Chemoenzymatic synthesis, nanotization and anti-*Aspergillus* activity of optically enriched fluconazole analogues

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Abstract

Despite recent advances in diagnostic and therapeutic advances in antifungal research, aspergillosis still remains a leading cause of morbidity and mortality. One strategy to address this problem is to enhance the activity spectrum of known antifungals, and we now report the first successful application of *Candida antarctica* lipase (CAL) for the preparation of optically enriched fluconazole analogs. Anti-*Aspergillus* activity was observed for an optically enriched derivative, *(-)-S*-2-(2',4'-difluorophenyl)-1-hexyl-amino-3-(1''',2''',4''') triazol-1'''-yl-propan-2-ol, which exhibits MIC values of 15.6 µg/mL and 7.8 µg/disc in microbroth dilution and disc diffusion assays, respectively. This compound is tolerated by mammalian erythrocytes and cell lines (A549 and U87) at concentrations of up to 1000 µg/mL. When incorporated into dextran nanoparticles, the novel, optically enriched fluconazole analog exhibited improved antifungal activity against *Aspergillus fumigatus* (MIC = 1.63 µg/mL). These results not only demonstrate the ability of biocatalytic approaches to yield novel, optically enriched fluconazole derivatives but also suggest that enantiomerically pure fluconazole derivatives, and their nanotised counterparts, exhibiting anti-*Aspergillus* activity may have reduced toxicity.

Aspergillosis remains a significant threat to public health, and, in spite of continuous efforts to improve timely diagnosis and clinical therapies, mortality caused by this disease remains unacceptably high [1, 2]. Current therapeutic options for treating *Aspergillus*-induced disorders include antifungal agents such as polyenes, azoles and echinocandins [3, 4]. Thus the discovery of new antifungal compounds remains important given the need to address the development of drug resistance in pathogenic fungi [5-7]. One approach to accomplishing this goal is to prepare new derivatives of existing drugs with broad spectrum activity and enhanced pharmacokinetic properties. As part of our on-going efforts to use lipases [8-12], which catalyze reactions with high degree of chemo-, regio- and stereoselectivity in organic synthesis, we became interested in preparing new antifungals using biocatalysis.

Fluconazole, introduced in 1990, is a bis-triazole antifungal drug which possesses interesting pharmacokinetic properties, such as low plasma binding affinity, good water solubility, low first pass metabolism, high oral bioavailability and a long half-life, all of which should make it a drug of choice for treating fungal infections [13, 14]. On the other hand, fluconazole has been reported to exhibit only limited activity against *Aspergillus* infections [15], which has led to many reports concerning the synthesis of various types of fluconazole derivatives and their chiral separation/resolution into constituent enantiomers [16-19]. We now report the use of *Candida antarctica* lipase (CAL-B) in catalysing the addition of amines to an achiral epoxide to yield optically enriched fluconazole analogues in which one of the triazole rings is replaced by *n*-alkylamino and cycloalkylamino substituents. To the best of our knowledge, the work reported herein is the first direct synthesis of optically

enriched fluconazole analogues using biocatalytic methods. *In vitro* assays show that the optically enriched analogues exhibit more potent antifungal activity than the corresponding racemic mixtures. Interestingly, this bioactivity can be enhanced by their encapsulation in dextran-based nanoparticles [20].

