



## Synthesis and antifungal activity investigation of a novel clotrimazole derivative

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**Abstract:** Azole antifungal agents disrupt fungal ergosterol synthesis that is essential for the formation of fungal cell membrane by preventing 14- $\alpha$ -demethylase enzyme from binding to its substrate. Clotrimazole is one of the first generations of azole antifungal agents. To discover a novel azole antifungal agent, biphenyl derivative was synthesised together with clotrimazole by multistep linear synthesis. Structures of synthesised azole agents have been validated by spectral analysis and potential antifungal activity of both compounds was determined on a yeast, *E.coli* and *M.luteus* by using a disk diffusion method. Clotrimazole and its biphenyl derivative were active against yeast but a novel compound resulted less activity than clotrimazole. Antibacterial effect was not observed for either azole agents.

**Keywords:** Clotrimazole, biphenyl derivative, azole antifungals

### INTRODUCTION

Azole antifungals, including clotrimazole, act as an inhibitor to the fungal ergosterol synthetic pathway. Ergosterol is an essential compound for fungal cell wall integrity and requires that the C-14 methyl group of sterol to be removed if it is to maintain its integrity and normal functionality. Azole antifungals target the haeme protein, which is an active catalytic site of 14- $\alpha$ -demethylase (CYP51) enzyme that catalyses 14- $\alpha$ -demethylation of lanosterol in the ergosterol synthetic route. The inhibition of 14- $\alpha$ -demethylase enzyme leads to the accumulation of demethylated compounds, hence results in the death of fungal cell [1 - 3]. Clotrimazole is, one of the first generations of azole drugs, was first introduced by Bayer Ag (Germany) in 1967. Since that time this compound has been extensively studied. Clotrimazole has a broad spectrum of activity and is effective against *Candida* and *Aspergillus* species [4, 5].

As antifungal resistance develops in fungi and other factors occur that affect the result of fungal infection treatment, scientists invent new antifungals. A number of structure-activity relationship studies of antifungal agents have been conducted over the past years. The main target of azole antifungals is CYP51 (14- $\alpha$ -demethylase) of p450 enzymes and many studies focused on the computer aided structure modelling of this enzyme in relation to the activity of

antifungals. Some structure-activity relationship studies of azole derivatives with the aid of molecular modelling of the CYP51 enzyme active site and the substrate binding mode suggest that conjugated aromatic moieties, including biphenyl and pyrrolylphenyl residues, confer better antifungal activity due to the favourable steric interaction within the enzyme's active site cavity. Also, if an electron withdrawing group is placed in the para position of the phenyl group, then antifungal activity was increased [6]. In this study we aimed to design novel azole antifungal agents based on clotrimazole core structure.

### EXPERIMENTAL

**General:** All reactions were conducted under nitrogen. Solvent concentration was performed on a Buchi rotary evaporator R-210. Flash column chromatography was conducted on silica 40-60  $\mu$ , 60 E with ethyl acetate and petrol as eluent unless specified otherwise. Melting points were determined by a Stuart SMP11 apparatus. IR spectra were obtained on a Varian 800 FT-IR. <sup>1</sup>H and <sup>13</sup>C NMR spectra were obtained on a BRUKER AVANCE-300 and a JEOL ECS-400. Mass spectra were recorded on a Waters LCT-Premier ESI high resolution mass spectrometer.

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**Preparation of (2-chlorophenyl)diphenylmethanol**

**(2):** Bromobenzene (1.6 g, 10.5 mmol) was added to magnesium turnings (0.25 g, 10.5 mmol). The reaction mixture was stirred at reflux and then at room temperature for a further 6 hours. Ketone **(1)** (1.5 g, 7 mmol) was added and the reaction mixture was left stirring at room temperature for overnight. The reaction mixture was quenched with saturated ammonium chloride solution (30 mL). The aqueous layer was separated. Combined organic layers were washed with water and dried over  $\text{MgSO}_4$  and then removed. The crude yellow oily mass obtained was purified by flash chromatography to give 0.75 g of alcohol **(2)** (36% yield) as white crystals. mp 83-85 °C (lit 83 °C)[7];  $R_f = 0.45$ ; IR (neat)  $\nu_{\text{max}}/\text{cm}^{-1}$  3566, 3058, 1466, 1010;  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  (ppm): 7.40 (dd, 1H,  $J = 1.6, 8.8$  Hz), 7.24-7.35 (m, 11H), 7.01 (dt, 1H,  $J = 1.2, 6.3$  Hz), 6.70 (dd, 1H,  $J = 1.5, 6.3$  Hz), 4.46 (s, 1H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  (ppm): 145.6, 143.8, 133.3, 131.4, 131.3, 128.9, 127.9, 127.7, 127.2, 126.3, 82.5;  $m/z$  calcd for  $\text{C}_{19}\text{H}_{14}\text{Cl}$   $[\text{M}-\text{OH}]^+$  277.0784, found 277.0782.

