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Design, Synthesis and Bio-evaluation of C-1 Alkylated Tetrahydro-β-carboline Derivatives as Novel Antifungal Lead Compounds

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ABSTRACT

The field of antifungal agent has become static and development of resistance by the pathogen as well as limited clinical efficacy of marketed drugs demand the constant development of new antifungals. The presence of hydrocarbon chain of specific length linked with various different heterocycles was found to be an important structural feature in various antifungal lead compounds. Based on the prominent antimicrobial activity of β-carboline derivatives, a set of C1 alkylated tetrahydro-β-carboline derivatives were proposed to be active against fungi. To validate and confirm the role of suitable alkyl chains linked to a β-carboline scaffold, few related analogues having C1 aryl substituents were also synthesized in one step *via* classic Pictet-Spengler reaction. The synthesized library was evaluated for its antifungal activity against *C. albicans*, *C. krusei*, *C. parapsilosis*, *C. kefyr*, *C. glabrata*, *C. tropicalis* and *C. neoformans.* One of the library members (compound **12c**), with *n*-alkyl chain of eight carbons exhibited potent antifungal activity against *C. glabrata* and *C. kefyr*. The lead compound, being selectively toxic also demonstrated prominent synergy enhancing the potency of antifungal drugs up to 10-fold. The time kill kinetic studies confirmed the efficacy of compound **12c**, where the results obtained were comparable to that of Amp B. FE-SEM analysis revealed the increased asymmetry, disintegration and roughness of cell surface which could be because of the possible interaction of compound **12c** at membrane level or interference in cell wall structure. Apoptosis/necrosis detection assay confirmed the significant apoptotic activity in *C. glabrata* cells after **12c** treatment which was responsible for the rapid killing of *C. glabrata* cells.

Key words: Antifungals, Pictet-Spengler, β-carboline, *Candida glabrata*, Synergistic effect, SAR, Apoptosis

In recent years, fungal infections have emerged as a major cause of death in immunocompromised patients and cause ~ 1.4 million death globally per years [1]. Most common human fungal pathogens include *Candida albicans* and non-albicans species namely, *Candida krusei* (*C. krusei*), *Candida parapsilosis* (*C. parapsilosis*), *Candida kefyr* (*C. kefyr*), *Candida tropicalis* (*C. tropicalis*), *Candida glabrata* (*C. glabrata*) and *Cryptococcus neoformans* (*C. neoformnas*) representing 4th leading cause of nosocomial disease and accounting for ~75% of all fungal infections [2]. Although a large number of antifungal agents are discovered, the pathogenic fungi are constantly developing resistance to these drugs [3]. Overall, the field of antifungal agent has become static and there are only four major groups of antifungals currently available in the market namely; azoles, polyenes, allylamines and echinocandins [4]. Many of these antifungal agents are associated with drawbacks such as development of resistance, side effects, limited clinical efficacy and poor bioavailability [5] which demand the constant development of new antifungals. Recently well-known clinical antifungals such as amphotericin B and fluconazole were also found less potent due to the development of resistance against the most common fungal pathogens like *C. albicans*, *C. glabrata*, and *C. neoformans*. [6].

During our recent investigation towards the synthesis of tetrahydro-β-carboline (THβC) derivatives as potent indoleamine 2,3-dioxygenase (IDO1) inhibitors [7-8], it was observed that few IDO1 inhibitors demonstrated promising antifungal activities. In particular, it was interesting to observe potent antifungal activity of novel alkylated azole **1** which demonstrated the inhibition of 14α-demethylase enzyme involved in ergosterol biosynthesis. [9]. The presence of such hydrocarbon chain of specific length linked with various different heterocycles was found to be an important structural feature in various other antifungal lead compounds. These structures were proposed to be active using structure-based de novo

design based on the structure of lanosterol 14α-demethylase (CYP51) of fungi. Most active analogues in this series include 7-hydroxy-2-heptyl-4-oximinochromane **2** [10], 2-decyl-tetrahydroisoquinoline-6,7-diol **3** [11] and 5-methoxy-*N*-nonyl-tetrahydronaphthalen-2-amine **4** [12] **(Figure 1).**