Results

Synthesis of fluconazole analogues. A series of linear and cyclic alkylamines was screened for reaction with the epoxide ring of (±)-1-[2-(2, 4-difluorophenyl)-oxiranylmethyl]-1*H*-[1,2,4]-triazole (**1,** Figure 1) in a number of different organic solvents. Three different immobilized lipases were also evaluated for their ability to catalyze this reaction: *Candida rugosa* lipase (CRL), porcine pancreatic lipase (PPL) and CAL-B. Although the ring-opening reactions catalysed by CRL and PPL were of no practical utility, when the reaction was performed in the presence of CAL-B in tetrahydrofuran (THF) as solvent, the desired products (**3a-j**, **5a** and **5b**) were obtained with good yields in optically enriched forms (Figure 1, and Tables S1 and S2 in Supporting Information). Very importantly, all of the twelve novel fluconazole analogues formed in the lipase-catalyzed reactions were optically active showing that aminolysis of the racemic starting epoxide (±)-1 had proceeded in an enantioselective fashion (Table S1). These twelve compounds could also be prepared in racemic form, as viscous oils in 75-80 % yields, by direct reaction of the alkylamines with the racemic epoxide precursor (±)-**1** in THF at 55 ^{o}C. The time taken for complete consumption of aliphatic amines **2a-j**, **4a** and **4b** in the CAL-B catalyzed reaction varied between 18h and 28h, which was considerably shorter than the 48-56 h required for the chemical addition of the amines (Table S2 in Supporting Information). The structures of all twelve fluconazole analogues were

unambiguously established on the basis of spectroscopic data (IR, ^{1}H- and ^{13}C NMR, and mass spectra), and by comparison to literature data for known compounds **3b**, **3c**, **5a** and **5b** [21, 22].

Although the enantiomeric enrichment of the fluconazole analogues prepared by lipase-catalyzed addition was not established, we were able to assign the absolute configuration of the major enantiomer using the optical activity of the unreacted epoxide isolated from the reaction mixture. These samples rotated polarized light in a positive (+) direction, meaning that the recovered, unreacted epoxide was enriched in the enantiomer for which the stereogenic centre has the (*S*) configuration (Table S1 in Supporting Information) [23]. CAL-B therefore preferentially employs (-)-*R*-**1** in the aminolysis reaction and, assuming a standard S_N2 mechanism for reaction of the amine with the epoxide, we can deduce that the fluconazole analogues must be enriched in the (-)-*S*-enantiomer (Figure 1).

Antifungal activities of the fluconazole analogs Pathogenic *Aspergillus* strains (*Aspergillus fumigatus* ITCC 6604, *Aspergillus flavus* ITCC 5192, and *Aspergillus niger* ITCC 0004) were used to determine the *in vitro* antifungal efficacy of the fluconazole analogues, in both their optically enriched and racemic forms. These experiments used standard microbroth dilution (MDA), disc diffusion (DDA) and spore germination inhibition (PSGI) assays [24, 25]. We note that the MDA assay is based on the same basic principle as that used in the CLSI micro-dilution protocol. The only difference between the two assays is that CLSI uses RPMI medium to prepare diluted drug solutions rather than the Sabouraud dextrose broth (a medium used to culture *Aspergillii* in the laboratory) used by us to determine the MIC of the fluconazole derivatives. As recommended in CLSI protocols, we carefully

monitored MDA parameters with respect to preparation of the test compounds, medium preparation, temperature, inoculum size, incubation time, minimum inhibitory concentration (MIC)/endpoint determination, data recording and interpretation of results to ensure the validity and quality of our results. On this point, we note that a previous study from our laboratory [26] showed that results with RPMI 1640 or RPMI 1640 containing glucose were not different from those obtained by using Sabouraud dextrose broth.

On the basis of their MIC values, all the compounds exhibited moderate to good anti-*Aspergillus* activities, with the analogue *(-)-S*-**3d** being more potent than the commercially available fluconazole (Table 1). We also observed that optically enriched mixtures of *(-)-S*-**3a**, *(-)-S*-**3c**, *(-)-S*-**3d**, *(-)-S*-**3e** and *(-)-S*-**5b** were more active than the corresponding racemates. These data also confirm that introducing a linear aliphatic alkyl side chain is important for imparting antifungal activity, as reported previously [23, 24]. On the other hand, when additional, "distal" N-substituted alkyl groups were present, as in compounds *(-)-S*-**3g** and *(-)-S*-**3h**, antifungal activity was completely lost (Table 1). Compounds **3j** and **5a** exhibited no biological activity in microbroth dilution assays and were not studied further. Our work also shows that the length of the alkyl side chain is an important factor in determining activity, i.e. the compound *(-)-S*-**3d**, containing an n-hexyl moiety, has higher activity than *(-)-S*-**3a**, *(-)-S*-**3b** and *(-)-S*-**3c**, which contain ethyl, *n*-propyl and *n*-butyl groups, respectively (Table 1). Decreasing the linker chain length also led to higher activity. Optically enriched *(-)-S*-**3d** was the most potent compound against *Aspergillus fumigatus* (Table 1) and was therefore used to examine how encapsulation in dextran nanoparticles might impact anti-fungal activity.

