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Toxicity of some Bis Mannich Bases and Corresponding Piperidinols in the Brine Shrimp (*Artemia salina*) Bioassay

H. Inci Gul,[1,3]* Mustafa Gul[2] and Ercin Erciyas[3]

[1] Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Ege University, Izmir, Turkey
[2] Department of Physiology, Faculty of Medicine, Ataturk University, Erzurum, Turkey
[3] Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Ataturk University, Erzurum, Turkey

Key words: Mannich bases; brine shrimp bioassay; toxicity; partition coefficient.

Some acetophenone-derived bis Mannich bases were synthesized: bis[β-benzoylethyl]ethylamine hydrochloride (IIa), bis[β-(*p*-methylbenzoyl)ethyl]ethylamine hydrochloride (IIb), bis[β-(*p*-chlorobenzoyl)ethyl]ethylamine hydrochloride (IId), bis[(2-thienylcarbonyl)ethyl]ethylamine hydrochloride (IIe); some corresponding piperidinol derivatives: 3-benzoyl-1-ethyl-4-phenyl-4-piperidinol hydrochloride (IIIa), 1-ethyl-3-(*p*-methylbenzoyl)-4-(*p*-methylphenyl)-4-piperidinol hydrochloride (IIIb), 1-ethyl-3-(*p*-methoxybenzoyl)-4-(*p*-methoxyphenyl)-4-piperidinol hydrochloride (IIIc), 1-ethyl-3-(*p*-chlorobenzoyl)-4-(*p*-chlorophenyl)-4-piperidinol hydrochloride (IIId), 1-ethyl-4-(2-thienyl)-3-(2-thienylcarbonyl)-4-piperidinol hydrochloride (IIIe); and some representative quaternary piperidinols: 3-benzoyl-1-ethyl-4-hydroxy-1-methyl-4-phenylpiperidinium iodide (IIIf), 1-ethyl-4-hydroxy-1-methyl-3-(*p*-methylbenzoyl)-4-(*p*-methylphenyl)piperidinium iodide (IIIg). Toxicity was tested by the brine shrimp bioassay as an intermediate test before further *in vivo* animal experiments. Piperidine derivatives were found to be more potent than bis Mannich bases. Quaternary piperidine derivatives IIIf and IIIg and also non-quaternary piperidine derivatives IIIb, IIIe, IIIc and IIId were more toxic than 5-fluorouracil in brine shrimp bioassay. Except for IIe, bis Mannich bases were not effective. Quaternization and conversion of bis Mannich bases to corresponding piperidines improved the toxicity. The lipid solubility of the compounds may not affect the toxicity. From these findings the quaternary piperidine derivatives IIIf and IIIg could be used in further drug development and also for *in vivo* experiments.
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INTRODUCTION

Mannich bases have various biological activities, including analgesic (Pilli *et al*., 1992), anti-inflammatory (Palaska *et al*., 1995), antimicrobial (Erciyas *et al*., 1994; Gul *et al*., 2001a; Gul *et al*., 2002a), cytotoxic (Gul *et al*., 2000, 2002b,c) and anticonvulsant (Dimmock *et al*., 1992; Gursoy *et al*., 1996; Gul *et al*., in press a) activities. Biological activities of Mannich bases have been attributed to alkylation of cellular nucleophiles, especially thiols after undergoing deamination. We showed previously that some Mannich bases are thiol alkylators, which may exert their toxicity by thiol alkylation (Erciyas *et al*., 1994; Gul *et al*., 2002b), and that they alter cellular glutathione levels and the activities of some glutathione-related enzymes (Gul *et al*., 2001b; Gul *et al*., 2002d).

We have synthesized some bis Mannich bases derived from acetophenone, *p*-substituted acetophenones and 2-acetylthiophene, namely bis(β-aroylethyl)ethylamine hydrochlorides **IIa**, **IIb**, **IIc**, **IId** and **IIe** (Fig. 1) and their corresponding piperidine derivatives, 3-aroyl-4-aryl-1-ethyl-4-piperidinol salts **IIIa**–**g** (Fig. 2), respectively, and reported their cytotoxic activities against Jurkat cells (Gul *et al*., 2002b). Aryl functions were phenyl for **IIa** and **IIIa**, *p*-methylphenyl for **IIb** and **IIIb**, *p*-chlorophenyl for **IId** and **IIId**, 2-thienyl for **IIe** and **IIIe** and *p*-methoxyphenyl for **IIIc**. The aromatic parts were phenyl and *p*-methylphenyl for **IIIf** and **IIIg**, respectively. Hydrochloride was replaced with methyl iodide in **IIIf** and **IIIg**, which are quaternary derivatives. In this study, we tested the toxic activities of these compounds by a brine shrimp bioassay that is reported to be a rapid, reliable, sensitive, inexpensive method not requiring complicated laboratory equipment (Meyer *et al*., 1982; McLaughlin *et al*., 1991). Furthermore, it does not require the animal serum that is needed for cell-based studies. The brine shrimp bioassay is an effective general bioassay used to screen for chemicals that are toxic to zoological systems. Brine shrimps have been utilized previously for analyses of mycotoxins (Eng-Wilmot and Martin, 1979), morphine-like compounds (Richter and Goldstein, 1970), the carcinogenicity of phorbol esters (Kinghorn *et al*., 1977) and toxicants in marine environments (Vanhaecke *et al*., 1981). We have shown recently that acetophenone-derived Mannich bases are effective in this bioassay (Gul *et al*., 2002c). The eggs of the brine shrimp *Artemia*

