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DESIGN, SYNTHESIS AND EVALUATION OF NEW AZOLES AS ANTIFUNGAL AGENTS: A MOLECULAR HYBRIDIZATION APPROACH

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Two benzimidazole derivatives and one nitroimidazole derivative were designed using molecular hybridization approach. The designed compounds were synthesized and their *in vitro* antifungal activities were tested against *Candida albicans* and *Saccharomyces cerevisiae* strains. Based on the antifungal activity data, compound **9** containing 2-methyl-5-nitroimidazole moiety proved to be the most potent compound. It was more active than the reference drugs fluconazole and ketoconazole against *S. cerevisiae*.

Keywords: benzimidazole, nitroimidazole, azole, antifungal activity.

INTRODUCTION

Research and development of potent and effective antifungal agents represents one of the most important advances in therapeutics, not only in the control of serious infections, but also in the prevention and treatment of some infectious complications of other therapeutic modalities such as cancer chemotherapy and surgery. Over the past decade, fungal infection became an important complication and a major cause of morbidity and mortality in immunocompromised individuals such as those suffering from tuberculosis, cancer or AIDS and in organ transplant cases [1]. In clinical practice, antifungal agents that can be used for treating life-threatening fungal infections are limited. These drugs fall under five major classes: azoles, allylamines, polyenes, fluropyrimidines and thiocarbamates [2]. Among these, azoles are the most widely used antifungal agents because of their high therapeutic index.

Azoles (Fig. 1) are a large and relatively new group of synthetic compounds, of which imidazoles and triazoles are two clinically beneficial families employed in the treatment of systemic fungal infections as well as in agriculture [3 – 6]. Azole antifungal agents inhibit the cytochrome P450 sterol 14α-demethylase (14DM, CYP51) by a mechanism in which the heterocyclic nitrogen atom (N-3 of imidazole and N-4 of triazole) binds to the heme iron atom in the binding site of fungal enzyme [7, 8]. Oxyconazole (Fig. 1) is one of the famous azole antifungal agents containing the imidazole ring with oxyimino moiety that has been widely used in the treatment of fungal infections [9].

In addition, some nitroimidazole- and benzimidazole-containing drugs such as metronidazole, thiabenazole, parbendazole, benomyl and carbendazim (Fig. 2) were shown to have very potent antifungal activity [10]. Recently, a number of new benzimidazoles have been synthesized and screened for their antifungal activities (Fig. 3) [11 – 14]. Some evidence suggests that the benzimidazole moiety present in some compounds (Fig. 2) exhibit a pharmacophore character for the inhibition of fungal activity [15]. Metronidazole is one of the nitroimidazole-containing compounds with antibacterial and antifungal activity.

Here we report the design and synthesis of novel imidazole and benzimidazole derivatives (compounds **7 – 9**) and their screening for antifungal activity. As shown in Fig. 4, the proposed design is based on the molecular hybridization approach, with hybridization of two imidazole (oxyconazole and benzimidazole) pharmacophores capable of acting as antifungal agents. Therefore, we explored this idea further based on design, synthesis and pharmacological evaluation of the new series of compounds containing benzimidazole and nitroimidazole with oxyimino moiety. To