Characterization of (-)-S-3d release from O-alkylated dextran nanoparticles. Dextran nanoparticle-based drug delivery systems are biocompatible, biodegradable, possess low immunogenicity [20], and can be used for controlled release of pharmacologically active substances [27]. We therefore encapsulated optically enriched *(-)-S*-**3d** into three types of dextran nanoparticles, derivatized with *O*-hexadecyl, *O*-decyl and *O*-heptyl chains to ensure amphiphilicity, and examined their effect on anti-*Aspergillus* activity. After trapping *(-)-S*-**3d** within each of the nanoparticles by self-assembly (encapsulation efficiencies for the *O*-hexadecyl, *O*-decyl and *O*-heptyl nanoparticles were 50 ± 4 %, 22 ± 2 % and 30 ± 2 %), the resulting particle size distributions were determined using dynamic light scattering (DSL). These measurements showed that the sizes of the *O*-hexadecyl-, *O*-decyl- and *O*-heptyl- derivatized nanoparticles were 140 ± 16 nm, 187 ± 13.16 nm and 183 ± 14.73 nm, respectively, and that all of the samples had a low polydispersity index (< 0.3) (Supporting Information). Examination of the rate at which the fluconazole analogue (-)-(*S*)-**3d** was released from each of the three types of nanoparticles, showed an initial burst for the *O*-hexadecyl- and *O*-decyl-derivatized nanoparticles (Figure 2).

Anti-Aspergillus activity and cytotoxicity of (-)-S-3d encapsulated in O-alkylated dextran nanoparticles. We next examined the effect of nanoparticle encapsulation on the activity of *(-)-S*-**3d** against *Aspergillus fumigatus* using a microbroth dilution assay (Figure 3). After 48 h of incubation (approximately 80 % release), *(-)-S*-**3d** encapsulated in *O*-decyl-derivatized nanoparticles inhibited the growth of *Aspergillus fumigatus* at an effective concentration of 3.16 µg/mL. Perhaps more importantly, when the optically enriched fluconazole analogue was

encapsulated in *O*-hexadecyl nanoparticles, complete inhibition of *Aspergillus fumigatus* growth was achieved at an effective concentration of 1.63 µg/mL (41.3 % release at an initial concentration of 3.95 µg/mL). In addition, nanoparticle-encapsulated *(-)-S*-**3d** exhibits activity at a lower concentration when compared to both fluconazole and free *(-)-S*-**3d**. Although we believe that this effect is associated with sustained release of the compound over time, it is also possible that drug uptake is more efficient because the drug in its encapsulated form is more efficiently captured by the cells. The general importance of this observation is also evident from the fact that the MIC of amphotericin B was decreased from 1.95 µg/mL to 0.97 µg/mL when the drug was encapsulated in *O*-heptyl nanoparticles.

