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.

44 Aspergillosis remains a significant threat to public health, and, in spite of

45 continuous efforts to improve timely diagnosis and clinical therapies, mortality

46 caused by this disease remains unacceptably high [1, 2]. Current therapeutic

47 options for treating *Aspergillus*-induced disorders include antifungal agents

48 such as polyenes, azoles and echinocandins [3, 4]. Thus the discovery of new

49 antifungal compounds remains important given the need to address the

50 development of drug resistance in pathogenic fungi [5-7]. One approach to

51 accomplishing this goal is to prepare new derivatives of existing drugs with

52 broad spectrum activity and enhanced pharmacokinetic properties. As part of

53 our on-going efforts to use lipases [8-12], which catalyze reactions with high

54 degree of chemo-, regio- and stereoselectivity in organic synthesis, we

55 became interested in preparing new antifungals using biocatalysis.

56   Fluconazole, introduced in 1990, is a bis-triazole antifungal drug which

57 possesses interesting pharmacokinetic properties, such as low plasma binding

58 affinity, good water solubility, low first pass metabolism, high oral

59 bioavailability and a long half-life, all of which should make it a drug of choice

60 for treating fungal infections [13, 14]. On the other hand, fluconazole has been

61 reported to exhibit only limited activity against *Aspergillus* infections [15],

62 which has led to many reports concerning the synthesis of various types of

63 fluconazole derivatives and their chiral separation/resolution into constituent

64 enantiomers [16-19]. We now report the use of *Candida antarctica* lipase

65 (CAL-B) in catalysing the addition of amines to an achiral epoxide to yield

66 optically enriched fluconazole analogues in which one of the triazole rings is

67 replaced by *n*-alkylamino and cycloalkylamino substituents. To the best of our

68 knowledge, the work reported herein is the first direct synthesis of optically

69 enriched fluconazole analogues using biocatalytic methods. *In vitro* assays

70 show that the optically enriched analogues exhibit more potent antifungal

71 activity than the corresponding racemic mixtures. Interestingly, this bioactivity

72 can be enhanced by their encapsulation in dextran-based nanoparticles [20].

73

74 **Results**

75 ***Synthesis of fluconazole analogues.*** A series of linear and cyclic alkylamines

76 was screened for reaction with the epoxide ring of (±)-1-[2-(2, 4-difluorophenyl)-

77 oxiranylmethyl]-1*H*-[1,2,4]-triazole (**1,** Figure 1) in a number of different organic

78 solvents. Three different immobilized lipases were also evaluated for their ability to

79 catalyze this reaction: *Candida rugosa* lipase (CRL), porcine pancreatic lipase (PPL)

80 and CAL-B. Although the ring-opening reactions catalysed by CRL and PPL were of

81 no practical utility, when the reaction was performed in the presence of CAL-B in

82 tetrahydrofuran (THF) as solvent, the desired products (**3a-j**, **5a** and **5b**) were

83 obtained with good yields in optically enriched forms (Figure 1, and Tables S1 and

84 S2 in Supporting Information). Very importantly, all of the twelve novel fluconazole

85 analogues formed in the lipase-catalyzed reactions were optically active showing

86 that aminolysis of the racemic starting epoxide (±)-1 had proceeded in an

87 enantioselective fashion (Table S1). These twelve compounds could also be

88 prepared in racemic form, as viscous oils in 75-80 % yields, by direct reaction of the

89 alkylamines with the racemic epoxide precursor (±)-**1** in THF at 55 ^{o}C. The time

90 taken for complete consumption of aliphatic amines **2a-j**, **4a** and **4b** in the CAL-B

91 catalyzed reaction varied between 18h and 28h, which was considerably shorter

92 than the 48-56 h required for the chemical addition of the amines (Table S2 in

93 Supporting Information). The structures of all twelve fluconazole analogues were

94 unambiguously established on the basis of spectroscopic data (IR, ^{1}H- and ^{13}C NMR,

95 and mass spectra), and by comparison to literature data for known compounds **3b**,

96 **3c**, **5a** and **5b** [21, 22].