**Preparation of 1-((2-chlorophenyl)diphenylmethyl)-1H-imidazole (4)**

Thionyl chloride (0.82 g, 6.9 mmol) was added into a solution of alcohol **(2)** (0.69 g, 2.3 mmol) and the mixture stirred at 0 °C for 1 hour. The reaction mixture was left at reflux stirring for overnight. The organic solvent was evaporated and the residue washed with acetonitrile (2 x 20 mL) to afford compound **(3)** as a yellow oily mass that was submitted for the further reaction directly without purification and structure elucidation. Imidazole (0.3 g, 4.4 mmol) with  $\text{Et}_3\text{N}$  (0.6 g, 6.6 mmol) was added to the solution of compound **(3)** (0.7 g, 2.2 mmol). The reaction mixture was left at room temperature at reflux stirring for 72 hours. The organic solvent was removed and  $\text{EtOAc}$  (20 mL) with water (20 mL) was added to the residue. The aqueous layer was extracted with  $\text{EtOAc}$  (2 x 20 mL). Combined organic layers were washed with water and dried over  $\text{MgSO}_4$  prior to evaporation. The crude product was purified by flash chromatography to afford 0.69 g of compound **(4)** as a white crystal (yield 91%). mp 130-133 °C;  $R_f = 0.37$ ; IR (neat)  $\nu_{\text{max}}/\text{cm}^{-1}$  3064, 1489, 1443, 1210, 750;  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  (ppm): 7.48 (s, 1H), 7.41-7.44 (m, 1H), 7.32-7.37 (m, 7H), 7.26-7.29 (m, 1H), 7.19-7.23 (m, 4H), 7.07 (s, 1H), 6.92 (dd, 1H,  $J = 1.5, 6.3$  Hz), 6.76 (s, 1H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$  (ppm): 151.1, 150.5, 148.9, 144.5, 140.3, 138.0, 137.7, 137.3, 135.5, 135.2, 135.1, 133.8, 127.0, 68.9;  $m/z$  calcd for  $\text{C}_{19}\text{H}_{14}\text{Cl}$   $[\text{M}-\text{imid}]^+$  277.0784, found 277.0780.

**Preparation of [1,1'-biphenyl]-4-yl(phenyl)methanol**

**(6):** Bromobenzene (3.14 g, 20 mmol) was added to magnesium turnings (0.48 g, 20 mmol). The reaction mixture was stirred at reflux, then at room temperature for an additional 4 hours. Aldehyde **(5)** (1.82 g, 10 mmol) was added to the reaction and the

mixture left stirring at room temperature for overnight. The reaction mixture was quenched with a saturated ammonium chloride solution (40 mL). The aqueous layer was separated and extracted with  $\text{Et}_2\text{O}$ . The combined organic layers were washed with water then dried over  $\text{MgSO}_4$  and then removed. The crude yellow crystals obtained were purified by flash chromatography to give 1.34 g of Alcohol **(6)** (73% yield) as weak yellow crystals. mp 79-83 °C (lit 90-92 °C)[8];  $R_f = 0.21$ ; IR (neat)  $\nu_{\text{max}}/\text{cm}^{-1}$  3256, 3028, 1487, 1016;  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  (ppm): 7.57-7.60 (m, 3H), 7.28-7.49 (m, 10H), 5.92 (d, 1H,  $J = 3.0$  Hz), 2.25 (d, 1H,  $J = 3.0$  Hz);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$  (ppm): 143.8, 142.9, 140.8, 140.5, 128.8, 128.6, 127.7, 127.4, 127.3, 127.2, 127.0, 126.6, 76.1;  $m/z$  calcd for  $\text{C}_{19}\text{H}_{15}$   $[\text{M}-\text{OH}]^+$  243.1174, found 243.1165.