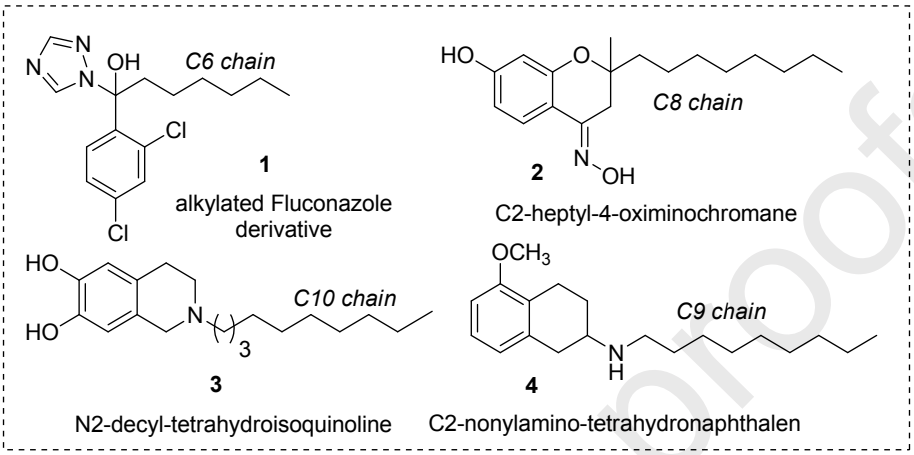


Figure 1. Alkylated heterocycles showing antifungal activities.

It was also observed that, various β-carboline derivatives are also known for their antifungal activities. Specifically, the 9-(4-chlorophenoxy)propyl)-6-fluoro-tetrahydro-β-carboline **5** (**Figure 2**) showed promising in vitro antifungal activity and also exhibited good fungicidal activity against both fluconazole sensitive and resistant *C. albicans* and demonstrated potent inhibition against *C. albicans* hyphal growth and biofilm formation [13].