97   Although the enantiomeric enrichment of the fluconazole analogues prepared

98 by lipase-catalyzed addition was not established, we were able to assign the

99 absolute configuration of the major enantiomer using the optical activity of the

100 unreacted epoxide isolated from the reaction mixture. These samples rotated

101 polarized light in a positive (+) direction, meaning that the recovered, unreacted

102 epoxide was enriched in the enantiomer for which the stereogenic centre has the (*S*)

103 configuration (Table S1 in Supporting Information) [23]. CAL-B therefore

104 preferentially employs (-)-*R*-**1** in the aminolysis reaction and, assuming a standard

105 S_N2 mechanism for reaction of the amine with the epoxide, we can deduce that the

106 fluconazole analogues must be enriched in the (-)-*S*-enantiomer (Figure 1).

107

108 ***Antifungal activities of the fluconazole analogs*** Pathogenic *Aspergillus*

109 strains (*Aspergillus fumigatus* ITCC 6604, *Aspergillus flavus* ITCC 5192, and

110 *Aspergillus niger* ITCC 0004) were used to determine the *in vitro* antifungal efficacy

111 of the fluconazole analogues, in both their optically enriched and racemic forms.

112 These experiments used standard microbroth dilution (MDA), disc diffusion (DDA)

113 and spore germination inhibition (PSGI) assays [24, 25]. We note that the MDA

114 assay is based on the same basic principle as that used in the CLSI micro-dilution

115 protocol. The only difference between the two assays is that CLSI uses RPMI

116 medium to prepare diluted drug solutions rather than the Sabouraud dextrose broth

117 (a medium used to culture *Aspergillii* in the laboratory) used by us to determine the

118 MIC of the fluconazole derivatives. As recommended in CLSI protocols, we carefully

119   monitored MDA parameters with respect to preparation of the test compounds,

120   medium preparation, temperature, inoculum size, incubation time, minimum

121   inhibitory concentration (MIC)/endpoint determination, data recording and

122   interpretation of results to ensure the validity and quality of our results. On this point,

123   we note that a previous study from our laboratory [26] showed that results with RPMI

124   1640 or RPMI 1640 containing glucose were not different from those obtained by

125   using Sabouraud dextrose broth.

126   On the basis of their MIC values, all the compounds exhibited moderate to

127   good anti-*Aspergillus* activities, with the analogue *(-)-S*-**3d** being more potent than

128   the commercially available fluconazole (Table 1). We also observed that optically

129   enriched mixtures of *(-)-S*-**3a**, *(-)-S*-**3c**, *(-)-S*-**3d**, *(-)-S*-**3e** and *(-)-S*-**5b** were more

130   active than the corresponding racemates. These data also confirm that introducing a

131   linear aliphatic alkyl side chain is important for imparting antifungal activity, as

132   reported previously [23, 24]. On the other hand, when additional, "distal" N-

133   substituted alkyl groups were present, as in compounds *(-)-S*-**3g** and *(-)-S*-**3h**,

134   antifungal activity was completely lost (Table 1). Compounds **3j** and **5a** exhibited no

135   biological activity in microbroth dilution assays and were not studied further. Our

136   work also shows that the length of the alkyl side chain is an important factor in

137   determining activity, i.e. the compound *(-)-S*-**3d**, containing an n-hexyl moiety, has

138   higher activity than *(-)-S*-**3a**, *(-)-S*-**3b** and *(-)-S*-**3c**, which contain ethyl, *n*-propyl and

139   *n*-butyl groups, respectively (Table 1). Decreasing the linker chain length also led to

140   higher activity. Optically enriched *(-)-S*-**3d** was the most potent compound against

141   *Aspergillus fumigatus* (Table 1) and was therefore used to examine how

142   encapsulation in dextran nanoparticles might impact anti-fungal activity.

