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Synthesis and Evaluation of Antimicrobial Activity of Some 1,5(6)-H/or -Methyl-2-subtituted Benzimidazole Derivatives

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In this study, the investigation of structure-activitiy relationships for some newly synthesized benzimidazole structures was aimed. For this purpose, eight compounds having 1,5(6)-H/or -CH_3 and 2-substituted benzimidazole structures were synthesized. Subsequently, their antimicrobial activities were examined. These compounds were tested *in vitro* against three Gram positive (*Enterococcus faecalis*, *Staphylocccus aureus* and *Staphylocccus epidermidis*) and two Gram negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*). In addition, they were screened *in vitro* for their antifungal activities. All of these compounds inhibited the growth of Gram positive and Gram negative bacteria at MIC values between 12.5 and 200 µg/mL and had MIC values between 50 and 200 µg/mL for the following fungi (*Candida albicans*, *Candida krusei*, *Candida glabrata*, *Candida tropicalis* and *Candida parapsilosis*).

Key Words: Benzimidazole, Antimicrobial, Antibacterial and Antifungal activity.

INTRODUCTION

The frequency of microbial infection in humans has increased dramatically because of multi-drug resistant microbial isolates (*e.g.*, fungi and bacteria)[1-4]. The increasing clinical significance of drug-resistant bacterial pathogens has lent additional urgency to microbiological and antibacterial research. It is well established that benzimidazoles exhibit a wide variety of pharmacological properties such as antimicrobial[5], antitumor[6,7] and inhibition of nucleic acid synthesis[8]. Many anticancer drugs (such as Imet 3393 (cytostasan)[9], oncodazole[10], Hoechst 33258 (pibenzimol)[6] have a benzimidazole ring in their structures. In addition, the discovery of imidazole and related structures in biologically important molecules (such as

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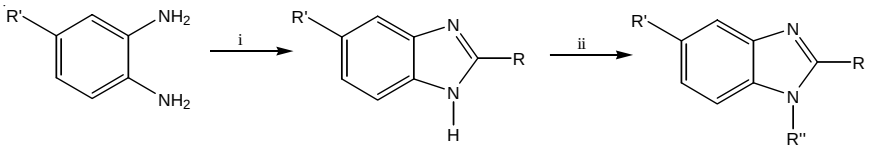
ionheme systems, vitamin B_{12} and its derivatives), there has been increased interest in imidazole chemistry. Benzimidazole derivatives and their metal complexes have been investigated as model compounds for a number of biological molecules including metalloenzymes and serine proteases[11]. Benzimidazole is found in a variety of naturally occuring compounds such as vitamin B_{12} and its derivaties and it is structurally similar to purin bases.

The overall object of the study was to achieve better understanding the antimicrobial activity of the benzimidazole ring at some fungi and bacteria. In this study, eight benzimidazole compounds having 1,5(6)-H/or-CH_3 and 2-substituent (-CH_2OH, -CH_2NH_2, -CH_3, -C_2H_5, -CH_2SH) benzimidazoles structures were synthesized (Fig. 1). In particular, the role of free N-H group in the benzimidazole ring with respect to antimicrobial activity was investigated through the synthesis of a series of compounds with and without this group. Synthesis of the compounds (**1-8**) has previously been reported by our research group[12-16]. These compounds were investigated for microbiological activity against bacterial and yeast isolates. Their antibacterial activities were investigated using three Gram positive (*Enterococcus faecalis, Staphylocccus aureus* and *Staphylocccus epidermidis*) and two Gram negative (*Escherichia coli, Pseudomonas aeruginosa*) bacteria and employing broth microdilution. Subsequently, their antifungal inhibitory activities were investigated against yeast-like fungi (*Candida albicans, Candida krusei, Candida glabrata, Candida tropicalis* and *Candida parapsilosis*).

Structural elucidation of these compounds was performed by IR, ^{1}H NMR and elemental analysis.



Comp.	R	R'	Comp.	R	R"
1	-CH_2OH	-H	3	-CH_2OH	-H
2	-CH_2OH	-CH_3	6	-CH_3	-CH_3
4	-CH_2NH_2	-H			
5	-CH_3	-H			
7	-C_2H_5	-H			
8	-CH_2SH	-H			

Reagents: (i) RCOOH, % 5.5 N HCl, reflux 2-30 h (ii) NaH, MeI, DMF, reflux, 24 h

Fig. 1. General synthesis scheme of the compounds

Structures and formulas, purification procedures, physical and spectral data of the compounds are shown in Table-1 respectively.