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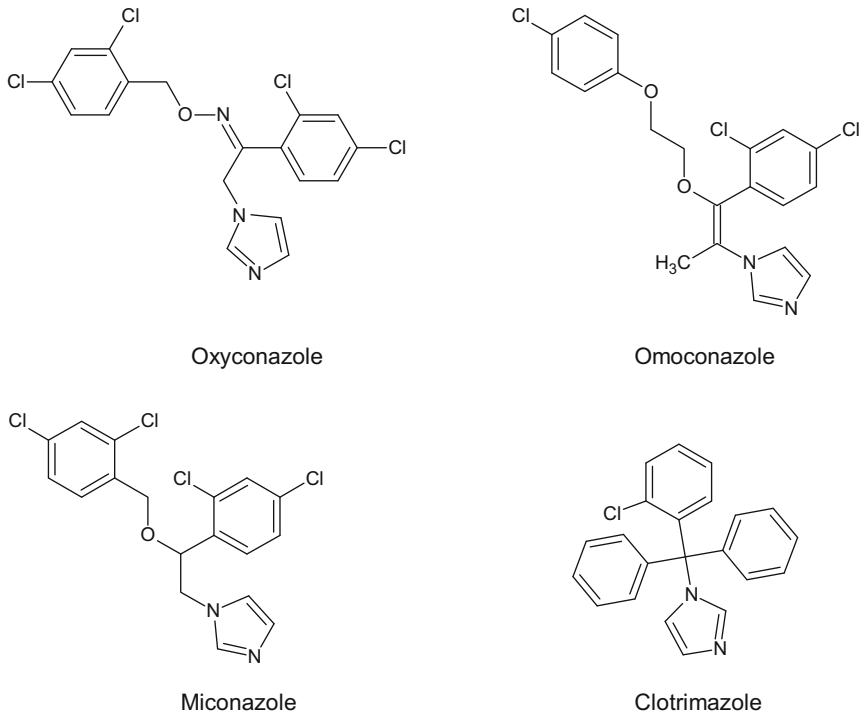

Fig. 1. Structures of azole antifungal agents.

achieve a good structure – activity relationship (SAR) in this series of compounds, the oxyimino part was kept like oxyconazole and the azole part was changed with nitroimidazole and various benzimidazoles.

EXPERIMENTAL

Chemistry

Nitroimidazole and benzimidazole derivatives (compounds **7 – 9**) were synthesized according to scheme in Fig. 5. These compounds were prepared by reaction of nitroimidazole and benzimidazoles (compounds **2** and **3**) [16] with 2-chloro-1-(2, 4-dichlorophenyl)-*N*-hydroxyethanimine and 2, 4-dichlorobenzylchloride in two steps [17 – 19].

All compounds were characterized by TLC, followed by IR, elemental analysis and, proton NMR. Melting points were determined using a Thomas-Hoover capillary apparatus and were uncorrected. The ^{1}H NMR spectra were recorded on a Bruker FT-500 spectrometer, using TMS as an internal standard. The IR spectra were measured on a Nicolet 550 FT-IR spectrometer. Elemental analysis was carried out with a Perkin-Elmer model 240-C apparatus. The results of elemental analysis (C, H, N) were within ± 0.4% of the calculated values.

2-Chloro-1-(2,4-dichlorophenyl)-N-hydroxyethanimine (1). An aqueous solution of hydroxylamine hydrochloride (1.86 g, 26.7 mmol) was added to a solution of 2-chloro-1-(2,4-dichlorophenyl)ethanone (2 g, 8.8 mmol) in methanol (30 mL) and the reaction mixture was stirred for 72 h at room temperature. The reaction product was precipitated by addition of water (50 mL), filtered, and dried to obtain the target compound as white crystals; yield, 91%; MP, 75 – 78°C; IR (KBr; ν, cm^{-1}): 3200 (OH), 1590 (C=N); ^{1}H NMR ($CDCl_3$; δ, ppm): 9.07 (bs, 1H, OH), 7.40 – 7.50 (m, 1H, phenyl), 7.25 – 7.30 (m, 2H, phenyl), 4.61 (s, 2H, CH_2); $C_8H_6Cl_3NO$.

1H-Benzimidazole (2). This compound was prepared according to the literature [16]; MP, 171 – 172°C; IR (KBr; ν, cm^{-1}): 3110 (NH); ^{1}H NMR ($CDCl_3$; δ, ppm): 8.05 (bs, 1H, NH), 7.58 – 7.70 (m, 3H, H-2, H-4 and H-7 benzimidazole), 7.28 – 7.39 (m, 2H, H-5 and H-6 benzimidazole); $C_7H_6N_2$

2-Methyl-1H-benzimidazole (3). This compound was prepared according to the literature [16]; MP, 176°C; IR (KBr; ν, cm^{-1}): 3120; (NH); ^{1}H NMR ($CDCl_3$; δ, ppm): 7.35 – 7.55 (m, 2H, H-4 and H-7 benzimidazole), 7.05 – 7.30 (m, 2H, H-5 and H-6 benzimidazole), 6.30 (bs, 1H, NH), 2.59 (s, 3H, CH_3); $C_8H_8N_2$.