**Preparation of [1,1'-biphenyl]-4-yl(phenyl)methanone (7)**

Solution of alcohol **(6)** (1 g, 3.8 mmol) was added to pyridinium chlorochromate (1.2 g, 5.7 mmol) and celite (4 g) and the reaction mixture stirred at reflux for 3 hours at room temperature. The reaction mixture was filtered and the collected organic solution was dried over  $\text{MgSO}_4$  and concentrated to afford 1.11 g (100% yield) of ketone **(7)** as a white crystal. mp 87-90 °C (lit 99-100 °C) [9],  $R_f = 0.47$ ; IR (neat)  $\nu_{\text{max}}/\text{cm}^{-1}$  3052, 1643, 1597, 1445;  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  (ppm): 8.58-8.62 (m, 2H), 8.51-8.55 (m, 2H), 8.35-8.38 (m, 2H), 8.21-8.31 (m, 3H) 8.06-8.13 (m, 4H), 7.97-8.02 (m, 1H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$  (ppm): 196.4, 145.3, 140.0, 137.8, 136.3, 132.4, 130.8, 130.1, 129.0, 128.4, 128.2, 127.4, 127.0;  $m/z$  calcd for  $\text{C}_{19}\text{H}_{15}\text{O}$   $[\text{M}]^+$  259.1123, found 259.1130.

**Preparation of [1,1'-biphenyl]-4-yl(4-fluorophenyl)(phenyl)methanol (8)**

Solution of 4-fluoro-1-bromobenzene (0.9 g, 5.4 mmol) was added to magnesium turnings (0.13 g, 5.4 mmol). The reaction mixture was stirred at reflux, then at room temperature for further 6 hours. A solution of ketone **(7)** (0.7 g, 2.7 mmol) was added and the reaction mixture left stirring at room temperature for overnight. The reaction mixture was quenched with a saturated ammonium chloride solution (30 mL). The aqueous layer was separated and extracted with  $\text{Et}_2\text{O}$  (2 x 20 mL). The combined organic layers were washed with water then dried over  $\text{MgSO}_4$  and evaporated. Purification of the crude product was conducted by flash chromatography to give 0.73 g of alcohol **(8)** as a colourless oil (76% yield).  $R_f = 0.35$ ; IR (neat)  $\nu_{\text{max}}/\text{cm}^{-1}$  3477, 3030, 1600, 1506, 1224, 1158;  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  (ppm): 8.34-8.37 (m, 2H), 8.26-8.30 (m, 2H), 8.11-8.16 (m, 2H), 7.95-8.05 (m, 10H), 7.54-7.62 (m, 2H) 2.91 (s, 1H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$  (ppm): 179.1, 176.1, 158.6, 157.2, 153.6, 150.8, 150.3, 137.5, 137.4, 136.3, 135.8, 135.7, 135.3, 135.1, 134.7, 134.5, 134.1, 133.6, 118.7, 118.5, 77.2;  $m/z$  calcd for  $\text{C}_{25}\text{H}_{18}\text{F}$   $[\text{M}-\text{OH}]^+$  337.1393, found 337.1401.

**Preparation of 1-[[1,1'-biphenyl]-4-yl(4-fluorophenyl)(phenyl)methyl]-1H-imidazole (10):**

Thionyl chloride (0.7g, 6 mmol) with dimethyl formamide (0.1 mL) was added to a solution of alcohol (**8**) (0.7 g, 2 mmol) in anhydrous dichloromethane (20 mL) and the mixture stirred at 0°C for 1 hour. The reaction mixture was left at 40°C at reflux stirring for overnight. The organic solvent was evaporated under reduced pressure and the residue was washed with acetonitrile (2 x 20 mL) to afford compound (**9**) as a yellow oily mass and was submitted for the further reaction immediately with no purification and the structure elucidation. A solution of imidazole (0.2 g, 3.5 mmol) with Et<sub>3</sub>N (0.5g, 5.2 mmol) was prepared in anhydrous acetonitrile (20 mL) at 0°C and slowly added to a solution of compound (**10**) (0.7g, 1.7 mmol) in anhydrous acetonitrile (20 mL). The reaction mixture was left at 80°C at reflux stirring for 72 hours. The organic solvent was evaporated and EtOAc (20 mL) with water (20 mL) was added to the residue. The aqueous layer was extracted with EtOAc (2 x 20 mL). The combined organic layers were washed with brine solution (30 mL) then dried over MgSO<sub>4</sub> prior to evaporation. The crude product was purified by flash chromatography to afford 0.3g of compound (**10**) as a white crystal (47% yield). mp 97-99 °C; R<sub>f</sub> = 0.38; IR (neat)  $\nu_{\max}/\text{cm}^{-1}$  3031, 1735, 1600, 1507, 1233, 1164; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  (ppm): 8.17-8.24 (m, 4H), 8.11 (s, 1H), 8.01-8.06 (m, 2H), 7.91-7.96 (m, 4H), 7.66-7.73 (m, 6H), 7.62 (s, 1H), 7.49-7.57 (m, 2H), 7.30 (s, 1H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>)  $\delta$  (ppm): 179.3, 176.2, 152.9, 151.6, 151.2, 149.9, 148.7, 148.0, 139.6, 139.5, 137.7, 136.2, 135.7, 135.4, 135.3, 134.7, 133.9, 133.4, 127.1, 119.0, 118.7, 68.3; *m/z* calcd for C<sub>25</sub>H<sub>18</sub>F [M-Imid]<sup>+</sup> 337.1393, found 337.1401.

