Antifungal Evaluation of Bis Mannich Bases Derived from Acetophenones and Their Corresponding Piperidinols and Stability Studies

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The development of resistance to current antifungal therapeutics drives the search for effective new agents. The fact that some acetophenone-derived Mannich bases had shown antifungal activities in our previous studies led us to design and synthesize acetophenone-derived bis Mannich bases, B1—B5, bis(β-aroylethyl)methylamine hydrochlorides, to evaluate their antifungal activity. These bis Mannich bases were then converted to the corresponding piperidinols, C1—C5, which are structural isomers of bis derivatives, 3-aroyl-4-aryl-1-methyl-4-piperidinol hydrochlorides, to see alterations in biological activity. A stability study of B1 and C1 was also carried out to estimate whether they alkylate the thiols. All compounds studied have shown antifungal activity, especially against dermatophytes (*Trichophyton rubrum*, *Trichophyton mentagrophytes*, *Trichophyton tonsurans*, and *Microsporum canis*), in the concentration range studied (2—128 μg/ml). The activity was especially apparent against *T. tonsurans*. All compounds had at least equal antifungal activity compared with the reference compound amphotericin-B against *T. tonsurans*. Bis Mannich bases were generally found to be more potent compounds than their structural isomer piperidinols. The results of our stability studies suggest that thiol alkylation may contribute to the antifungal activity of the Mannich bases synthesized. Even though all compounds showed antifungal activity against dermatophytes, bis Mannich bases B1, B2, B4, and B5 appear to have potential for developing novel antifungal agents against dermatophytes.

Key words acetophenone; antifungal activity; dermatophyte; mannich base

Mannich bases have several biological activities such as antimicrobial,[1—5] cytotoxic,[6—8] anticancer,[9,10] analgesic,[11] diuretic,[12,13] and anticonvulsant activities.[14—17] Deamination is important in evoking biological responses. An amino ketone possessing at least one activated hydrogen atom at the β position to an amino group can undergo deamination *in vivo* or under simulated *in vitro* conditions to generate the corresponding α,β-unsaturated ketone.[18] The biological activities of Mannich bases have been attributed to these liberated α,β-unsaturated ketones that can alkylate nucleophiles, especially thiol groups.[1,6,19,20] It has been shown that there is a relationship between the increased antimicrobial activity and an increased breakdown of Mannich bases.[21]

The development of resistance to current antifungal therapeutics continues to drive the search for more effective new agents. It is reported that Mannich bases such as those of conjugated styryl ketones,[4] and isatin N-Mannich bases[22] have antifungal activity. The fact that several acetophenone-derived Mannich bases had shown remarkable antifungal activities in our previous studies,[2,3] led us to design and synthesize some acetophenone-derived bis Mannich bases, **B1**—**B5**, bis(β-aroylethyl)methylamine hydrochlorides, to evaluate their antifungal activity against some yeasts and dermatophytes. Later these bis Mannich bases were converted to the corresponding piperidinols, **C1**—**C5**, which are structural isomers of bis derivatives, 3-aroyl-4-aryl-1-methyl-4-piperidinol hydrochlorides, to see alterations in their biological activity with modifications in their chemical structure. A stability study of the **B1** representing bis Mannich bases and **C1** representing piperidines was also carried out to estimate whether these compounds alkylate the thiols, which may contribute to their possible mechanism of action.

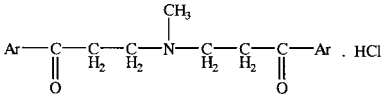
MATERIALS AND METHODS

Chemistry The synthesis of acetophenone-derived bis Mannich bases **B1**—**B5** and some corresponding piperidinols **C1**, **C4** (Table 1) were reported in our previous study.[16]

Synthesis of **C2** and **C5** was as follows: 1-Methyl-3-*p*-methylbenzoyl-4-*p*-methylphenyl-4-piperidinol hydrochloride (**C2**): A mixture of 13.64 g of bis[β-(*p*-methylbenzoyl)ethyl]-methylamine hydrochloride synthesized according to our previous study[16] and 40 ml of NaOH solution (5%) was stirred vigorously at 35 °C until the oily base solidified, which was first obtained in 30 min. The mixture was stirred at room temperature overnight. The solid was filtered and crystallized from ethanol to give a piperidine derivative, which was a crude base of **C2** (8.55 g). This base was solved in dry ether and treated with gaseous hydrogen chloride to obtain com-