2-(1H-Benzimidazol-1-yl)-1-(2,4-dichlorophenyl)-N-hydroxyethanimine (4). Compound **1** (1 g, 4.19 mmol) was gradually added to a solution of compound 2 (1.5 g, 12.7 mmol) in DMF (10 mL) and the resulting mixture was stirred for 10 h at room temperature. The reaction product was precipitated by addition of water (20 mL), filtered, washed, and dried to obtain the target compound; yield, 79%; MP, 200 – 201°C; IR (KBr; ν, cm^{-1}): 3205 (OH), 1592

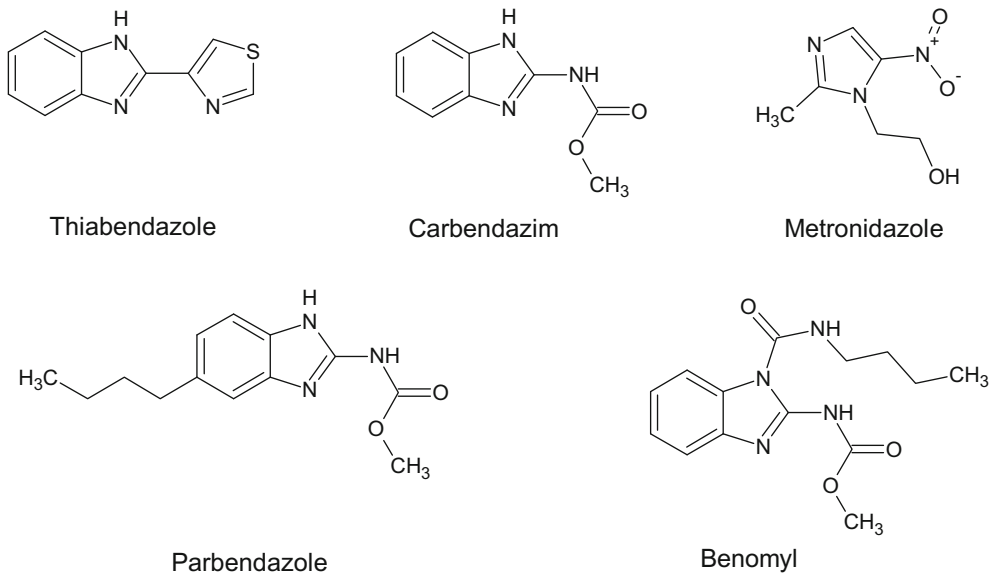

Fig. 2. Structures of some nitroimidazole and benzimidazole based antifungal agents.

(C=N).; ^{1}H NMR ($CDCl_3$; δ, ppm): 7.55 – 7.80 (m, 2H, H-4 and H-7 benzimidazole), 7.00 – 7.50 (m, 4H, H-5, H-6 benzimidazole and H-3, H-6 phenyl), 6.73 (d, J= 8.1 Hz, 1H, H-5 phenyl), 5.25 (s, 2H, CH_2); $C_{15}H_{11}Cl_2N_3O$.