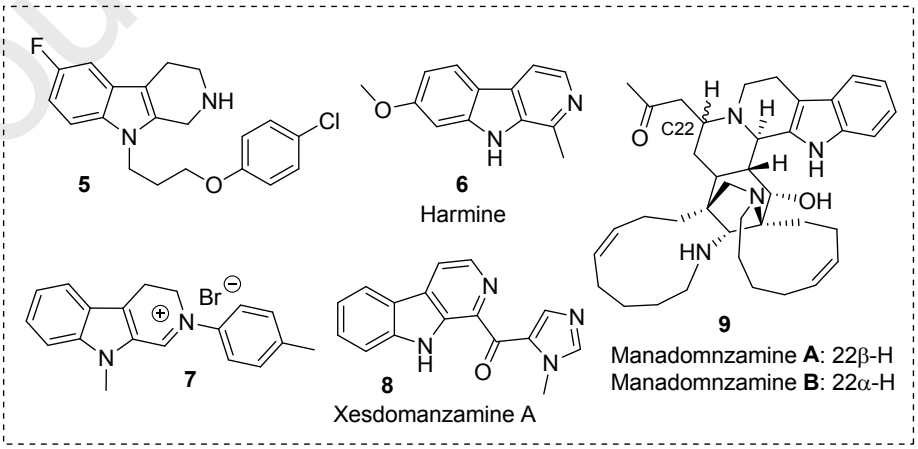


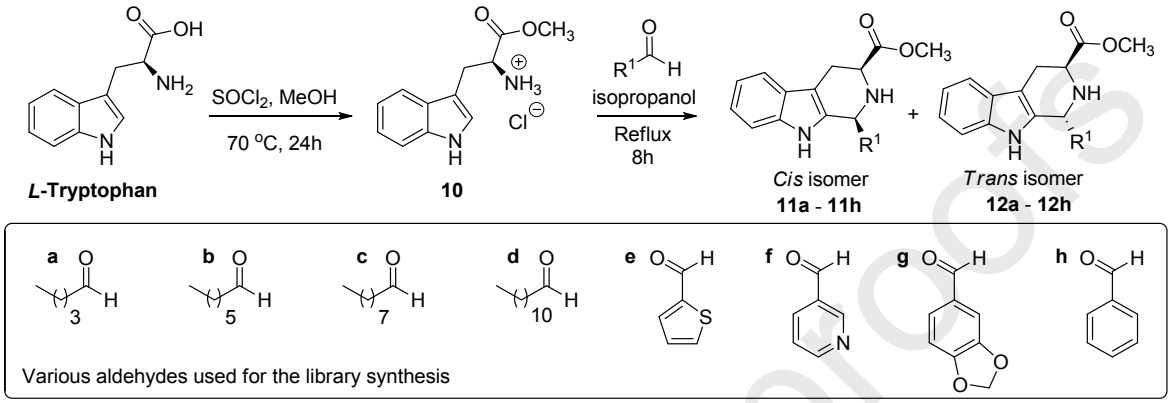
Figure 2. β-Carboline derivatives showing antifungal activities.

Harmine **6** was recommended as new antimicrobial biorational by Nenaah based on its potent activity against *Proteus vulgaris*, *Bacillus subtilis* and *C. albicans* [14]. Recently, Zhou *et al.* demonstrated 2-phenyl-3,4-dihydroisoquinolin-2-ium **7** as promising lead compound for the development of new antifungal agents [15]. Xestomanzamine A (**8**) and two novel manzamine type alkaloids, named Manadomanzamines A (**9A**) and B (**9B**), isolated from an Indonesian sponge Acanthostrongylophora *spp.* (Haplosclerida: Petrosiidae) having β-carboline skeleton also demonstrated potent antimicrobial activities [16]. Interestingly, the C22β-H isomer **9A** was found to be selectively active against *C. albicans,* whereas the C22α-H isomer **9B** was active against *C. neoformans*.

Considering the importance of β-carboline scaffold, specific stereochemistry and heterocycle linked with appropriate alkyl chain for determining the antifungal activity, a set of C1 alkylated tetrahydro-β-carboline derivatives were proposed to be active against fungi. The construction of tetrahydro-β-carboline chemotype from L-tryptophan also provide an opportunity to synthesize *cis*- and *trans* diastereomers to understand the effect of specific stereochemistry on the biological activity.

Tetrahydro-β-carboline (THβC) framework is observed in various indole based natural products. Specifically, C-1 substituted optically active THβC derivatives have attracted considerable interest of synthetic chemist owing to the presence of this scaffold in various biologically active natural products and marketed drugs [17]. Pictet Spengler reaction discovered in 1911 is one of the most efficient, reliable and shortest routes to synthesize optically active C-1 substituted THβC framework [18]. This reaction proceeds through the formation of imine intermediate between tryptamine and aldehyde followed by intramolecular cyclisation through spiroindole which leads to the generation of THβC adduct

[19]. We planned to synthesize various C-1 substituted THβC derivatives wherein, L-tryptophan methyl ester was used instead of Tryptamine, so as to obtain both *cis* and *trans* THβCs to study the effect of specific stereochemistry at C1 and C3 position on the anti-fungal activity.



Scheme 1. Synthesis of **11a-11h** (*cis*) and **12a-12h** (*trans*).

L-Tryptophan was refluxed with $SOCl_2$ in methanol to provide L-tryptophan methyl ester hydrochloride **10** in 95% yield (**Scheme 1**) [20]. Having the compound **10** in hand, next we envisioned to synthesize both *cis* and *trans* isomers of THβC derivatives in one step with high yield and approximate equal ratio (*cis*:*trans* ~ 50/50) for the ease of chromatographic separation. In the Pictet-Spengler reaction of L-Tryptophan methyl ester with aldehydes or ketones, the *cis* isomer is predominantly formed under kinetically controlled condition. Whereas, the selectivity is transferred towards *trans* isomer under thermodynamically controlled condition, which exclusively depend upon the nature of reagents, solvents, reaction time and temperature [21]. We recently observed that the Pictet-Spengler reaction of L-tryptophan methyl ester hydrochloride with various aldehydes in isopropanol in 8 hours of reflux quantitatively resulted in the formation of both the isomers with low cis/trans selectivity [20]. Following the optimized protocol, a library of THβC derivatives (**11a** – **11h** and **12a** – **12h**) was synthesized in moderate to good yield (50-70%) and with almost equal

propositions. The diastereomers were purified by column chromatography using 230 - 400 mesh silica gel and the formation of desired product was confirmed by IR, ^{1}H-NMR,^{13}C-NMR and mass spectrometry. The purity of the synthesized library was confirmed by HPLC.