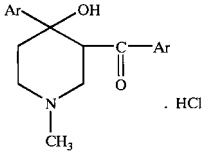
Table 1. Compounds Studied for Antifungal Activity

Bis Mannich bases



Ar: **B1** (C_6H_5), **B2** (*p*-$CH_3C_6H_4$), **B3** (*p*-$CH_3OC_6H_4$), **B4** (*p*-ClC_6H_4), **B5** (C_4H_3S)

Piperidinols



Ar: **C1** (C_6H_5), **C2** (*p*-$CH_3C_6H_4$), **C4** (*p*-ClC_6H_4), **C5** (C_4H_3S)

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pound **C2**. This compound was crystallized from methanol–ether (4.80 g, 35% yield), mp: 200 °C. The analytical data of the synthesized compound were as follows: ^{1}H-NMR (CD_3OD) δ: 1.94 (1H, dt, J=2.4 Hz, J=−14.8 Hz, H5a), 2.22 (3H, s, Ar-CH_3), 2.38 (3H, s, Ar-CH_3), 2.77 (1H, m, H5eq), 2.87 (3H, s, N-CH_3), 3.47 (4H, m), 5.15 (1H, d, J=2.8 Hz, OH), 5.45 (1H, dd, J=4.1 Hz, J=10.9 Hz, H3), 7.04 (2H, d, J=7.9 Hz, Ar-H), 7.26 (2H, d, J=8.54 Hz, Ar-H), 7.40 (2H, d+m, J=8.3 Hz, Ar-H), 8.01 (2H, d+m, J=6.8 Hz, Ar-H). ^{13}C-NMR (CD_3OD) δ: 200.95 (s), 146.36 (s), 140.94 (s), 137.20 (s), 132.27 (s), 129.94 (d), 129.30 (d), 129.27 (d), 124.36 (d), 71.26 (s), 52.73 (d), 50.94 (t), 46.04 (t), 43.36 (q), 36.91 (t), 21.81 (q), 20.88 (q). IR (KBr) cm^{-1} 1650 (C=O). UV λ_{max} (H_2O) nm (log ε): 265 (4.13). ESI-MS m/z: 325, 324 (M^++1), 306. *Anal.* Calcd for $C_{21}H_{26}ClNO_2$: C, 70.09; H, 7.28; N, 3.89. Found: C, 70.49; H, 7.25; N, 3.91.

1-Methyl-4-(2-thienyl)-3-(2-thienylcarbonyl)-4-piperidinol hydrochloride (**C5**): The same experimental procedure used for **C2** was applied to obtain **C5** (1.4 g, 27.45% yield, mp: 194 °C). A mixture of 5.10 g (0.015 mol) of **B5** and 35 ml of a 10% solution of NaOH in 110 ml of distilled water were stirred at 40 °C overnight. The final compound, **C5** was crystallized using methanol.

The analytical data of the synthesized compound were as follows: ^{1}H-NMR (CD_3OD) δ: 2.18 (1H, dt, J=2.5 Hz, J=−15.1 Hz, H5a), 2.73 (1H, m, H5 eq), 2.90 (3H, s, N-CH_3), 3.44 (3H, m), 3.54 (1H, dd, J=3.8 Hz, J=−11.53 Hz, 6eq), 5.30 (1H, dd, J=3.9 Hz, J=12.2 Hz, H3), 5.40 (1H, d, J=2.7 Hz, OH), 6.85 (1H, dd, J=3.6 Hz, J=5.1 Hz, Ar-H), 6.98 (1H, dd, J=1.2 Hz, J=3.2 Hz, Ar-H), 7.12 (1H, dd, J=1.2 Hz, J=5.1 Hz, Ar-H), 7.20 (1H, dd, J=4.0 Hz, J=4.9 Hz, Ar-H), 7.75 (1H, dd, J=1.1 Hz, J=4.9 Hz, Ar-H), 8.42 (1H, dd, J=0.9 Hz, J=4.0 Hz, Ar-H). ^{13}C-NMR (CD_3OD) δ: 193.1 (s), 149.1 (s), 142.2, 137.6 (d), 136.7 (d), 129.6 (d), 127.3 (d), 124.7 (d), 122.9 (d), 70.6 (s), 52.9 (d), 50.7 (t), 48.5 (t), 43.4 (q), 38.0 (t). IR (KBr) cm^{-1} 1640 (C=O). UV λ_{max} (H_2O) nm (log ε): 235 (4.07), 270 (4.03), 295 (4.00). ESI-MS m/z: 310, 309, 308 (M^++1), 290, 247. *Anal.* Calcd for $C_{15}H_{18}ClNO_2S_2$: C, 52.39; H, 5.28; N, 4.07. Found: C, 52.15; H, 5.08; N, 4.09.

