the solvent peak CDCl_3 (77.16 ppm) or DMSO_d6 (39.52 ppm). All compounds were prepared with analytical purity up to accepted standards for new organic compounds (>98%) which was checked by high field NMR analysis.

N-alkylpiperidin-4-one (10 mmol) was dissolved in 20 mL of ethanol, benzaldehyde derivative (20 mmol) and 40% of NaOH (2 equiv.) were then added at 0°C . The solution was stirred at room temperature overnight. The yellow precipitate was filtrated off, washed with cold ethanol and dried under vacuum (48-86% yield). Spectral data, structure, and NMR spectra of the compounds are given in the supplementary materials.

4.2. Antimicrobial Activity

The antifungal effect of compounds was measured following the reference methods for yeasts (NCCLS M27-A) and molds (M38-P) as previously described. [32] Reference yeast strains tested were *Candida albicans* (DSM 10697), *C. auris* (DSM 21092), *C. glabrata* (DSM 11226), *C. tropicalis* (DSM 9419), and *Cryptococcus neoformans* (DSM11959). Filamentous fungi tested were either human pathogens (i.e. *A. fumigatus* (DSM 819) and *Trichophyton rubrum* (DSM16111)) or plant pathogens (i.e. *Aspergillus flavus* (DSM 1959), *Colletotrichum graminicola* (DSM 63127), *Fusarium graminearum* (DSM 1095), *Magnaporthe oryzae* (gift from Richard O'Connell UMR Bioger, Paris Saclay, (DOI: 10.1094/Phyto-82-421)), *Microdochium bolleyi* (DSM 62073), and *Penicillium verrucosum* (DSM 12639)). Yeast suspensions were prepared by resuspending colonies collected from PDA plates in sterile NaCl 0.9% solution. Yeasts were then diluted at $1-2 \times 10^3$ yeasts/mL in RMPI media supplemented with glucose (1,8%) buffered with MOPS (final concentration of 0.165 mol/L (pH 7.0)). For filamentous fungi, conidi were collected from mycelium grown on PDA plates using sterile solution of NaCl 0.9% supplemented with Tween at 0.1%. After counting under microscope, dilution at $2-3 \times 10^4$ conidi/mL were also prepared in MOPS-buffered RMPI media supplemented with glucose. Diluted yeast or fungi were then exposed to increasing concentrations of compounds (1/2 serial dilution) in 96-wells plates, pure DMSO (maximal concentration of 1%) being used as negative control. Plates were incubated at 35°C for 24-48 h for yeasts and filamentous fungi infecting humans (i.e. *A. fumigatus* and *T. rubrum*) or 25°C for 48-72 h for the other filmantous fungi from the environment or infecting plants. Minimum Inhibiting Concentrations (MIC) were determined as the lowest concentrations of compounds totally inhibiting the growth of the fungi. The antibacterial effect of the compounds was measured through liquid MIC determination using Gram-negative (*Acinetobacter baumannii* (DSM 30007), *Enterobacter cloacae* (DSM 30054), *Klebsiella pneumoniae* (DSM 26371), and *Pseudomonas aeruginosa* (ATCC 9027) and Gram-positive (*Enterococcus faecalis* (DSM 2570), *Enterococcus faecium* (DSM 20477), and *Staphylococcus aureus* (ATCC 6538) bacteria from the ESKAPE group following the National Committee of Clinical Laboratory Standards (NCCLS, 1997) procedure as previously described. [33] In all case, MIC were determined in n=2-3.

4.3. Cytotoxicity Studies

The toxicity of the compounds was tested using human cells (i.e. A498 (human kidney cell line), BEAS-2B (normal human airway epithelial cells), Caco-2 (human intestinal cell line), HaCaT (normal human skin cells), and HepG2 (human liver cell line)) as previously described [34]. Cells were maintained at 37°C in a 5% CO₂ incubator in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal calf serum (FCS), 1% L-glutamine and 1% antibiotics (all from Invitrogen). Cells grown on 75 cm² flasks were detached using trypsin-EDTA solution (ThermoFisher), counted using Mallasez's chamber, and seeded into 96-well cell culture plates (Greiner Bio-one) at approximately 10⁴ cells per well. After 24-48h, when the cells reached 80-90 % confluence, wells were aspirated and increasing concentrations of compounds (from 0 to 1000 µM, ½ dilution) were added to the cells, DMSO (maximal concentration of 1%) being used as negative control. After 48 h incubation at 37°C in a 5% CO₂ incubator, wells were aspirated, and the cell viability was measured by adding 100 µL of resazurin solution at 0.03 mg/mL final in phosphate buffer saline with calcium and magnesium chloride (PBS⁺⁺). After 1 h incubation at 37°C, the fluorescence intensity of the wells was measured using a microplate reader (SynerMix, Biotek, Ex 530 nm/Em 590 nm). The fluorescence values were normalized by the negative controls (DMSO treated cells) and expressed as the percentage of cell viability. The cytotoxic concentrations 50, (i.e. CC₅₀) of compounds corresponding to the concentrations causing a reduction of 50% of the cell viability were calculated using GraphPad® Prism 8 software (n=3).

4.4. Molecular Docking

All the ligands were fully optimized with the Gaussian16 Rev. A03 package at the HF/6-31G(d) level of theory. The atomic charges were computed at the HF/6-31G(d) level of theory with the RESP scheme. For docking studies, X-ray crystal structure of sterol 14 α -demethylase (CYP51) from *Trypanosoma cruzi* in complex with inhibitor fluconazole was obtained from the Protein Data Bank (PDB), (PDB code: 3KHM). The enzyme was prepared by removing fluconazole, then adding hydrogen atoms and atomic charges with AutoDock Tools software. The molecular docking studies were carried out with the AutoDock 4.2.6 software. The docking box set at 70 × 70 × 70 Å and was centered at x = 2.270 Å, y = -23.537 Å and z = 16.924 Å (center of fluconazole). The Lamarckian Genetic Algorithm with default parameters was applied for simulation. The number of docking runs was 20. The pose drawings and interaction calculations were performed with ProteinPlus and BIOVIA Discovery Studio. The 3D active sites were drawn with PyMOL. Details of molecular docking analysis are given in the supplementary materials.

5. Conclusions

This study highlights the potential of 1,5-diarylidene-4-piperidones as effective antifungal agents, particularly against *Cryptococcus neoformans* and other clinically significant fungi. Among the synthesized compounds, **3h** emerged as the most promising candidate, displaying superior antifungal potency. Its ability to inhibit fluconazole-resistant strains further underscores its therapeutic value. Docking studies suggest that these compounds act by targeting sterol 14 α -demethylase, disrupting ergosterol biosynthesis in fungal cells. Additionally, their broad-spectrum antifungal activity and low cytotoxicity support their potential application in both medical and agricultural settings. Further in vivo studies and clinical evaluations are necessary to fully explore their therapeutic potential and to address the growing need for new antifungal drugs to combat resistant fungal pathogens.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Supplementary File S1: Spectral data, NMR spectra and Molecular Docking. References [x-x] are cited in the supplementary materials

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