143

144   ***Characterization of (-)-S-3d release from O-alkylated dextran***

145   ***nanoparticles.*** Dextran nanoparticle-based drug delivery systems are

146   biocompatible, biodegradable, possess low immunogenicity [20], and can be used

147   for controlled release of pharmacologically active substances [27]. We therefore

148   encapsulated optically enriched *(-)-S-***3d*** into three types of dextran nanoparticles,

149   derivatized with *O*-hexadecyl, *O*-decyl and *O*-heptyl chains to ensure amphiphilicity,

150   and examined their effect on anti-*Aspergillus* activity. After trapping *(-)-S-***3d*** within

151   each of the nanoparticles by self-assembly (encapsulation efficiencies for the *O*-

152   hexadecyl, *O*-decyl and *O*-heptyl nanoparticles were 50 ± 4 %, 22 ± 2 % and 30 ± 2

153   %), the resulting particle size distributions were determined using dynamic light

154   scattering (DSL). These measurements showed that the sizes of the *O*-hexadecyl-,

155   *O*-decyl- and *O*-heptyl- derivatized nanoparticles were 140 ± 16 nm, 187 ± 13.16 nm

156   and 183 ± 14.73 nm, respectively, and that all of the samples had a low

157   polydispersity index (< 0.3) (Supporting Information). Examination of the rate at

158   which the fluconazole analogue (-)-(*S*)-**3d** was released from each of the three types

159   of nanoparticles, showed an initial burst for the *O*-hexadecyl- and *O*-decyl-

160   derivatized nanoparticles (Figure 2).

161

162   ***Anti-Aspergillus activity and cytotoxicity of (-)-S-3d encapsulated in O-***

163   ***alkylated dextran nanoparticles.*** We next examined the effect of nanoparticle

164   encapsulation on the activity of *(-)-S-***3d*** against *Aspergillus fumigatus* using a

165   microbroth dilution assay (Figure 3). After 48 h of incubation (approximately 80 %

166   release), *(-)-S-***3d*** encapsulated in *O*-decyl-derivatized nanoparticles inhibited the

167   growth of *Aspergillus fumigatus* at an effective concentration of 3.16 μg/mL. Perhaps

168   more importantly, when the optically enriched fluconazole analogue was

169 encapsulated in *O*-hexadecyl nanoparticles, complete inhibition of *Aspergillus*

170 *fumigatus* growth was achieved at an effective concentration of 1.63 μg/mL (41.3 %

171 release at an initial concentration of 3.95 μg/mL). In addition, nanoparticle-

172 encapsulated *(-)-S*-**3d** exhibits activity at a lower concentration when compared to

173 both fluconazole and free *(-)-S*-**3d**. Although we believe that this effect is associated

174 with sustained release of the compound over time, it is also possible that drug

175 uptake is more efficient because the drug in its encapsulated form is more efficiently

176 captured by the cells. The general importance of this observation is also evident from

177 the fact that the MIC of amphotericin B was decreased from 1.95 μg/mL to 0.97

178 μg/mL when the drug was encapsulated in *O*-heptyl nanoparticles.

179 The cytotoxicity of (-)-*S*-**3d** and amphotericin B when encapsulated in

180 derivatized nanoparticles was also evaluated using haemolysis and MTT-based

181 assays (Figure 4). Perhaps unsurprisingly, given that erythrocytes and cell lines

182 treated with empty dextran nanoparticles (> 90 % cell viability at concentrations of 2

183 mg/mL) remained completely viable up to 1 mg/mL, the encapsulated, optically

184 enriched fluconazole analogue (-)-*S*-**3d** exhibited similar cytotoxicity to that of the

185 free compound. Thus, essentially no toxicity to two human cell lines (Figures 4b and

186 4c) was seen when the compound was present at concentrations similar to the MIC

187 values observed for its anti-fungal activity. The optically enriched fluconazole

188 analogue (-)-*S*-**3d** was also considerably less cytotoxic than free amphotericin B in

189 all assays (Figure 4). It is therefore interesting to note that encapsulating

190 amphotericin B into *O*-hexadecyl derivatized nanoparticles lowered the cytotoxicity of

191 this antifungal agent in both the hemolysis and MTT-based assays. Nevertheless,

192 cell viability was reduced for amphotericin B-containing nanoparticles relative to

193 derivatized nanoparticles containing fluconazole analogue (-)-*S*-**3d**.