**Antifungal activity evaluation of compounds 4 and 10:** Test samples (10 mg) were dissolved in DMSO (1 mL) to prepare 1 mg/mL solutions, respectively. Test microorganisms *E.coli*, *M.luteus* and yeast were suspended in a Ringer solution (1.5 mL) within an eppendorf tube (1.5 mL) using an inoculation loop. UV spectra were calibrated with McFarland standard solution No. 0.5 (described below). The suspension was diluted until the absorbance of UV spectroscopy indicated approximately 0.1. Test bacteria and a yeast were inoculated using sterile cotton-wool swab on Mueller Hinton agar (MHA) and potato dextrose agar (PDA) plates (90 mm), respectively. Solutions of test compounds were placed on blank disk filter

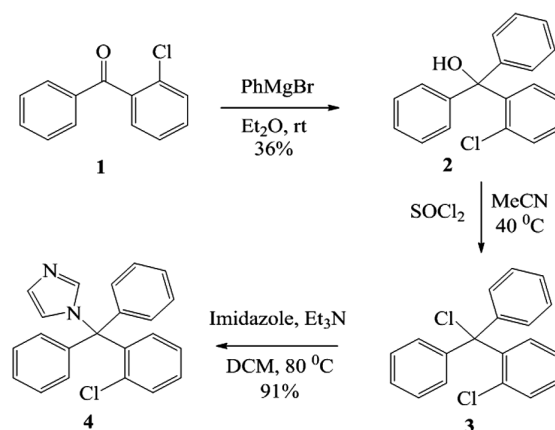
papers (7 mm) and the disks were transferred onto plates. Bacteria and yeast were allowed to grow 24 and 48 hours, respectively. Inhibition zones of test samples and standard drug were measured in millimetre.

McFarland Standard No. 0.5:

- 1.0 % Barium chloride (ml) 0.05
- 1.0 % Sulfuric acid (ml) 9.95
- Approx. cell density (1 x 10<sup>8</sup> CFU ml<sup>-1</sup>) 1.5
- % Transmittance 74.3
- Absorbance 0.132

**RESULTS AND DISCUSSION**

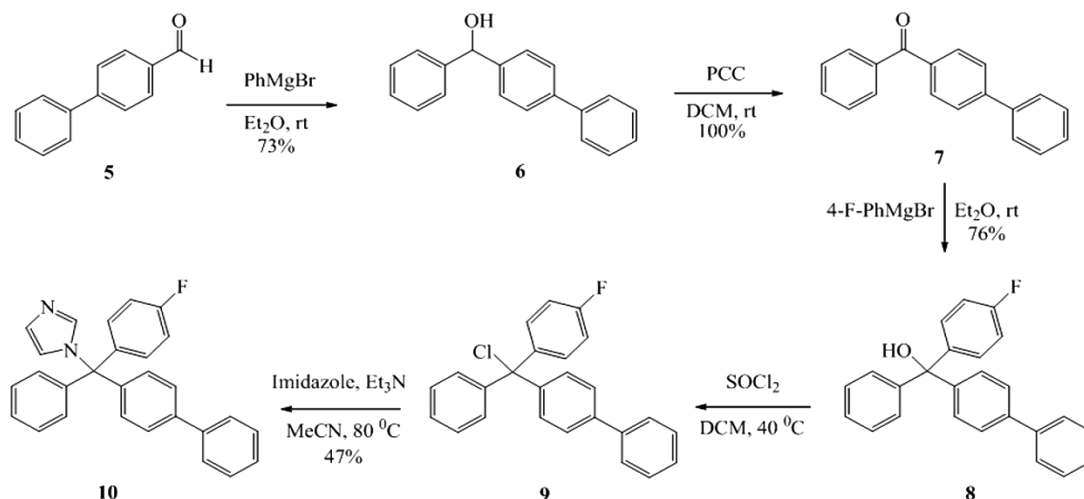
The antifungal agent clotrimazole (**4**) was synthesised in three steps (Scheme-1).