To check the validity of our hypothesis, C1-substituted THβC derivatives **11a-h** and **12a-h** were tested for their *in vitro* antifungal activity against seven fungal strains i.e *C. albicans*, *C. krusei*, *C. parapsilosis*, *C. kefyr*, *C. glabrata*, *C. tropicalis*, *C. neoformans* using amphotericin B (Amp B) as a positive control (**Table 1**). The preliminary *in vitro* antifungal activity confirmed that the trans-isomer **12c** bearing an eight-carbon linear aliphatic chain at C1 position exhibited significant antifungal activity. The respective *cis*-isomer (**11c**) was also found to be active but was active against only *C. neoformans* having MIC value 39.02 μM. Compound **12c** exhibited multi-spectrum antifungal activity against seven fungal strains and showed promising activity against *C. kefyr* and *C. glabrata* with MIC values of 9.70 μM for both. This observation confirmed the effect of specific stereochemistry at C1 and C3 position on the observed biological activity. The antifungal activity of other C1 aliphatic chain bearing compounds **11a**, **12a**, **11b**, **12b** (with C1 substituent chain length of less than eight carbons) and **11d**, **12d** (with C1 substituent chain length of more than eight carbons) was much lower than **11c** and **12c** suggesting the importance of substituent with appropriate chain length at C1 position. Haitao *et al.* [22] previously described the presence of narrow hydrophobic cleft at S3 subunit of CYP51, making stronger non-covalent interaction with lipophobic *n*-alkyl chains. Our observation suggested the possibility of interaction of compound **12c** at the S3 subunit of CYP51. No antifungal activity was observed for compounds bearing C1 aromatic and heteroaromatic substituents (**11e-h** and **12e-h**) confirming the significance of C1 aliphatic substituent for the antifungal activity.

Table 1. *In-vitro* antifungal activity. MIC in μM (μg/mL) of **11a-11h** (c*is*) and **12a-12h** (t*rans*).