TABLE-1
PHYSICAL AND ANALYTICAL DATA OF THE COMPOUNDS

Comp. No	m.f. (m.w.)	Purification Method	Yield (%)	m.p. (ºC)	NMR (δ ppm) (DMSO-d_6)	IR (KBr) cm^{-1}
1	$C_8H_8N_2O$ (148.16)	Crystallization EtOH	34	169	4.68 (s, 2H, -C$\underline{H}_2$OH), 5.53 (bs, 1H, -O$\underline{H}$), 7.07-7.16 (m, 2H) 7.45-7.47 (d, J=1.4, 2H), 12.17 (s, 1H, -N-$\underline{H}$)	3600-2300 (N-H, O-H, =C-H aromatic,) 2910 (-CH aliphatic) 1615 (C=N), 1600-1500 (C=C).
2	$C_9H_{10}N_2O$ (162.18)	Crystallization EtOH	34	198	2.38 (s, 3H, -C$\underline{H}_3$), 4.64 (s, 2H, -C$\underline{H}_2$OH), 5.50 (bs, 1H, -O$\underline{H}$), 6.91-6.95 (d, J=7.8 H^6, 1H), 7.21-7.41 (m, H^4, H^7, 2H)	3600-2200 (N-H, O-H, =C-H aromatic,) 2910 (-CH aliphatic) 1612 (C=N), 1600-1500 (C=C).
3	$C_9H_{10}N_2O$ (162.19)	Crystallization H_2O	50	143	3.83 (s, 3H, -N-C$\underline{H}_3$), 4.64 (s, 2H, -C$\underline{H}_2$OH), 4.72 (s, 1H, -O$\underline{H}$), 7.02-7.36(dtd, J=7.11, 6.78 and 1.1 Hz, H^5, H^6, 2H), 7.51-7.53 (d, J=7.84 Hz, H^7, 1H), 7.57-7.59 (d, J=7.84 Hz, H4, 1H),	3650-2300 (O-H,-CH aliphatic) 1650 (C=N), 1600-1400 (C=C).
4	$C_8H_{11}N_3Cl_2$ (220.10)	Crystallization EtOH	75	255	4.51 (s, 2H, -C$\underline{H}_2$), 7.45 (dd, J=6.1 and 3.1 Hz, 2H), 7.80 (dd, J=9.3 and 3.2 Hz, 2H), 9.10 (s, 3H, NH_3^+/NH_2)	3600-2300 (N-H, =C-H aromatic, -CH aliphatic), 1615 (C=N), 1600-1400 (C=C).
5	$C_8H_8N_2$ (132.16)	Crystallization EtOH	59	174	2.48 (s, 3H, -C$\underline{H}_3$), 7.44 (dd, J=9.0 and 3.2 Hz, 2H), 7.09 (dd, J=9.2 and 3.2 Hz, 2H), 12.25 (brs, 1H, -N$\underline{H}$)	3300-2300 (N-H, =C-H aromatic, -CH aliphatic), 1612 (C=N), 1600-1300 (C=C).
6	$C_9H_{10}N_2$ (146.19)	Crystallization H_2O	57	112	2.52 (s, 3H, -C$\underline{H}_3$), 3.72 (s, 3H, -N-C$\underline{H}_3$),7.50 (d, J=7.3 Hz, 1H), 7.45 (dd, J=7.5 Hz, 1H), 7.15 (dtd, J=7.5, 6.04 and 1.2 Hz, 2H)	3044-2300 (=C-H aromatic) 2904 (-CH aliphatic), 1616 (C=N), 1600-1300 (C=C).
7	$C_9H_{10}N_2$ (146.19)	Crystallization EtOH- H_2O	69	172	1.20-1.24 (t, J= 7.61 Hz, 3H, -C$\underline{H}_3$), 2.70-2.75 (q, J=7.60 Hz, 2H, -C$\underline{H}_2$), 6.98-7.02 (dd, J=9.4, 2H and 3.2 Hz, H^5, H^6), 7.36 (m, 2H , H^4, H^7), 12.40 (s, 1H, -N-$\underline{H}$)	3600-2300 (N-H, =C-H aromatic, -CH aliphatic), 1618 (C=N), 1600-1400 (C=C).
8	$C_8H_8N_2S$ (164.22)	Crystallization EtOH	60	158-159	4.01-4.37 (s, 2H, -C$\underline{H}_2$SH), 7.17-7.18 (m, H^5, H^6, 2H) 7.54 (m, H^4, H^7), 12.57 (brs, 1H, -N-$\underline{H}$)	3600-2300 (N-H, =C-H aromatic, -CH aliphatic), 2537-2578 (-SH), 1617 (C=N), 1600-1400 (C=C).