The cytotoxicity of (-)-*S*-**3d** and amphotericin B when encapsulated in derivatized nanoparticles was also evaluated using haemolysis and MTT-based assays (Figure 4). Perhaps unsurprisingly, given that erythrocytes and cell lines treated with empty dextran nanoparticles (> 90 % cell viability at concentrations of 2 mg/mL) remained completely viable up to 1 mg/mL, the encapsulated, optically enriched fluconazole analogue (-)-*S*-**3d** exhibited similar cytotoxicity to that of the free compound. Thus, essentially no toxicity to two human cell lines (Figures 4b and 4c) was seen when the compound was present at concentrations similar to the MIC values observed for its anti-fungal activity. The optically enriched fluconazole analogue (-)-*S*-**3d** was also considerably less cytotoxic than free amphotericin B in all assays (Figure 4). It is therefore interesting to note that encapsulating amphotericin B into *O*-hexadecyl derivatized nanoparticles lowered the cytotoxicity of this antifungal agent in both the hemolysis and MTT-based assays. Nevertheless, cell viability was reduced for amphotericin B-containing nanoparticles relative to derivatized nanoparticles containing fluconazole analogue (-)-*S*-**3d**.

Conclusions

Reacting alkylamines with a racemic epoxide precursor (Figure 1) in the presence of immobilized lipase CAL-B in THF provides a simple approach for the preparation of optically enriched fluconazole analogs, which appear to exhibit better antifungal activity against *Aspergillus* than fluconazole. Although the extent to which the enzyme catalyzes the coupling reaction in an enantioselective manner remains to be determined, we have been able to assign the (*S*)-configuration to the stereogenic centre of the enantiomer that exhibits biological activity, assuming that (i) the aminolysis reaction proceeds with its usual chemical mechanism, and (ii) only one enantiomer has antifungal activity. Given the difficulty of single-step chemical strategies for the preparation of chiral fluconazoles in optically enriched form, we anticipate that the enzymatic methodology reported herein will have significant impact in this approach to obtaining novel variants of existing antifungal drugs.

The most active analogue prepared in this study, (-)-*S*-**3d**, is more potent against *Aspergillus fumigatus* than fluconazole, having MIC values of 8-16 μg/mL in a series of *in vitro* assays. Perhaps more importantly for drug discovery, the anti-*Aspergillus* potency of this compound is enhanced (MIC 1.6-4.0 μg/mL) by encapsulation in derivatized nanoparticles, with minimal *in vitro* cytotoxic effects at concentrations of up to 2 mg/mL against human erythrocytes and cell lines of human origin.

Materials and Methods

General procedure for the CAL-B catalysed synthesis of optically enriched fluconazole analogues. CAL-B immobilized on accurel beads (300

mg) was added to a solution of the epoxide (±)-**1** (5.0 mmol) and the appropriate amine (**2a-j**, **4a** or **4b**, 2.5 mmol) dissolved in THF, and the mixture incubated at 55 °C. The extent of the reaction was monitored by TLC and the enzyme was removed by filtration when the amine was consumed. After removal of THF at reduced pressure, the residue was subjected to column chromatography using chloroform/ methanol as eluent to afford optically enriched samples of pure fluconazole analogues *(-)-S*-**3a-3j**, *(-)-S*-**5a** or *(-)-S*-**5b** and the unreacted epoxide (+)-*S*-**1**.

***(-)-S*-2-(2',4'-Difluorophenyl)-1-hexylamino-3-(1''',2''',4''')triazol-1'''-yl-propan-2-ol (3d)** was obtained as a viscous oil in 80% yield. $[\alpha]_D^{20}$ -20.3 (c 0.01, $CHCl_3$); IR spectrum (film) μ_{max}: 3315 (OH and NH), 2979, 1620, 1508, 1415, 1267, 1145, 960 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 0.83 (3H, t, J = 7.63 Hz), 1.17-1.33 (8H, m), 2.43 (2H, t, J = 6.87 Hz), 2.81 (1H, d, J = 12.97 Hz), 3.12 (1H, d, J = 12.21 Hz), 4.49 (1H, d, J = 14.50 Hz), 4.58 (1H, d, J = 13.73 Hz), 6.74-6.82 (2H, m), 7.50-7.55 (1H, m), 7.77 (1H, s) and 8.10 (1H, s). ^{13}C NMR (100 MHz, $CDCl_3$): δ 13.92, 22.46, 26.55, 29.83, 31.49, 50.00, 54.14 (d, J_{CF}= 3.83 Hz), 55.98 (d, J_{CF}= 4.79 Hz), 72.96 (d, J_{CF}= 5.75 Hz), 104.12 (d, J_{CF}=26.84 Hz), 111.38 (d, J_{CF}= 20.61 Hz), 125.05 (d, J_{CF} = 13.42 Hz), 129.79 (d, J_{CF} = 6.71 Hz), 144.60, 151.09, 158.92 (d, J_{CF} = 237.78 Hz) and 162.29 (d, J_{CF} = 249.20 Hz). HRMS: m/z 339.1991 ($[M+H]^+$, $C_{17}H_{25}F_2N_4O$ calcd. 339.1969).