Comp.	*Candida albicans*	*Candida krusei*	*Candida Tropicalis*	*Candida parapsilosis*	*Candida kefyr*	*Candida glabrata*	*Cryptococcus neoformans*
11a	>250 (>80.70)	>250 (>80.70)	>250 (>80.70)	>250 (>80.70)	>250 (>80.70)	>250 (>80.70)	>250 (>80.70)
12a	>250 (>80.70)	156.25 (50.70)	>250 (>80.70)	156.25 (50.40)	156.25 (50.44)	>250 (>80.70)	>250 (>80.70)
11b	>250 (>87.72)	>250 (>87.72)	>250 (>87.72)	>250 (>87.72)	>250 (>87.72)	>250 (>87.72)	156.25 (54.82)
12b	>250 (>87.72)	>250 (>87.72)	>250 (>87.72)	>250 (>87.72)	>250 (>87.72)	>250 (>87.72)	>250 (>87.72)
11c	>250 (>94.73)	>250 (>94.73)	>250 (>94.73)	>250 (>94.73)	62.5 (23.68)	62.5 (23.68)	**39.06 (14.80)**
12c	**39.06 (14.80)**	**19.57 (7.41)**	**19.57 (7.41)**	**39.06 (14.80)**	**9.70 (3.67)**	**9.70 (3.67)**	**19.57 (7.41)**
11d	>250 (>101.74)	>250 (>101.74)	>250 (>101.74)	>250 (>101.74)	>250 (>101.74)	>250 (>101.74)	>250 (>101.74)
12d	>250 (>101.74)	>250 (>101.74)	>250 (>101.74)	>250 (>101.74)	>250 (>101.74)	>250 (>101.74)	>250 (>101.74)
11e	>250 (>78.09)	>250 (>78.09)	>250 (>78.09)	>250 (>78.09)	>250 (>78.09)	>250 (>78.09)	>250 (>78.09)
12e	>250 (>78.09)	>250 (>78.09)	>250 (78.09)	>250 (78.09)	>250 (78.09)	>250 (78.09)	>250 (78.09)
11f	>250 (>76.84)	>250 (>76.84)	>250 (>76.84)	>250 (>76.84)	>250 (>76.84)	>250 (>76.84)	>250 (>76.84)
12f	>250 (>76.84)	>250 (>76.84)	>250 (>76.84)	>250 (>76.84)	>250 (>76.84)	>250 (>76.84)	>250 (>76.84)
11g	>250 (>87.64)	>250 (>87.64)	>250 (>87.64)	>250 (>87.64)	>250 (>87.64)	>250 (>87.64)	>250 (>87.64)
12g	>250 (>87.64)	>250 (>87.64)	>250 (>87.64)	>250 (>87.64)	>250 (>87.64)	>250 (>87.64)	>250 (>87.64)
11h	>250 (>76.59)	>250 (>76.59)	>250 (>76.59)	>250 (>76.59)	>250 (>76.59)	>250 (>76.59)	>250 (>76.59)
12h	>250 (>76.59)	>250 (>76.59)	>250 (>76.59)	>250 (>76.59)	125 (>76.59)	>250 (>76.59)	>250 (>76.59)
Amp B	1.56 (1.44)	0.78 (0.72)	0.78 (0.72)	1.56 (11.55)	12.50 (11.55)	0.39 (0.36)	0.78 (0.72)

The repeated treatment or long time use of antifungal drugs in clinic has led to the development of resistance against various fungal strains. A combination therapy with synergistic effect is an emerging approach to tackle this prevailing problem, in which potency of a particular drug/compound can be altered under the influence of another compound. *C. glabrata* have emerged as the second most common cause of candidacies after *C. albicans*.

Therefore, the synergistic activity of the lead compound (**12c**) was studied with known antifungals amphotericin B (Amp B), fluconazole (FLC) and ketoconazole (KTZ) against *C. glabrata* by checkerboard method. *In vitro* activity of **12c** was evaluated with combination of marketed antifungal drugs such as Amp B, KTZ and FLC against *C. glabrata* strain (**Table 2**). The effect is considered to be synergistic if fractional inhibitory concentration index [FICI = (MIC of drug in combination/MIC of drug alone + MIC of tested compound in combination/MIC of tested compound alone)] ≤ 0.5. Interestingly, approximately 8- and 11-fold enhancement of antifungal activity was observed for **12c** and drug Amp B against *C. glabrata*, respectively with FICI value of 0.24, suggesting a strong synergy between both **12c** and Amp B. The result observed is quite interesting as very low dosage of highly toxic Amp B is required in the combination. In case of Fluconazole (FLC), the MIC value of **12c** and FLC was lowered to ~4 fold, and FIC value of 0.48 also indicated the effective synergy between FLC and compound **12c**. In the combination of **12c** and KTZ, the drug required to inhibit the growth of *C. glabrata* decreased to ~9 fold and the amount of lead compound **12c** required was reduced to ~4 fold. The calculated fractional inhibitory concentration of 0.38 for the combination of KTZ and **12c** also confirmed the effective synergy among these drugs.