Scheme 1. Synthesis of clotrimazole

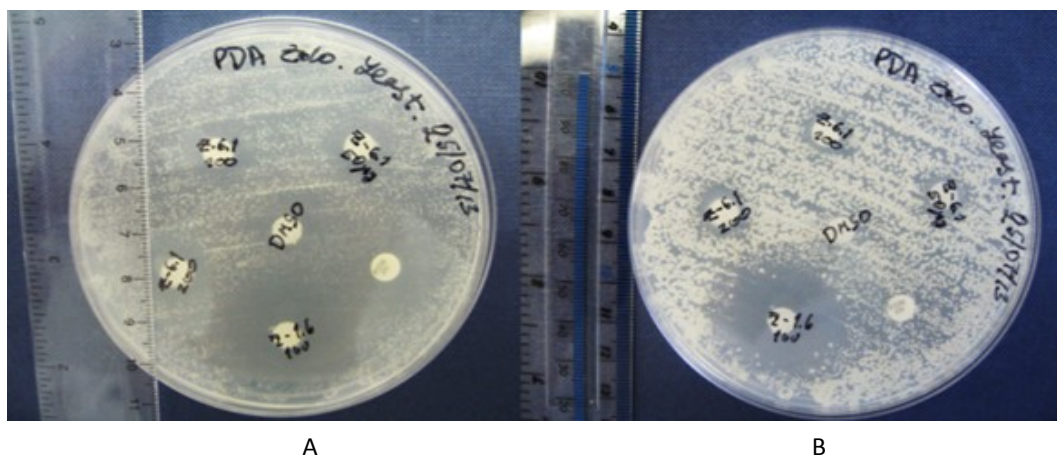
The synthesis started with commercially available ketone (**1**) and preparation of alcohol (**2**) was successful (36% yield). Chlorinated compound (**3**) has been prepared and submitted for further substitution reaction immediately without purification, characterisation and the structure elucidation due to instability of chlorinated compound (**3**) as this was previously reported[10]. Characterisation of clotrimazole (**4**) was achieved with spectral analysis including 2D (<sup>1</sup>H-<sup>1</sup>H) COSY NMR, MS and IR to confirm the structure.

Synthesis of a novel azole biphenyl derivative (**10**) was carried out in 5 steps linear synthetic route (Scheme 2). The overall yield of the entire synthesis was low at 26 %. There was no previous analytical data of the structure of compound (**10**), the spectral analysis supported the formation of a new compound.

Scheme 2. Synthetic route of the biphenyl derivative (**10**)

The antifungal activity of the biphenyl derivative (**10**, Z-6.1) together with clotrimazole (Z-1.6) was determined using Kirby-Bauer agar diffusion method. Fluconazole - 25  $\mu\text{g}$  (FCN) standard test sample and a DMSO dissolved disk were used as control agents. Similar inhibition zones were observed for the biphenyl derivative (**10**) and DMSO dissolved disks

excluding clotrimazole and fluconazole on yeast after growing 24 hours (Figure 1, A). This result was found to be related to solvent we used to dissolve our test samples for the bioassay and therefore we decided to grow yeast further 24 hours. After growing 48 hours, different inhibition zones were observed as indicated in Table 1 (Figure 1, B).



A B  
Fig. 1. Yeast plates. A - after 24 hours, B - after 48 hours

Table 1. Inhibition zones on an yeast plate

Samples	Concentration ( $\mu\text{g}$ )	Inhibition zone (mm)	
		24 hours after	48 hours after
Z-6.1	50	15	11
	100	15	11
	200	17	13
Z-1.6	100	31	27
FCN	25	23	0
DMSO	-	13	0

The novelazole derivative (**10**) had the highest activity at 200  $\mu\text{g}$ , whereas the clotrimazole showed more activity at twice as less concentration compared to the novelazole. Moreover, the compound (**10**) was not active against both *M.luteus* and *E.coli* after 24 hours of growing.

## CONCLUSIONS

We successfully synthesised clotrimazole (**4**) and a novel biphenyl derivative as 1-([1,1'-biphenyl]-4-yl(4-fluorophenyl)(phenyl)methyl)-1H-imidazole (**10**) through multistep synthesis. The overall yield of novel clotrimazole derivative (**10**) was comparably low at 26

% We found that the compound (**10**) was active against a yeast but less than compound (**4**). DMSO was believed not to be an appropriate solvent for antifungal testing by virtue of its physical property (high boiling point). Therefore, it was impossible to test compounds in higher concentration due to limited absorption of the disks we used. The newly synthesised compound (**10**) has been proven not to be a potential candidate for further antifungal agent development.

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