1-(2,4-Dichlorophenyl)-N-hydroxy-2-(2-methyl-1H-benzimidazol-1-yl)ethanimine (5). Compound **1** (0.97g, 4.06 mmol) was gradually added to a solution of compound 3 (1.6 g, 12.20 mmol) in DMF (10 mL) and the resulting mixtrue was stirred for 10 h at room temperature. The reaction product was precipitated by addition of water (20 mL), filtered, washed, and dried to obtain the target compound; yield 71%; MP, 240 – 241°C; IR (KBr; ν, cm^{-1}): 3480 (OH), 1610 (C=N); ^{1}H NMR (DMSO-d_6; δ, ppm): 11.43 (s, 1H, OH), 7.04 – 7.60 (m, 7H, aromatic), 5.21 (s, 2H, CH_2), 2.40 (s, 3H, CH_3); $C_{16}H_{13}Cl_2N_3O$.

1-(2,4-Dichlorophenyl)-N-hydroxy-2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethanimine (6). Compound **1** (0.5g, 2.09 mmol) was gradually added to a solution of 2-methyl-5-nitroimidazole (0.8 g, 6.3 mmol) in DMF (7 mL) and the resulting mixture was stirred for 48 h at room temperature. The reaction product was precipitated by addition of water (15 mL), filtered, washed, and dried to obtain the target compound; yield, 74%; MP, 220 – 221°C; IR (KBr; ν, cm^{-1}): 1602 (C=N), 1535 and 1369 (NO_2); ^{1}H NMR (DMSO-d_6; δ, ppm): 7.59 (s, 1H, imidazole), 7.50 (d, J = 2.4 Hz, 1H, H-3 phenyl), 7.31 (dd, J = 8.4 Hz, J = 2.4 Hz,

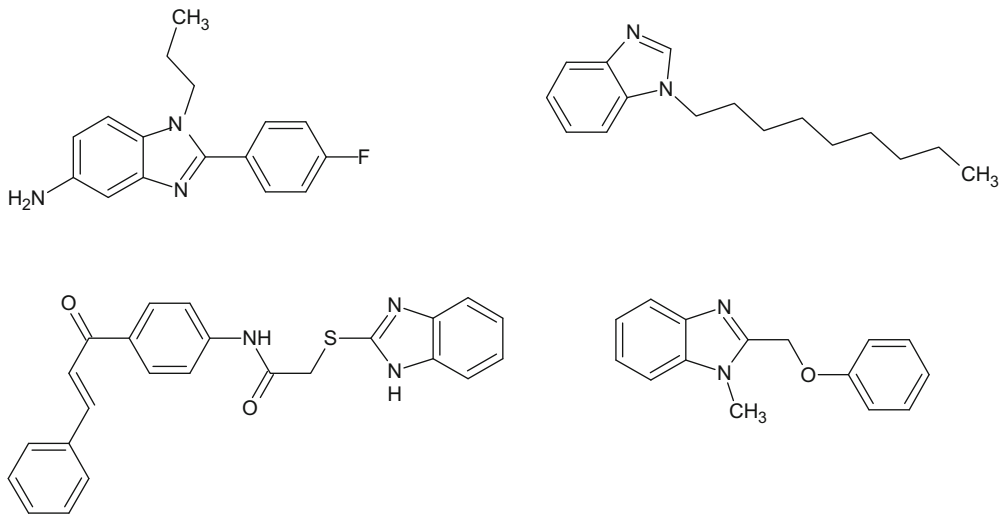

Fig. 3. Some recently synthetized benzimidazole-containing antifungal agents.

H-5 phenyl), 6.90 (d, J = 8.4 Hz, 1H, H-6 phenyl), 5.18 (s, 2H, CH_2), 2.45 (s, 3H, CH_3); $C_{12}H_{10}Cl_2N_4O_3$.