Table 2. Synergistic study of compound **12c** with known antifungals (Amp B = Amphotericin B, FLC = Fluconazole, KTZ = Ketoconazole) against *C. glabrata*

MIC (μM)	MIC (μM)	MIC (μM)	FIC index	Effect
Amp B 0.78	**12c** 9.71	**Amp B + 12c** 0.07 + 1.21	0.24	Synergy
FLC 13.07	**12c** 9.71	**FLC + 12c** 3.26 + 2.42	0.48	Synergy
KTZ 15.06	**12c** 9.71	**KTZ + 12c** 1.63 + 2.42	0.35	Synergy

Antimicrobial compounds are intended to have selective toxicity against the microbes under investigation. Mammalian cell toxicity of lead compound **12c** (up to 50 μM concentration)

was evaluated against normal human embryonic kidney cell lines (HEK-293) and cervical cancer cell lines (HeLa) by performing MTT assay. At 10 µM, the cytotoxicity of compound **12c** was observed to be 10% and 14% against normal HEK-293 and HeLa cell lines, respectively (**Figure 3**). Even at higher concentration range of **12c** (20µM-50µM), 70-80% of HeLa and HEK-293 cells were viable. It is noteworthy to mention that in the combination study, maximum 2.5 µM of **12c** was used at which more than 95% HEK-293 cells were viable, demonstrating selective toxicity of the lead compound against the fungal strains.

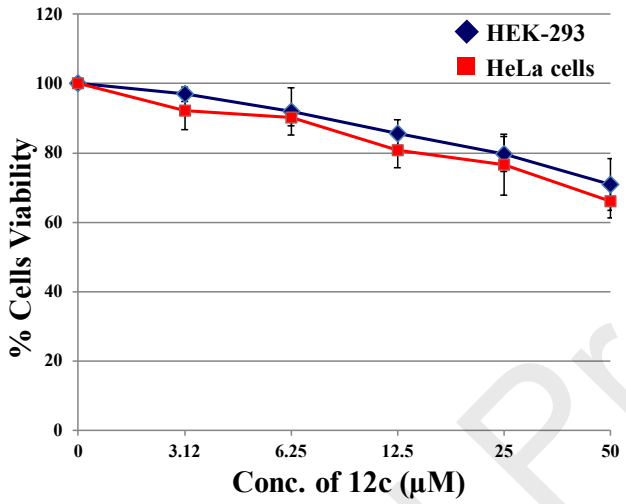


Figure 3. Cell toxicity study of **12c** against HEK-293 and HeLa cell lines.

Time kill assay demonstrate the efficacy of drug/compound by determining the time required to kill the targeted microorganism at MIC. Time kill kinetic study of **12c** was carried against *C. glabrata* at MIC = 9.20 µM (**Figure 4**). Compound **12c** rapidly inhibited the growth of *C. glabrata* and the results were comparable to that of Amp B. In particular, **12c** took 12 hrs to kill the complete colony of *C. glabrata,* on the other hand Amp B took about 4 hrs to achieve the same.

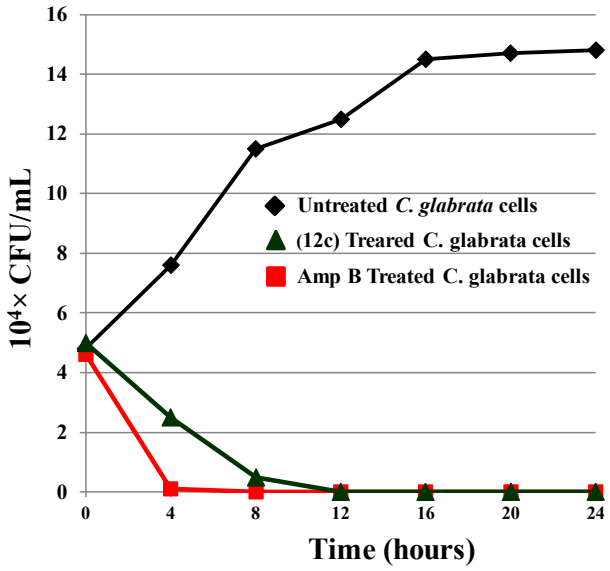


Figure 4. Time kill kinetic study of **12c** and **Amp. B** against *C. glabrata*