Microbroth dilution assay Various concentrations of different derivatives in the range of 0.24-1000.0 µg/mL were prepared in 96 well culture plates (Nunc, Roskilde, Denmark) by serial dilution in Sabouraud dextrose broth. Wells were inoculated with 1 x 10^6 spores (conidia) of *Aspergillus* in 10 µL of spore suspension. Negative

controls were solvent in medium and spores only, with amphotericin B and fluconazole being used as positive controls. Plates were incubated at 37 ºC using a BOD incubator (Calton, NSW, India) and examined macroscopically after 48 h for the growth of *Aspergillus* mycelia. The activity of the analogues was defined as positive if the medium appeared clear without any growth of *Aspergillus* mycelia, and the minimum concentration of compounds inhibiting growth was reported as MIC (Table 1).

Disc diffusion assay Autoclaved Sabouraud dextrose agar (SDA) was poured into radiation-sterilized petri dishes (10.0 cm diameter). A suspension of conidia of *Aspergillus* was prepared and overlaid on the agar plates. Different concentrations of the fluconazole analogues were impregnated on 5.0 mm diameter sterilized discs (Whatman No. 1) and placed on the agar. Control discs containing solvent, amphotericin B or fluconazole were also included in the assay. Plates were incubated at 37 ºC and the zone of inhibition determined after 72 h. MICs reported for this assay (Table 1) correspond to fluconazole analogue concentrations giving a zone of inhibition of at least 6.0 mm diameter from the centre of the plate.

Percent spore germination inhibition assay Serial dilutions, ranging from 0.24-1000.0 µg/mL, of each fluconazole analogue dissolved in Sabouraud dextrose broth were placed in radiation-sterilized petri dishes (10.0 cm diameter), with each dish then being inoculated with 100 ± 5 *Aspergillus* conidia. After incubation for 16 h at 37 ºC, wells were examined for spore germination using an inverted microscope (Nikon Diphot, Japan), and the number of germinated, and non-germinated, spores

recorded. MICs in this assay (Table 1) correspond to fluconazole analog concentrations resulting in inhibition of spore germination.

In vitro cytotoxicity assays. Two approaches were performed to assess the cytotoxicity of the fluconazole analogues. First, using a standard haemolytic assay [28], erythrocytes from healthy individuals were suspended in phosphate buffered saline (PBS) to give a 2 % suspension (v/v). These cells were then incubated with various concentrations of each compound for 1 h at 37 °C before being pelleted by centrifugation at 3000 x *g* for 10 min. The percentage haemolysis was then calculated from the optical density at 450 nm of the supernatant (Figure 4a). The effect of solvent and PBS on erythrocyte viability was also checked. Triton X-100 (Sigma Chemicals, USA) was used for complete haemolysis of the erythrocytes.