2-(1H-Benzimidazol-1-yl)-N-((2,4-dichlorobenzyl)oxy)-1-(2,4-dichlorophenyl)ethanimine (7). To a suspension of compound **4** (1 g, 3.125 mmol) and potassium carbonate (0.43 g, 3.125 mmol) in 15 mF of dry DMF was gradually added 0.5 mL (0.6 g, 3.15 mmol) of 2,4-dichlorobenzyl chloride and the reaction mixture was stirred for 72 h at room temperature. Then, 40 mL water was added to the mixture and it was extracted with ethylacetate (3 × 20 mL). The solvent was removed under reduced pressure and the oily residue was purified by TLC (silica gel; ethyl acetate – chloroform, 5:1) to give the title compound as a white solid; yield, 70%; MP, 132 – 134.5°C; IR (KBr; ν, cm^{-1}): 1601 (C=N); ^1H NMR ($CDCl_3$; δ, ppm): 7.85 (d, J = 8 Hz, 1H, aromatic), 7.37 (d, J = 2.4 Hz, 1H, aromatic), 7.32 (t, J = 7.6 Hz, 2H, aromatic), 7.08 (m, 5H, aromatic), 6.90 (dd, J = 8.4 Hz, J = 2.0 Hz, 1H, aromatic), 6.10 (d, J = 8.8 Hz, 1H, aromatic), 5.27 (s, 2H, OCH_2), 4.28 (s, 2H, CH_2); $C_{22}H_{15}Cl_4N_3O$.

N-((2,4-Dichlorobenzyl)oxy)-1-(2,4-dichlorophenyl)-2-(2-methyl-1*H*-benzimidazol-1-yl)ethanimine (8). To a suspension of compound **5** (1 g, 2.99 mmol) and potassium carbonate (0.41 g, 2.99 mmol) in 15 mL dry DMF was gradually added 0.4 mL (0.58 g, 2.99 mmol) of 2,4-dichlorobenzyl chloride and the reaction mixture was stirred for 72 h at room temperature. Then, 40 mL water was added and the mixture was extracted with ethylacetate (3 × 20 mL). The solvent was removed under reduced pressure and the oily residue was purified by column chromatography (silica gel, chloroform) to obtain the title compound as a white solid; yield, 63%; MP 124 – 125.5°C; IR (KBr; ν, cm^{-1}): 1590 (C=N); ^1H NMR ($CDCl_3$; δ, ppm): 7.68 (d, J = 8 Hz, 1H, aromatic), 7.13 – 7.44 (m, 7H, aromatic)7.07 (dd, J = 8.4 Hz, J = 1.6 Hz, 1H, aromatic), 6.40 (d, J = 8.4 Hz, 1H, aromatic), 5.15 (s, 2H, CH_2O), 5.05 (s, 2H, CH_2), 2.35 (s, 3H, CH_3); $C_{23}H_{17}Cl_4N_3O$.

N-((2,4-Dichlorobenzyl)oxy)-1-(2,4-dichlorophenyl)-2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethanimine (9). To a suspension of compound **6** (0.7 g, 2.208 mmol) and potassium carbonate (0.3 g, 2.208 mmol) in 8 mL dry DMF was