* Correspondence to: H. Inci Gul, Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Ataturk University, 25240 Erzurum, Turkey.
E-mail: incigul@atauni.edu.tr

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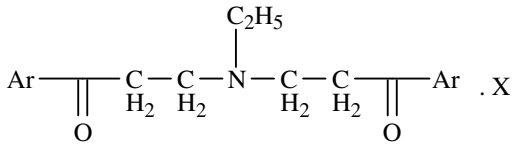
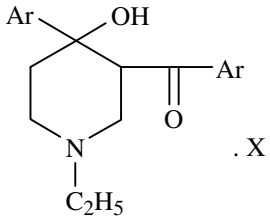


	Ar	X
IIa	C_6H_5	HCl
IIb	*p*-$CH_3C_6H_4$	HCl
IId	*p*-ClC_6H_4	HCl
IIe	C_4H_3S	HCl

Figure 1. Synthesized bis Mannich bases, namely bis(β-aroylethyl)ethylamine hydrochlorides **IIa**, **IIb**, **IId** and **IIe**.

salina (Leach) are available at low cost in pet shops as a food for tropical fish, and they remain viable for years in the dry state. Upon being placed in seawater, the eggs hatch within 48 h, providing large numbers of larvae (nauplii) to be used in the bioassay. This bioassay easily utilizes a large number of organisms for statistical validations and a relatively small amount of sample (2–20 mg). Because brine shrimp nauplii (whole organisms) were used in the brine shrimp bioassay, it may serve as an intermediate test before further *in vivo* animal experiments are carried out on a large scale. Once effective compounds have been determined in this bioassay, specific and more sophisticated bioassays can be employed. The effects of chemical modifications of the compounds, such as structural isomerism (bis Mannich bases with open chain and piperidinol derivatives with cyclic structure are structural isomers), quaternization and ring replacement in the aromatic part of the chemical structures, and partition coefficients of the compounds were tested on their toxicity in this bioassay.



	Ar	X
IIIa	C_6H_5	HCl
IIIb	*p*-$CH_3C_6H_4$	HCl
IIIc	*p*-$CH_3OC_6H_4$	HCl
IIId	*p*-ClC_6H_4	HCl
IIIe	C_4H_3S	HCl
IIIf	C_6H_5	CH_3I
IIIg	*p*-$CH_3C_6H_4$	CH_3I

Figure 2. Synthesized piperidinol derivatives, namely 3-aroyl-4-aryl-1-ethyl-4-piperidinol salts **IIIa–g**.

MATERIALS AND METHODS

Syntheses

Synthesis of the compounds (Figs 1 and 2) was reported in our previous study (Gul *et al*., 2002b). Chemical structures of the synthesized compounds were confirmed by UV, IR, ^{1}H-NMR and ^{13}C-NMR-DEPT (Gul *et al*., 2002b), mass spectroscopies and elemental analysis. Microanalyses (C, H, N) were performed on a CHN rapid elemental analyser (Perkin-Elmer Instruments, Norfolk, CT, USA). The results of elemental analyses of the compounds were within ±0.4%.

Toxicity of the compounds in the brine shrimp bioassay

The bioassay was according to a published procedure (Meyer *et al*., 1982) as follows.

Sample preparation. Compounds to be tested were dissolved in chloroform–methanol (8 : 2) to prepare solutions at 1, 10, 100 and 1000 μg ml^{-1} concentrations. Three vials were prepared at each concentration. One vial was kept as a control, with a mixture of chloroform–methanol (8 : 2) only. 5-Fluorouracil (Roche, France) also was tested as the reference compound.

Hatching the shrimp. Brine shrimp eggs (*Artemia salina*, Leach; San Francisco Bay Brand Inc., Newark, CA 94560, USA) were hatched in a shallow rectangular plastic dish filled with artificial seawater prepared using a commercial salt mixture (Artemia Saltz; Hobby, Aus dem hause Doshe Aquaristik, Bonn) and double-distilled water according to the directions on the package (3.8 g of sea salt per 100 ml of distilled water). An unequal partition was made in the plastic dish with the help of a perforated device. Eggs were sprinkled into the large compartment, which was darkened, whereas the smaller compartment was illuminated by an overhead fluorescent lamp (36 W) at a distance of 40 cm. After 48 h, phototropic nauplii, which were separated from their shells in the divided tank, were collected from the illuminated side using a capillary tube.