Conclusions

194   Reacting alkylamines with a racemic epoxide precursor (Figure 1) in the

195

196   presence of immobilized lipase CAL-B in THF provides a simple approach for

197   the preparation of optically enriched fluconazole analogs, which appear to

198   exhibit better antifungal activity against *Aspergillus* than fluconazole. Although

199   the extent to which the enzyme catalyzes the coupling reaction in an

200   enantioselective manner remains to be determined, we have been able to

201   assign the (*S*)-configuration to the stereogenic centre of the enantiomer that

202   exhibits biological activity, assuming that (i) the aminolysis reaction proceeds

203   with its usual chemical mechanism, and (ii) only one enantiomer has antifungal

204   activity. Given the difficulty of single-step chemical strategies for the

205   preparation of chiral fluconazoles in optically enriched form, we anticipate that

206   the enzymatic methodology reported herein will have significant impact in this

207   approach to obtaining novel variants of existing antifungal drugs.

208   The most active analogue prepared in this study, (-)-*S*-**3d**, is more

209   potent against *Aspergillus fumigatus* than fluconazole, having MIC values of 8-

210   16 µg/mL in a series of *in vitro* assays. Perhaps more importantly for drug

211   discovery, the anti-*Aspergillus* potency of this compound is enhanced (MIC

212   1.6-4.0 µg/mL) by encapsulation in derivatized nanoparticles, with minimal *in*

213   *vitro* cytotoxic effects at concentrations of up to 2 mg/mL against human

214   erythrocytes and cell lines of human origin.

215

Materials and Methods

216

217   ***General procedure for the CAL-B catalysed synthesis of optically***

218   ***enriched fluconazole analogues.*** CAL-B immobilized on accurel beads (300

219   mg) was added to a solution of the epoxide (±)-**1** (5.0 mmol) and the appropriate

220   amine (**2a-j**, **4a** or **4b**, 2.5 mmol) dissolved in THF, and the mixture incubated at 55

221   °C. The extent of the reaction was monitored by TLC and the enzyme was removed

222   by filtration when the amine was consumed. After removal of THF at reduced

223   pressure, the residue was subjected to column chromatography using chloroform/

224   methanol as eluent to afford optically enriched samples of pure fluconazole

225   analogues *(-)-S*-**3a-3j**, *(-)-S*-**5a** or *(-)-S*-**5b** and the unreacted epoxide (+)-*S*-**1**.

226

227   ***(-)-S*-2-(2',4'-Difluorophenyl)-1-hexylamino-3-(1''',2''',4''')triazol-1'''-yl-prop-**

228   **an-2-ol (3d)** was obtained as a viscous oil in 80% yield. $[\alpha]_D^{20}$ -20.3 (c 0.01,

229   CHCl$_3$); IR spectrum (film) μ_{max}: 3315 (OH and NH), 2979, 1620, 1508, 1415, 1267,

230   1145, 960 cm^{-1}. ^{1}H NMR (400 MHz, CDCl$_3$): δ 0.83 (3H, t, J = 7.63 Hz), 1.17-1.33

231   (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

232   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-

233   7.55 (1H, m), 7.77 (1H, s) and 8.10 (1H, s). ^{13}C NMR (100 MHz, CDCl$_3$): δ 13.92,

234   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),

235   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

236   (d, J$_{CF}$ = 13.42 Hz), 129.79 (d, J$_{CF}$ = 6.71 Hz), 144.60, 151.09, 158.92 (d, J$_{CF}$ =

237   237.78 Hz) and 162.29 (d, J$_{CF}$ = 249.20 Hz). HRMS: m/z 339.1991 ([M+H]$^+$,