EXPERIMENTAL

Melting points were measured on an Electrothermal 9200 melting point apparatus and were uncorrected. Infrared spectra were recorded in KBr pellets on a Mattson 1000 FTIR spectrometer in the range 4000-400 cm^{-1}. Proton magnetic resonance (^1H NMR) spectra were recorded in DMSO-d_6 (Merck) on a Bruker 400 MHz spectrometer. All chemicals and solvents used were of reagent grade (Aldrich, Merck, Sigma) and were used without further purification. Thin-layer chromotography (TLC) was perfomed on pre-coated aluminium plates (Silica gel 60 F_{254}, Merck). Plates were visualized by UV light, Dragendorff reagent and iodine vapour.

General procedure for the synthesis of benzimidazoles (1-8)

1,5(6)-H/-methyl-2-substituted benzimidazoles were prepared according to the Phillips method[17]. Equimolar amounts of diamine (*o*-phenylenediamine, 3,4-diaminotoluene) and the corresponding carboxylic acid in 5.5N hydrochloric acid were refluxed for 2-30 h. The solution was cooled in an ice bath and neutralized with sodium bicarbonate. The resulting precipitate was filtered off, washed several times with water and purified by recrystallization. 1-Methyl benzimidazoles were prepared from the corresponding 1*H*-benzimidazoles by the reaction with methyl iodide in the presence of sodium hydride in DMF.

Preparation of the cell culture

To evaluate cytotoxicity of chemical synthesis for human cells, the HEp-2 cell line (HEp-2 cell line no: ATCC CCL23) was selected. The cell culture medium consisted of EMEM (Eagle's Minimum Essential Medium) with fetal calf serum (Seromed, Biochrom KG, Germany) at a ratio of 10 % as the growth factor. The cells were incubated in an atmosphere of 5 % carbon dioxide at 37°C.

In order to test the effect of the synthesized compounds on HEp-2 cells, 5×10^4 cells were seeded into each well of 12-well plates, cultured for 6 h at 28°C and the cells were allowed to grow for an additional 48 h. The synthesized compounds were diluted and decreasing amounts (1500, 1000, 800, 400, 200, 100, 75, 50, 25, 12.5 and 6.25 µg/mL) were placed per well. The cytotoxicity of the compounds was determined using a conventional haemocytometer and the trypan blue-exclusion method[18]. The highest noncytocidal (on HEp-2 cells) concentration of the synthesized compounds was determined to be 1000 µg/mL. Therefore, up to 1000 µg/mL was used for the determination of the antimicrobial activities.

Antimicrobial activity

Stock solutions of the synthesized compounds were prepared by dissolving in methanol and then diluting in Mueller-Hinton broth and

Sabouraud dextrose broth to give an initial concentration of 8 mg/mL. Further dilutions of the compounds and standard drugs in the test medium were prepared at the required quantities at concentrations of 800, 400, 200, 100, 75, 50, 25, 12.5 and 6.25 µg/mL. In order to ensure that the solvents had no effect on microbial growth, a control test was also performed containing inoculated broth supplemented with only methanol and dimethyl sulfoxide at the same dilutions used for the test compounds and was determined to be inactive. The minimal inhibitory concentrations (MIC) for each compound was investigated against standard bacterial strains; Escheri*chia coli* (ATCC 25922), *Enterococcus faecalis* (ATCC 29212), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 29213) and *Staphylococcus epidermidis* (ATCC 12228) and yeast-like fungi; *Candida albicans* (ATCC 90028), *Candida krusei* (ATCC 6258) and *Candida parapsilosis* (ATCC 22019), *Candida tropicalis* (ATCC 22019) and *Candida glabrata* (ATCC 32554) obtained from the Refik Saydam Hifzisihha Institute, Ankara, Turkey.

Flucanazole and ampicillin were used as control drugs. The observed data on the antimycotic activity of the compounds and the control drugs are given in Tables 2 and 3.