Antifungal Activity Assay The antifungal activity of these compounds was determined against yeasts and dermatophytes. Five species of yeasts (*Saccharomyces cerevisiae*, *Geotrichum* sp., *Candida krusei*, *Rhodotorula* sp., three strains of *Candida albicans*) and four species of dermatophytes (*Trichophyton rubrum*, *Trichophyton mentagrophytes*, *Trichophyton tonsurans*, and *Microsporum canis*) were used to test the antifungal activity of the compounds. *Candida albicans I* was ATCC strain 90028 and all the other fungi were isolated from clinical specimens. The agar dilution method[23] was used in susceptibility testing, as in our previous study.[1] The method was considered more suitable to screen the antimicrobial activity of several compounds than the broth dilution method described by the NCCLS,[24,25] which is the approved standard method for susceptibility determination of yeasts. The concentration range of the compounds tested in Sabouraud–dextrose agar (Difco, U.S.A.) was 2—128 μg/ml. Amphotericin-B (Fungizone, Bristol Myers Squibb) was used as a reference antifungal drug.

For the susceptibility test, fungi were grown in Sabouraud–dextrose broth at 28 °C, the yeasts for 2 and the dermatophytes for 7 d. For the inoculum, dermatophytes were used without dilution, while yeasts were diluted 1 : 10 in 0.9% NaCl. Ten microliters of each fungus was pipetted on the Sabouraud agar containing the compound to be tested. The microbes were incubated at 28 °C, the yeasts for 4 d and the dermatophytes for 10 d. The minimum inhibitory concentration (MIC) values reported were the lowest concentration of the compound (μg/ml) which inhibited the growth of the fungus. Antimicrobial tests were performed three times to verify the repeatability.

Stability Studies: Reactions of B1 and C1 with 2-Mercaptoethanol 2-Mercaptoethanol (1.17 g, 1.1 ml, 0.015 mol) was added to the solution of **B1** (4.97 g, 0.015 mol) in phosphate buffer (pH 7.4, 25 ml), and the mixture was incubated at 37 °C in a shaking, constant-temperature water bath for 24 h. The reaction was monitored by TLC using chloroform–methanol (9 : 1), and the disappearance of starting ketone was followed. The reaction mixture was extracted with chloroform (3×15 ml), the chloroform layer was dried over sodium sulfate and filtered, and solvent was removed *in vacuo*. The residue obtained from the reaction was passed through a column of silica gel by eluting with chloroform–methanol (9 : 1). Removal of the solvent gave 3-(2-hydroxyethylthio)-1-phenyl-1-propanone (2.3 g), which was a yellow liquid with a yield of 79%. In the case of **C1**, similar reaction conditions were carried out with **B1**. The residue obtained from the stability study of **C1** was purified by preparative TLC using the same developing system giving 3-(2-hydroxyethylthio)-1-phenyl-1-propanone in the yield of 30%.

Spectral Data of Compound 3-(2-Hydroxyethylthio)-1-phenyl-1-propanone: ^{1}H-NMR ($CDCl_3$) δ: 2.55 (1H, bs, OH), 2.77 (2H, t, J=7.5 Hz, $COCH_2$), 2.94 (2H, t, J=7.5 Hz, CH_2S), 3.28 (2H, t, J=7.5 Hz, SCH_2), 3.77 (2H, t, J=7.5 Hz, CH_2OH), 7.45 (2H, dd, J_1=7.5 Hz, J_2=1.5 Hz, Ar-H), 7.57 (1H, ddd, J_1=J_2=7.5 Hz, J_3=1.5 Hz, 1H, Ar-H), 7.95 (2H, dd, J_1=7.5 Hz, J_2=1.5 Hz, Ar-H). ^{13}C-NMR-DEPT ($CDCl_3$) δ: 198.01 (C=O), 136.31 (quaternary C),133.23, 128.54, 127.89 (CH), 60.54, 38.90, 35.71, 25.86 (CH_2). IR (KBr) cm^{-1}: 3400 (O–H), 1670 (C=O), 1190 (C–S stretching). UV ($CHCl_3$) nm (log ε): 245 (4.25). MS m/z: 211 (M^++1).