238   C$_{17}$H$_{25}$F$_2$N$_4$O calcd. 339.1969).

239

240   ***Microbroth dilution assay*** Various concentrations of different derivatives in the

241   range of 0.24-1000.0 μg/mL were prepared in 96 well culture plates (Nunc, Roskilde,

242   Denmark) by serial dilution in Sabouraud dextrose broth. Wells were inoculated with

243   1 x 10^6 spores (conidia) of *Aspergillus* in 10 μL of spore suspension. Negative

244   controls were solvent in medium and spores only, with amphotericin B and

245   fluconazole being used as positive controls. Plates were incubated at 37 ºC using a

246   BOD incubator (Calton, NSW, India) and examined macroscopically after 48 h for the

247   growth of *Aspergillus* mycelia. The activity of the analogues was defined as positive

248   if the medium appeared clear without any growth of *Aspergillus* mycelia, and the

249   minimum concentration of compounds inhibiting growth was reported as MIC (Table

250   1).

251

252   ***Disc diffusion assay*** Autoclaved Sabouraud dextrose agar (SDA) was poured into

253   radiation-sterilized petri dishes (10.0 cm diameter). A suspension of conidia of

254   *Aspergillus* was prepared and overlaid on the agar plates. Different concentrations of

255   the fluconazole analogues were impregnated on 5.0 mm diameter sterilized discs

256   (Whatman No. 1) and placed on the agar. Control discs containing solvent,

257   amphotericin B or fluconazole were also included in the assay. Plates were

258   incubated at 37 ºC and the zone of inhibition determined after 72 h. MICs reported

259   for this assay (Table 1) correspond to fluconazole analogue concentrations giving a

260   zone of inhibition of at least 6.0 mm diameter from the centre of the plate.

261

262   ***Percent spore germination inhibition assay*** Serial dilutions, ranging from 0.24-

263   1000.0 µg/mL, of each fluconazole analogue dissolved in Sabouraud dextrose broth

264   were placed in radiation-sterilized petri dishes (10.0 cm diameter), with each dish

265   then being inoculated with 100 ± 5 *Aspergillus* conidia. After incubation for 16 h at 37

266   ºC, wells were examined for spore germination using an inverted microscope (Nikon

267   Diphot, Japan), and the number of germinated, and non-germinated, spores

268 recorded. MICs in this assay (Table 1) correspond to fluconazole analog

269 concentrations resulting in inhibition of spore germination.

270

271 ***In vitro cytotoxicity assays.*** Two approaches were performed to assess the

272 cytotoxicity of the fluconazole analogues. First, using a standard haemolytic assay

273 [28], erythrocytes from healthy individuals were suspended in phosphate buffered

274 saline (PBS) to give a 2 % suspension (v/v). These cells were then incubated with

275 various concentrations of each compound for 1 h at 37 °C before being pelleted by

276 centrifugation at 3000 x g for 10 min. The percentage haemolysis was then

277 calculated from the optical density at 450 nm of the supernatant (Figure 4a). The

278 effect of solvent and PBS on erythrocyte viability was also checked. Triton X-100

279 (Sigma Chemicals, USA) was used for complete haemolysis of the erythrocytes.

280 In an alternate approach, an MTT-based assay [29] was used to examine the

281 cytotoxicity of the analogues against A549 (human pulmonary epithelial cells)

282 and U87 (primary glioblastoma cells) human cell lines, obtained from National

283 Centre for Cell Science, Pune, India (Figures 4b and 4c). Briefly, cells were

284 cultured in RPMI-1640 medium supplemented with L-glutamine and fetal calf

285 serum (10 % v/v), before being harvested at the log phase of confluency and

286 re-suspended in RPMI-1640 medium. Samples (2 x 10^4 cells in 100 µL) were

287 seeded into culture plates and allowed to grow overnight at 37 °C under 5 %

288 (v/v) CO_2. Fluconazole analogues were added at a variety of concentrations

289 and the cells were incubated under the same conditions for 24 h. Equivalent

290 amounts of solvent, amphotericin B and fluconazole were used as negative

291 and positive controls. The medium was removed from each well before the

292 addition of 50.0 µg of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

293   bromide (MTT) in PBS (100 µL). After incubation for a further period of 4 h at