TABLE-2
MIC VALUES (µg/mL) FOR BACTERIA OF THE TESTED COMPOUNDS

Compounds	*E. coli*	*P. aeruginosa*	*S. aureus*	*S. epidermidis*	*E. faecalis*
1	50	50	50	12.50	50
2	50	50	50	12.50	50
3	100	100	100	50.00	100
4	25	25	200	12.50	50
5	50	50	50	12.50	25
6	50	100	100	50.00	100
7	50	25	50	25.00	100
8	100	50	25	12.50	25
Ampicillin	6.25	31.2	3.12	1.56	6.25

Antibacterial assay

The cultures were prepared in Mueller-Hinton broth (Difco, MI, USA) for all the bacteria and incubated for 24 h at 37 ± 1°C. Testing was carried out in Mueller-Hinton broth at pH 7.4 and the twofold serial dilution technique was applied. The microorganisms were grown overnight in Mueller-Hinton broth at 37 ± 1°C and the final inoculum size was 10^5 CFU/mL for the antibacterial assay. A set of tubes containing only inoculated broth was kept as controls. After inculation for 24 h at 37 ± 1°C, the

lowest concentration that showed no growth of microorganism was recorded as the MIC expressed in µg/mL. These experiments were duplicated to define the MIC values.

TABLE-3
MIC VALUES (µg/mL) FOR YEAST-LIKE FUNGI OF THE TESTED COMPOUNDS

Compounds	*C. albicans*	*C. glabrata*	*C. krusei*	*C. parapsilosis*	*C. tropicalis*
1	50	50	200	100	200
2	50	50	50	100	100
3	50	50	100	100	200
4	50	50	50	50	50
5	100	100	50	50	100
6	100	100	100	100	100
7	50	50	100	50	100
8	50	50	100	200	100
Flucanazole	6.25	3.12	31.2	3.12	6.25

Antimycotic assay

The yeasts were maintained in Sabouraud dextrose broth (Difco, MI, USA, pH 7.4) and incubated for 24 h at 25 ± 1°C. The two fold serial dilution technique was applied. The microorganisms were grown overnight in Mueller-Hinton broth at 37 ± 1°C and the final inoculum size was 10^4 CFU/mL for the antifungal assay. A set of tubes containing only inoculated broth was kept as a control. After incubation for 48 h at 25 ± 1°C, the lowest concentration that showed no growth of yeast was recorded to represent the MIC expressed in µg/mL. Each experiment was duplicated in order to define the MIC values.

RESULTS AND DISCUSSION

The synthesized compounds were evaluated *in vitro* for antimicrobial activity against *E. coli, P. aeruginosa, S. aureus, S. epidermidis, E. faecalis, C. albicans, C. glabrata, C. krusei, C. parapsilosis, C. tropicalis*. Flucanazole and ampicillin were used as controls (Tables 2 and 3). All of the compounds were able to inhibit the growth of the screened microorganisms *in vitro* with MIC values between 12.5-100 µg/mL. Among the compounds tested, 1, 2, 4, 5, 8 showed potent activity against *S. epidermidis* (ATCC 12228) with MIC values of *ca.* 12.5 µg/mL which was one order of magnitude less than that of ampicillin (1.56 µg/mL). The other compounds 3, 6, 7 were relatively less active. In addition, the antibacterial activity of these compounds was more efficient against Gram positive bacteria than Gram negative bacteria.

The MIC values against *Staphylococcus aureus* (ATCC 29213) were of the order of 25-200 µg/mL. On the other hand, MIC values for *E. faecalis* (ATCC 29212) were 25-100 µg/mL. It was determined that these compunds possessed antibacterial activity against gram negative bacteria at similar levels (25-100 µg/mL) as obtained for *S. aureus* (ATCC 29213). Hence, the tested compounds may be regarded as highly active against *S. epidermidis* and less active against *E. coli, P. aeruginosa, S. aureus* and *E. faecalis*.

The antifungal assessment revealed that the compounds possess only moderate or slight activity. The MIC values are generally within the range of 50-200 µg/mL. By comparing their MIC values with flucanazole, the compounds were less active against *C. albicans, C. glabrata, C. krusei, C. parapsilosis* and *C. tropicalis*.

As we consider all results obtained from antifungal and antibacterial tests together and concluded that entire compounds tested more active towards bacteria then fungi. The reason for the lower antifungal activity of these compounds may be due to different structure of cell walls in fungi and bacteria. The fungal cell wall and cell membrane are fundamentally different from those of bacteria and other eukaryotes. Fungal cell walls are composed largely of chitin, a polymer of N-acetylglucosamine, rather than peptide-glican-a characteristic component of bacterial cell walls. The fungal membrane contains ergosterol rather than the cholesterol found in mammalian membranes[19].

When the compounds bearing –NH group are compared to those with $–N–CH_3$ substituents regarding antibacterial and antifungal activities, the ones with –NH group were found to be more active. Thus, this may indicate the necessity to have such functional groups for antibacterial and antifungal activities in this kind of compounds. However, it still requires further investigation; the experiments regarding the structure activity relationship are stil underway.

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