Many synthetic as well as natural compounds exhibited antifungal activity by inducing apoptotic/necrotic activity in *Candida spp.* Further, the inhibition of *Candida cells* growth and survival might result from induced apoptotic activity. Therefore, generation of apoptosis by compound **12c** was evaluated by the externalization of phosphatidylserine (PS, apoptosis marker) from the inner leaflet of *C. glabrata* cell membrane. The *C. glabrata* cells treated with compound **12c** for 4 hrs. were compared by using annexin V-FITC and PI staining with untreated cells (**Figure 5**). The annexinV-FITC specifically binds to externalized PS whereas the PI binds to *Candida* cell membrane. Both unstained (**Figure 5A**) and stained (**Figure 5B**) *C. glabrata* cells showed no significant necrosis and apoptosis compared to compound **12c** treated *C. glabrata* cells (**Figure 5C**). A significant apoptotic activity in *C. glabrata* cells after **12c** treatment was clearly observed leading to rapid killing of *C. glabrata* cells.

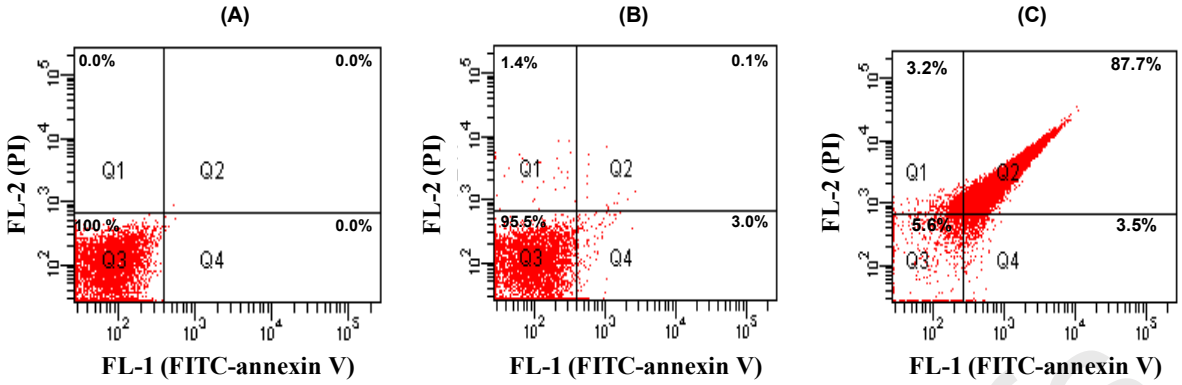


Figure 5. Detection of necrosis/apoptosis marker in compound **12c** treated *C. glabrata* by removal of phosphatidylserine from cell membrane. (A) Unstained *C. glabrata* cells. (B) *C. glabrata* cells + FITC-annexin V + PI and (C) *C. glabrata* cells + FITC-annexin V + PI + compound **12c** (at 09.71 µM). Both treated and untreated *C. glabrata* cells were incubated at 30ºC for 4hrs. in RPMI-1640 media.

Ergosterol, a component of fungal cell membranes, is essential for fungal cell growth. Most of the antifungals either inhibit ergosterol biosynthesis by targeting CYP51 enzyme of the fungi (such as azole drugs) or directly bind or interact with ergosterol (such as Amp B) interfering the functioning and integrity of the fungal cell membrane leading to alterations of cell morphology and subsequent death of fungal cells. The effect of compound **12c** treatment on the morphology of *C. glabrata* cells at MIC was observed using Field Emission Scanning Electron Microscopy (FE-SEM) (**Figure 6**). Increased asymmetry, disintegration and roughness of cell surface was clearly observed which could be because of the possible interaction of compound **12c** at membrane level or interference in cell wall structure.