294   37 °C, the MTT solution was removed and the cells were lysed using

295   isopropanol-HCl (100.0 µL). The absorption of each well (at 540 nm) was used

296   to determine the percentage cytotoxicity in a micro-plate reader (Spectra max

297   384 plus, Molecular Devices, USA).

298

299   **Acknowledgements**

305

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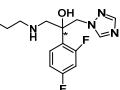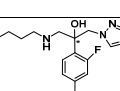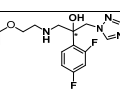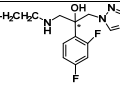
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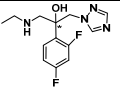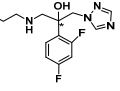
402   **Figure 1.** CAL-B catalyzed epoxide ring opening with open chain and cyclic aliphatic

403   amines. Note that samples of each compound could also be prepared in racemic

404   form by heating the epoxide and amine at 55 $^{\circ}$C in THF (see Supporting

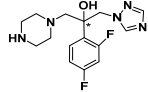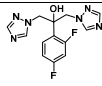405   Information). The new stereogenic centre is indicated by an asterisk.

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)

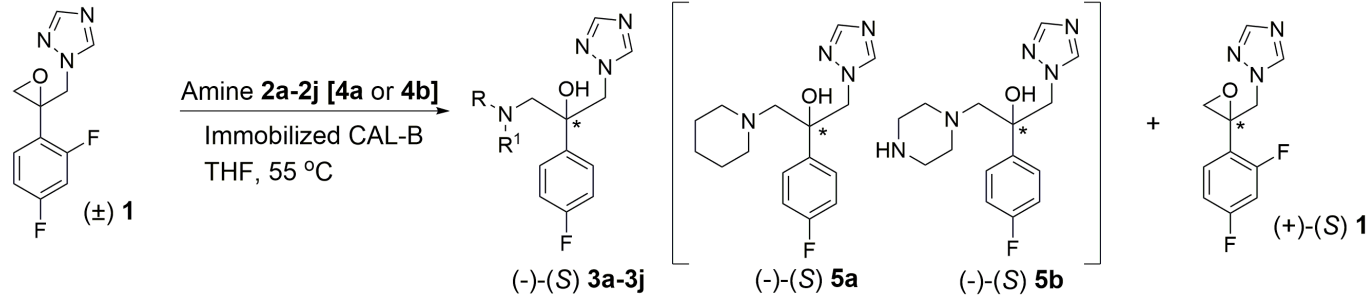418   Haemolytic assay; MTT-based assay using (b) A459 and (c) U87 cell lines.

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.

(±) 1

Amine **2a-2j** [**4a** or **4b**]
Immobilized CAL-B
THF, 55 °C

(-)-(*S*) **3a-3j**   (-)-(*S*) **5a**   (-)-(*S*) **5b**   +   (+)-(*S*) 1

2a or **3a:** R = H, R^1 = CH$_2$CH$_3$
2b or **3b:** R = H, R^1 = CH$_2$CH$_2$CH$_3$
2c or **3c:** R = H, R^1 = CH$_2$(CH$_2$)$_2$CH$_3$
2e or **3e:** R = H, R^1 = CH$_2$CH$_2$OPh
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$_2$)$_2$N(CH$_3$)$_2$
2h or **3h:** R = H, R^1 = CH$_2$(CH$_2$)$_2$N(CH$_2$CH$_3$)$_2$
2i or **3i:** R = H, R^1 = CH$_2$CH$_2$Ph
2j or **3j:** R = H, R^1 = CH$_2$(CH$_2$)$_2$CH$_2$Ph