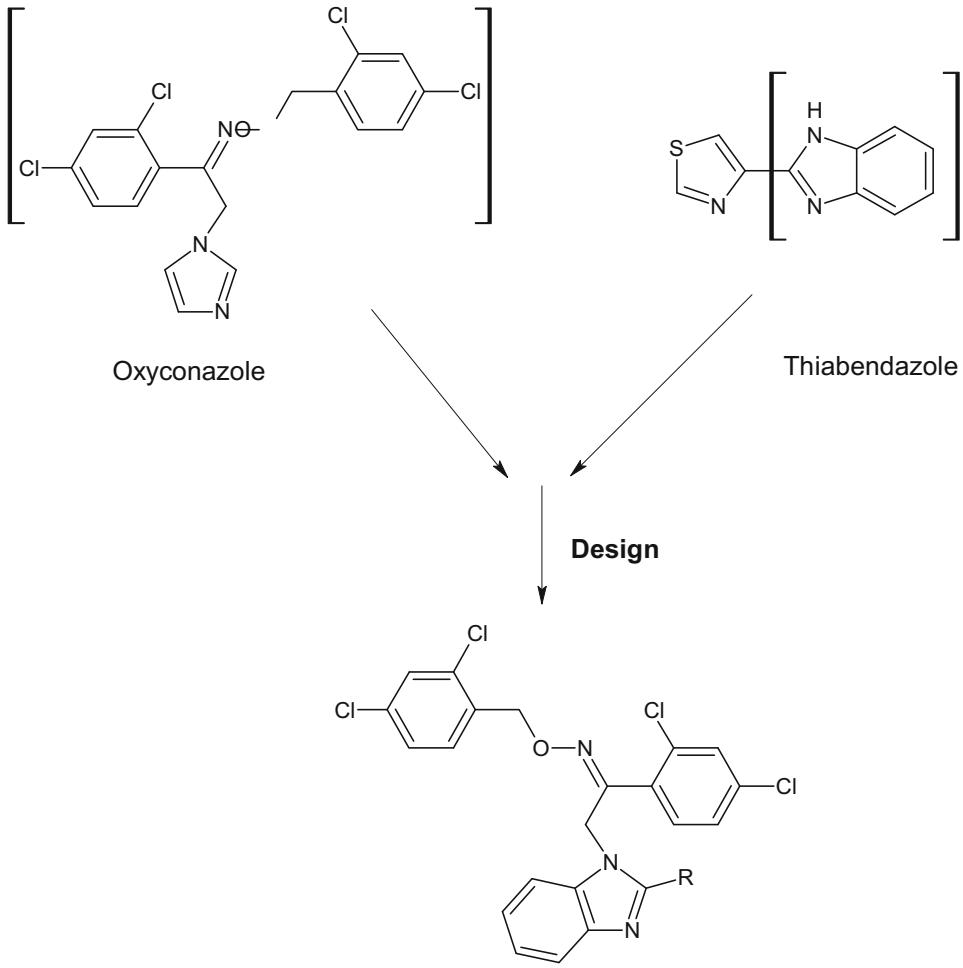

Fig. 4. Using molecular hybridization approach to design new antifungal agents.