RESULTS AND DISCUSSION

To our knowledge, compound **C5** is reported in this study for the first time. All compounds synthesized showed antifungal activity against dermatophytes at the concentration range of 2—128 μg/ml studied. The MIC values are presented in Table 2. The compounds were not effective against yeasts, except for compound **B2**. MIC values of the compounds against the yeasts; *S. cerevisiae*, *Geotrichum* sp., *C. krusei*, *Rhodotorula* sp. and three strains of *C. albicans*, were higher than 128 μg/ml. Only compound **B2** had MIC value of 128 μg/ml against *Geotrichum* sp. and one strain of *C. albicans* obtained from a clinical specimen. The reference compound (amphotericin-B) showed activity at 128, 128, >128, and 32 μg/ml against *S. cerevisiae*, *Geotrichum* sp., *C. krusei*, *Rhodotorula* sp., and at 4, 4, and 2 μg/ml against *C. albicans* strains, respectively. The MIC value of amphotericin-B for *C. albicans* ATCC 90028 was 2 μg/ml, which is within the MIC range given by the NCCLS.

Compounds **B1**, **B2**, **B4**, **B5**, **C2**, **C4**, and **C5** were more

Table 2. Antifungal Activity against Dermatophytes of the Synthesized Mannich Bases Presented as Minimal Inhibition Concentrations (MIC, μg/ml)

Dermatophyte	B1	B2	B3	B4	B5	C1	C2	C4	C5	Ref.
Trichophyton rubrum	64	32	128	32	64	128	128	64	128	16
Trichophyton mentagrophytes	32	16	128	32	32	128	128	128	128	32
Trichophyton tonsurans	32	16	128	64	16	128	64	64	32	128
Microsporum canis	64	32	128	32	32	128	16	32	64	16

Values represent the means of three independent experiments.

potent than the reference compound amphotericin-B against *T. tonsurans.* Compound **B2** was more potent than amphotericin-B against *T. mentagrophytes.* However, equal antifungal activity to that of amphotericin-B was found in compounds **B1**, **B4**, and **B5** against *T. mentagrophytes*, in compounds **B3** and **C1** against *T. tonsurans*, and in compound **C2** against *M. canis*.

All compounds synthesized in this study were less effective than the reference compound amphotericin-B against *T. rubrum*. Except its effect against *M. canis*, bis Mannich base **B2**, which contains an electron-donating CH_3 substituent, was the most potent compound against dermatophytes among all Mannich bases synthesized in this study. Apart from its effect against *M. canis*, **C4**, which contains an electron-accepting substituent chlorine, was the most potent piperidinol derivative against dermatophytes. Of the compounds synthesized, bis Mannich base **B3** and piperidinol derivative **C1** showed a nonspecific antifungal activity against all dermatophytes tested at 128 μg/ml concentration.

The effect of conversion of bis Mannich bases to the corresponding piperidinols were as follows: only the conversion of bis Mannich base **B2** to its corresponding piperidinol derivative **C2** increased the antifungal activity against *M. canis*. The conversion of bis Mannich base **B4** to its corresponding piperidinol derivative **C4** did not affect the antifungal activity against *M. canis* and *T. tonsurans*. However, the conversion of bis Mannich bases to their corresponding piperidinol derivatives generally decreased the antifungal activity against dermatophytes. Decreases in antifungal activity were greatest when bis Mannich bases **B1**, **B4**, and **B5** were converted to their corresponding piperidinol derivatives **C1**, **C4**, and **C5**, respectively, and bis derivative **B2** was converted to its corresponding piperidinol derivative **C2**.

The replacement of the benzene ring with its bioisoster thiophene ring increased the antifungal activity in bis Mannich bases and piperidinol derivatives against *T. tonsurans* and *M. canis*, while it did not affect the antifungal activity against *T. rubrum* and *T. mentagrophytes* in both types of Mannich base.

The MIC ranges of all compounds tested against different dermatophytes were between 16—128 μg/ml. The MIC range of amphotericin-B against the same dermatophytes was also between 16—128 μg/ml. Thus the compounds seemed to have equal antifungal activity compared with amphotericin-B. The MIC values of amphotericin-B obtained for dermatophytes were, however, higher compared with those in other studies, where MIC ranges were 0.03—16 μg/ml.[26] The method of testing influences the results. The agar dilution method was chosen to screen the antimicrobial activity of the compounds instead of the broth dilution method, which is the approved standard method to test the susceptibility of yeasts.[24,25] There is no approved standard method for filamentous fungi.