In an alternate approach, an MTT-based assay [29] was used to examine the cytotoxicity of the analogues against A549 (human pulmonary epithelial cells) and U87 (primary glioblastoma cells) human cell lines, obtained from National Centre for Cell Science, Pune, India (Figures 4b and 4c). Briefly, cells were cultured in RPMI-1640 medium supplemented with L-glutamine and fetal calf serum (10 % v/v), before being harvested at the log phase of confluency and re-suspended in RPMI-1640 medium. Samples (2 x 10^4 cells in 100 µL) were seeded into culture plates and allowed to grow overnight at 37 °C under 5 % (v/v) CO_2. Fluconazole analogues were added at a variety of concentrations and the cells were incubated under the same conditions for 24 h. Equivalent amounts of solvent, amphotericin B and fluconazole were used as negative and positive controls. The medium was removed from each well before the addition of 50.0 µg of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

bromide (MTT) in PBS (100 µL). After incubation for a further period of 4 h at 37 °C, the MTT solution was removed and the cells were lysed using isopropanol-HCl (100.0 µL). The absorption of each well (at 540 nm) was used to determine the percentage cytotoxicity in a micro-plate reader (Spectra max 384 plus, Molecular Devices, USA).

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401 **Figure Captions**

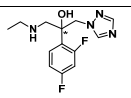
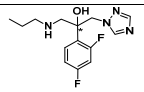
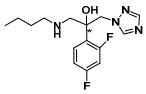
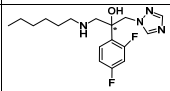
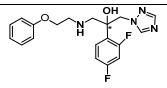
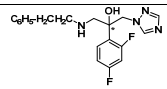
402 **Figure 1.** CAL-B catalyzed epoxide ring opening with open chain and cyclic aliphatic
403 amines. Note that samples of each compound could also be prepared in racemic
404 form by heating the epoxide and amine at 55 °C in THF (see Supporting
405 Information). The new stereogenic centre is indicated by an asterisk.

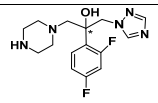
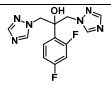
406 **Figure 2.** *In vitro* release of *(-)-S*-**3d** from *O*-hexadecyl- (blue triangles), *O*-decyl-
407 (red circles) and *O*-heptyl-derivatized (grey squares) dextran nanoparticles.

408 **Figure 3**. *In vitro* antifungal activity of *(-)-S*-**3d**, amphotericin B and their
409 dextran NPs. Lane a: Negative control; Lane b: Empty *O*-alkyl dextran
410 nanoparticles; Lane c: Amphotericin B; Lane d: Fluconazole; Lane e: *(-)-S*-**3d**;
411 Lane f: *O*-heptyl nanoparticles containing *(-)-S*-**3d**; Lane g: *O*-decyl
412 nanoparticles containing *(-)-S*-**3d**; Lane h: *O*-hexadecyl nanoparticles
413 containing *(-)-S*-**3d**; Lanei: *O*-heptyl nanoparticles containing Amphotericin B;
414 Lane j: *O*-decyl nanoparticles containing Amphotericin B; Lane k: *O*-hexadecyl
415 nanoparticles containing Amphotericin B.

416 **Figure 4**. *In vitro* cytotoxicity assays for optically enriched *(-)-S*-**3d** and amphotericin
417 B in both the free form and when encapsulated into dextran nanoparticles. (a)
418 Haemolytic assay; MTT-based assay using (b) A459 and (c) U87 cell lines.