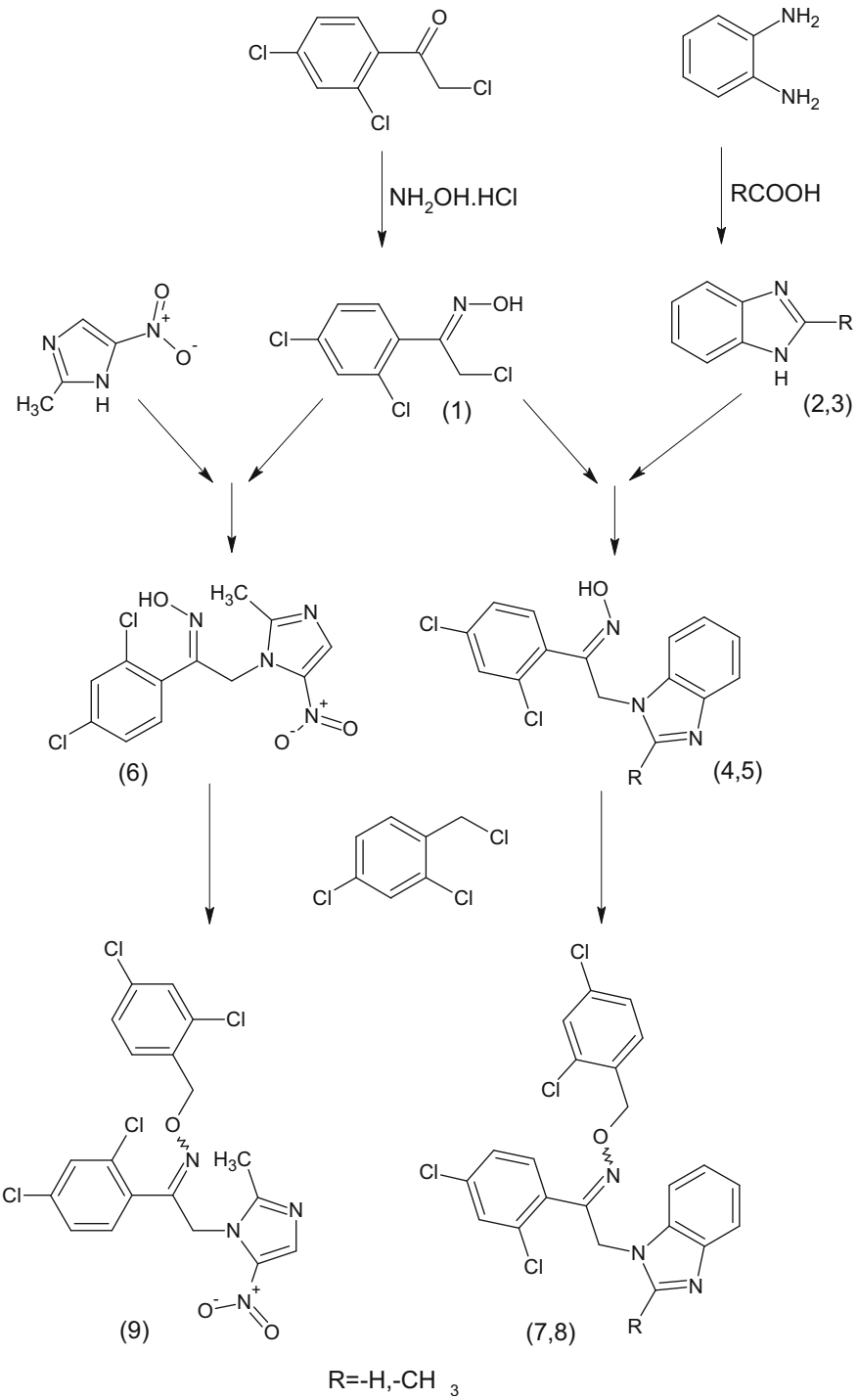

Fig. 5. Synthesis of nitroimidazole and benzimidazole derivatives.

gradually added 0.3 mL (0.43 g, 2.208 mmol) of 2,4-dichlorobenzyl chloride and the reaction mixture was stirred for 96 h at room temperature. Then, 30 mL water was added and the mixture was extracted with ethylacetate (3 × 20 mL). The solvent was removed under reduced pressure and the oily residue was purified by TLC (silica gel; ethyl acetate – chloroform, 3:1) to obtain the title compound as a white solid; yield 67%; IR (KBr; ν, cm^{-1}): 1615 (C=N), 1367 and 1530 (NO_2); ^1H NMR ($CDCl_3$; δ, ppm): 7.59 (s, 1H, imidazole), 7.56 (s, 1H, aromatic), 7.45 (s, 1H, aromatic), 7.36 (d, J = 6.8 Hz, 1H, aromatic), 7.30 (d, J = 8.4 Hz, 1H, aromatic), 7.24 (d, J = 8 Hz, 1H, aromatic), 6.94 (d, J = 8 Hz, 1H, aromatic), 5.25 (s, 2H, CH_2O), 4.95 (s, 2H, CH_2), 2.35 (s, 3H, CH_3); $C_{19}H_{14}Cl_4N_4O_3$.

TABLE 1. Antifungal Activity of Compounds **7 – 9** against *S. cerevisiae* PTCC 5052

Compound	R	MIC, μM	
		24 h	48 h
7	benzimidazole	>2086	>2086
8	2-methylbenz-imidazole	>2027	>2027
9	2-methyl-5-nitro-imidazole	64.01*	64.01*
Fluconazole		326.5	326.5
Ketoconazole		94.08	188.16
Amphotericin B		Not tested	Not tested
DMSO		10% v/v	10% v/v

* $p < 0.05$.