In our previous study,[2] antimicrobial activity was seen in the concentration range of 2—64 μg/ml using bis Mannich bases and piperidinol derivatives with similar chemical structures, except that the substituent on the nitrogen atom was ethyl instead of methyl. Two of the microorganisms used in that study (*T. rubrum* and *M. canis*) were also used in the present study. In our previous study,[2] piperidine derivatives [Ar: C_6H_5, *p*-$CH_3C_6H_4$, *p*-ClC_6H_4, 2-thienyl (C_4H_3S)] were found effective against these dermatophytes, while corresponding bis Mannich bases were ineffective. In the present study, antifungal activity was seen in the concentration range of 2—128 μg/ml using bis(β-aroylethyl)methylamine hydrochlorides as bis Mannich bases and 3-aroyl-4-aryl-1-methyl-4-piperidinol hydrochloride derivatives. In the present study, both bis Mannich bases and piperidine derivatives were effective against *T. rubrum* and *M. canis*. Replacement of the substituent ethyl to methyl located on the nitrogen atom increased the antifungal activity in bis Mannich bases, while it decreased the antifungal activity in piperidines against *T. rubrum* and *M. canis*. Differences in antifungal activity may result from differences in chemical structures, which may affect their interaction with receptors involved in the biological activity. In addition, differences in experimental procedures and origins of the dermatophytes used in the two studies might have contributed to the differences in antifungal activity. The dermatophytes, *T. rubrum* TEM and *M. canis* TEM were provided by the Aegean University Biology Department, Basic and Industrial Microbiology Section, Izmir, Turkey, in our previous study.[2] On the other hand, the dermatophytes used in the present study were from clinical specimens of Kuopio University Hospital, Kuopio, Finland.

In one of our previous studies,[3] acetophenone-derived mono Mannich bases, 3-amino-1-phenyl-1-propanone salts, and their corresponding bis derivatives, 3-amino-1-phenyl-2-aminomethyl-1-propanone salts, demonstrated remarkable antifungal activity against the same dermatophytes used in this study. This and other observations[2,3] suggest that acetophenone-derived Mannich bases have potential for developing novel antifungal agents against dermatophytes.

It is known that Mannich bases are able to liberate α,β-unsaturated ketones.[1,18,27] It is reported that the thiol group of the biomimetic nucleophiles can react with an unsaturated ketone much more quickly than amine- and hydroxyl-type nucleophiles under simulated physiological conditions.[1] 3-(2-Hydroxyethylthio)-1-phenyl-1-propanone was obtained as a result of stability study of compounds **B1** and **C1** with 2-mercaptoethanol in phosphate buffer (pH 7.4, 37 °C) in this study. This suggest that compounds **B1** and **C1** have undergone deamination and α,β-unsaturated ketones are produced.

The adduction of 2-mercaptoethanol to these unsaturated ketones *via* the thiol group to produce 3-(2-hydroxyethylthio)-1-phenyl-1-propanone suggests that these compounds are thiol alkylators. The compounds studied most probably exhibit their antifungal activity by this mechanism. Supporting this mechanism, we have previously shown that Mannich bases alter the level of the most abundant cellular thiol, glutathione, in Jurkat cells.[19,20] Dimmock *et al.*[28] have reported that Mannich bases of conjugated styryl ketones inhibit one or more of the following enzymes in the glutathione metabolic pathway: glutathione S-transferases, glutathione reductase, gamma-glutamyl transpeptidase, and glutathione peroxidase in *C. albicans*. It appears that the inhibition of the enzymes in glutathione metabolism may also be considered as a possible mechanism of action contributing to the antifungal activity.

CONCLUSIONS

Bis Mannich bases and their corresponding structural isomers, piperidinols, synthesized in this study were shown to have antifungal activity against dermatophytes, but not against the yeasts. The Mannich bases synthesized had generally equal or more potent antifungal activity compared with amphotericin-B against the dermatophytes. Bis Mannich bases were more potent than their corresponding structural isomers, the piperidinols, in terms of the antifungal activity against dermatophytes. Therefore conversion of bis Mannich bases to their corresponding piperidinols generally decreased antifungal activity against dermatophytes. The results of our stability studies suggest that thiol alkylation may contribute to the antifungal activity of the Mannich bases synthesised. Even though all compounds synthesized had generally equal antifungal activity against the dermatophytes compared with the reference compound, amphotericin-B, bis Mannich bases **B1**, **B2**, **B4**, and **B5** appear to have potential for developing novel antifungal agents against dermatophytes.

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