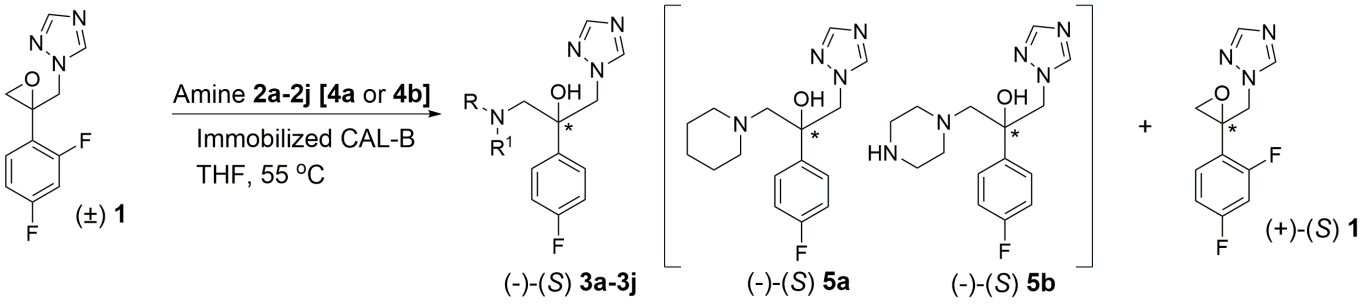
Table 1: *In vitro* activity of selected, optically enriched fluconazole analogues against *Aspergillus* species.[a,b]

	Analogue	*Aspergillus fumigatus*			*Aspergillus niger*			*Aspergillus flavus*		
		MDA (μg/ml)	DDA (μg/disc)	PSGI (μg/ml)	MDA (μg/ml)	DDA (μg/disc)	PSGI (μg/ml)	MDA (μg/ml)	DDA (μg/disc)	PSGI (μg/ml)
(-)-S-**3a**		125.0 (250.0)	62.50 (125.0)	125.0 (250.0)	250.0 (250.0)	125.0 (125.0)	250.0 (250.0)	250.0 -	125.0 -	250.0 -
(-)-S-**3b**		250.0 (250.0)	62.50 (125.0)	250.0 (250.0)	500.0 -	125.0 -	500.0 -	- -	- -	- -
(-)-S-**3c**		62.50 (125.0)	31.25 (31.25)	62.50 (125.0)	62.50 (500.0)	31.25 (125.0)	62.50 (500.0)	500.0 (1000.0)	125.0 (125.0)	500.0 (1000.0)
(-)-S-**3d**		15.62 (15.62)	7.81 (7.81)	15.62 (15.62)	62.50 (125.0)	15.62 (31.25)	62.50 (125.0)	125.0 (125.0)	15.62 (31.25)	125.0 (125.0)
(-)-S-**3e**		62.50 (125.0)	31.25 (62.50)	62.50 (125.0)	125.0 (500.0)	62.50 (125.0)	125.0 (500.0)	500.0 (500.0)	125.0 -	500.0 (500.0)
(-)-S-**3i**		500.0 (500.0)	250.0 (250.0)	500.0 (500.0)	500.0 -	125.0 -	500.0 -	500.0 -	250.0 -	500.0 -

*(-)-S-***5b**		62.50 (125.0)	31.25 (62.50)	62.50 (125.0)	125.0 (500.0)	62.50 (125.0)	125.0 (500.0)	500.0 (500.0)	125.0 (125.0)	500.0 (500.0)
	 Fluconazole	250.0	125.0	250.0	250.0	125.0	250.0	250.0	125.0	250.0
	Amphotericin B	1.95	0.97	1.95	1.95	0.97	1.95	1.95	0.97	1.95

[a]Values in parentheses are for the racemic form of the compound.

[b](-) shows no activity within the range of concentrations tested.



2a or **3a:** R = H, $R^1 = CH_2CH_3$
2b or **3b:** R = H, $R^1 = CH_2CH_2CH_3$
2c or **3c:** R = H, $R^1 = CH_2(CH_2)_2CH_3$
2e or **3e:** R = H, $R^1 = CH_2CH_2OPh$
2f or **3f:** R = CH_3, $R^1 = CH_2(CH_2)_{16}CH_3$
2g or **3g:** R = H, $R^1 = CH_2(CH_2)_2N(CH_3)_2$
2h or **3h:** R = H, $R^1 = CH_2(CH_2)_2N(CH_2CH_3)_2$
2i or **3i:** R = H, $R^1 = CH_2CH_2Ph$
2j or **3j:** R = H, $R^1 = CH_2(CH_2)_2CH_2Ph$