TABLE 2. Antifungal Activity of Compounds **7 – 9** against *C. albicans* ATCC 10231

Compound	R	MIC, μM	
		24 h	48 h
7	benzimidazole	>2086.89	>2086.89
8	2-methylbenzimid-azole	>2027.57	>2027.57
9	2-methyl-5-nitro-imidazole	128.02	128.02
Fluconazole		2.28	4.89
Ketoconazole		<1.3	1.3
Amphotericin B		<4.2	4.2
DMSO		5% v/v	5% v/v

Antifungal Activity Evaluation

The synthesized compounds were tested against *Candida albicans* (ATCC10231) and *Saccharomyces cervisiae* (PTCC 5052) fungal strains using broth microdilution assay. The broth microdilution method was implemented according to NCCLS guidelines [20, 21]. Both strains were sub-cultured on Sabouraud Maltose Agar (DIFCO, Becton, Dickinson, USA) growth media. *C. albicans* and *S. cervisiae* were suspended in sterile 0.9% NaCl solution. The turbidity of fungal suspensions was adjusted at 75 – 77% transmittance (*T*) using a spectrophotometer at 530 nm. The suspensions were diluted 1:1000 in Sabouraud maltose broth for testing. Serial dilutions of the test compounds with growth medium were prepared in microdilution wells, and 100 μL of diluted suspension was added to 100 μL of each test compounds solution. Microdilution trays were incubated at 35°C and examined after 24 and 48 h to determine MIC (minimum inhibitory concentration) values. Amphotericin B (AppliChem, Germany), fluconazole, and ketoconazole solutions and DMSO (Merck) 2.5% were used, respectively, as medium positive and negative controls [22].

Statistical Analysis

The one-way ANOVA statistical test was used to assess the significance of differences between various groups. In the case of significant *F* values, multiple comparison Tukey test was used to compare the means of different treatment groups. Results with $P < 0.05$ were considered to be statistically significant.

RESULTS AND DISCUSSION

Chemistry

Three new derivatives of imidazole and benzimidazole, compounds **7 – 9** (Fig. 5), were synthesized using dry DMF at room temperature and were purified by chromatography in good yield (63 – 70%). Chemical structures of synthesized compounds were confirmed using TLC followed by IR, elemental analysis, and proton NMR.

Antifungal Activity

The *in vitro* antifungal activities of the synthesized compounds **7 – 9** were tested against two representative pathogenic yeast fungi (*C. albicans* and *S. cerevisiae*) using broth microdilution assay (Tables 1 and 2). The MIC values were determined by comparison to amphotericin B, fluconazole, and ketoconazole as reference drugs.

Antifungal activity evaluation against *S. cerevisiae* (Table 1) showed that compounds **7** and **8** did not possess significant antimicrobial properties against tested pathogenic fungi, while compound **9** exhibited significant antifungal effect. MIC values of the synthesized compounds indicate that compounds **7** and **8,** which contain benzimidazole ring, are not active against *S. cerevisiae*. Compound **9**, which contains 2-methyl-5-nitroimidazolemoiety, is most active against *S. cerevisiae* (with a MIC value of 64.01 μM). In addition, the activity of this compound against *S. cerevisiae* after 24 h is higher than that of reference drugs fluconazole and ketoconazole (with MIC values 326.5 and 94.08 respectively). Our antifungal evaluation results showed that compound **9** was active against *S. cerevisiae* after 24 and 48 h, being about 5 and 1.5 times more active than fluconazole and ketoconazole after 24 h, respectively, and about 3 times more active than ketoconazole after 48 h.

Antifungal activity evaluation against *C. albicans* (Table 2) showed that compounds **7** and **8** also did not possess significant antifungal properties, while compound **9** exhibited a more noticeable antifungal effect. However, all these compounds were less active than the reference drugs fluconazole, ketoconazole, and amphotericin B.

Thus, based on the antifungal activities, the MIC values of tested compounds showed that only compound **9** was highly active against *S. cerevisiae* and this activity exceeded that of the reference drugs. The results of antimicrobial evaluation show that 2-methyl-5-nitroimidazole moiety is bioisoster of imidazole and triazole in azole-containing antifungal agents.

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