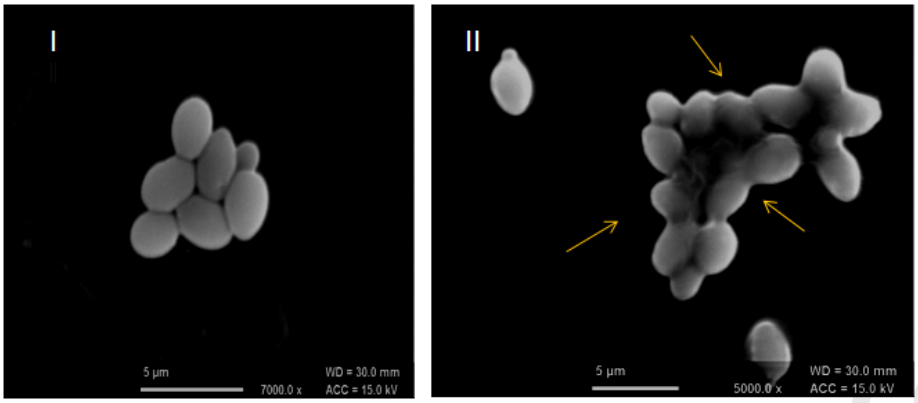


Figure 6. FE-SEM images of control (I) and **12c** treated (II) *C. glabrata*

Overall, a series of C1-alkyl/aryl THβC derivatives with both *cis* and *trans* stereochemistry were synthesized using Pictet-Spengler reaction between L-trytophan and various aliphatic/ aromatic aldehydes in moderate to good yield. A mixture of nitromethane and toluene (1:1) at reflux for 8 hrs was found to be the optimum condition for the desired PS cyclization. The synthesized derivatives were screened for antifungal activity against seven fungal strains. As envisioned, compound **12c** having specific stereochemistry (1R, 3S) at C1, C3 position with *n*-alkyl chain of eight carbons exhibited potent antifungal activity against all the fungal strains. Predominant activity was observed against *C. glabrata* and *C. kefyr* with MIC of 9.20 µM. The *in vitro* antifungal study of **12c** in combination with marketed drugs such as Amp B, KTZ, FLC demonstrated prominent synergy enhancing the potency of both active compound and antifungal drugs up to 10-fold. The FIC of **12c** with Amp, FLC and KTZ was 0.24, 0.48 and 0.35, respectively, confirming the strong synergistic effect. The lead compound **12c** also exhibited selective toxicity against the fungal strains. The cytotoxicity against normal mammalian cells (HEK-293) and cancer cell line (HeLa) was observed within the acceptable range. The time kill kinetic studies confirmed the efficacy of compound **12c**, which killed the given colony of *C. glabrata* in 12 hours and the results were comparable to that of Amp B. A significant apoptotic activity in *C. glabrata* cells after **12c** treatment was clearly observed

leading to rapid killing of fungal cells. FE-SEM analysis revealed the disintegration and roughness of the fungal cell surface. Further SAR investigation on this lead compound to improve the activity is currently ongoing.

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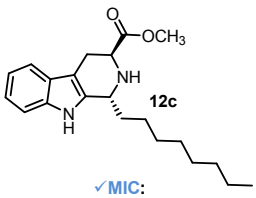
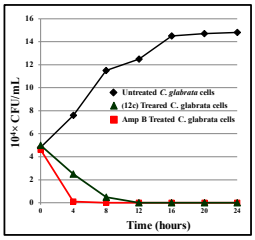
TOC GRAPHIC



✓MIC:
• *C. kefyr* = *9.70* µM (3.67 µg/mL)
• *C. glabrata* = *9.70* µM (3.67 µg/mL)

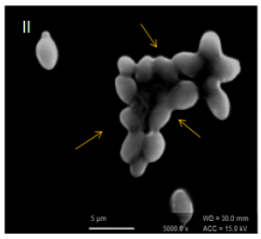
✓FIC Index (Synergy)
• 0.24 for 12c + Amp B
• 0.48 for 12c + FLC
• 0.35 for 12c + KTZ

✓ Time Kill Kinetics



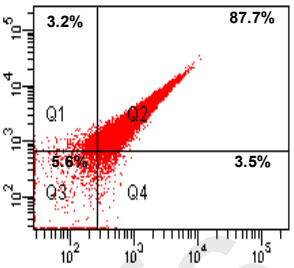
Compound 12c rapidly inhibited the growth of *C. glabrata*

✓ Cell Surface Morphology



after 12c treatment, increased asymmetry and damage of *C. glabrata* cells surface were observed.

✓ Apoptosis/Necrosis Evaluation



12c significantly induced apoptotic-like programmed cell death in *C. glabrata*.