Bioassay. The solvent mixture in each vial was evaporated under high vacuum and vials were left overnight in a fumehood. After 2 days, when the shrimp larvae were ready, 4 ml of seawater and ten shrimps were added to each vial and the volume was adjusted with seawater to 5 ml per vial. Final concentrations of the compounds tested were 1, 10, 100 and 1000 μg ml^{-1}.

After 24 h the number of survivors was counted for each vial. Data were analysed by a Finney (probit analysis method) computer program to determine the LD_{50} (The Finney computer program was obtained from Professor McLaughlin, Purdue University, West Lafayette, IN 47907, USA.)

Determination of the partition coefficients of the compounds

Determination of the maximum absorption wavelength (λ_{max}) and molar absorptivity (ε). The determinations

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were carried out using 1.2×10^{-5}–5.30×10^{-4} M solutions of the compounds in phosphate buffer (pH 7.4) at 37 °C. The values for the λ_{max} (nm) at which the partition coefficients were determined and molar absorptivity ε (cm^{-1} mol^{-1}) shown in Table 1 are the means of two experiments.

General procedure for the partition coefficient determinations. The partition coefficients of compounds were determined as described previously (Caccia *et al*., 1985), with some modifications. A weighed quantity of the appropriate compound was dissolved in phosphate buffer (pH 7.4), to give a concentration range of 1.2×10^{-5}–5.30×10^{-4} M. An aliquot of this stock solution (5 ml) was pipetted into a quick-fit flat-bottomed flask containing 5 ml of 1-octanol saturated with phosphate buffer. The flask and its contents were shaken horizontally at 37 °C in a constant-temperature circulating waterbath for 1 h and then left to separate and the organic and aqueous phases for 30 min at 37 °C. The volumes of each phase and the quantities of compounds used were predetermined in a preliminary experiment such that the solute concentration in the aqueous phase before and after distribution could be measured by UV spectroscopy. Appropriate dilutions were employed prior to the absorbance measurements of the aqueous phase before distribution. All absorbance measurements fell within the region of 0.1–1.0 absorbance readings, which is the region with the least error of measurements. The absorbance readings of the aqueous phase were estimated using Beer's Law, $A = \varepsilon\, l\, c$, where A = absorbance, ε = molar absorptivity (cm^{-1} mol^{-1}l), l = cell path length (cm) and, c = concentration (mol l^{-1}). The apparent partition coefficients (P) were calculated using the equation

$$P = (C_1 - C_2/C_2)(V_{buf}/V_{oct})$$

where C_1 and C_2 are the concentrations in the aqueous phase (buffer) before and after equilibration, respectively, and V_{buf} and V_{oct} are the volumes of buffer and 1-octanol used. The experiments were carried out in duplicate. Log P values, the concentrations studied, the wavelengths of maximum absorption (λ_{max}) and molar absorptivities (ε) of the compounds are shown in Table 1.

Table 1—Log P values, concentrations studied, wavelengths of maximum absorption (λ_{max}) and molar absorptivities (ε) of the compounds

Compound	Concentration (M)	λ_{max} (nm)	ε (cm^{-1} mol^{-1})	Log P
IIa	2.36×10^{-5}	254	15 636	0.3750
IIb	3.60×10^{-5}	265	6000	0.7924
IId	2.65×10^{-4}	263	2819	0.7129
IIe	3.10×10^{-4}	271	5055	0.1070
IIIa	3.10×10^{-4}	257	6935	0.6661
IIIb	3.20×10^{-4}	268	1844	0.8705
IIIc	3.60×10^{-5}	281	5278	−0.7256
IIId	5.30×10^{-4}	260	981	0.1187
IIIe	2.57×10^{-4}	278	1634	0.4527
IIIf	1.20×10^{-5}	250	21 667	−1.0414
IIIg	2.20×10^{-5}	261	14 273	0.3680

RESULTS

The toxicity results of the compounds synthesized (Figs 1 and 2) and of the reference compound 5-fluorouracil are given in Table 2. Partition coefficients of the compounds (as log P values) are shown in Table 1. The log P values of the compounds correlated positively with the log LD_{50} values but the relationship did not reach significance (Pearson's correlation coefficient = 0.532; $P = 0.092$).

DISCUSSION

Mannich bases synthesized in our study were toxic in the brine shrimp bioassay of this study and in the cell culture test using Jurkat cells in our previous study (Gul *et al*., 2002b).

Quaternary piperidine derivatives **IIIf** and **IIIg** were 6.41 and 4.70 times more toxic than 5-fluorouracil, respectively. Except for **IIIa**, non-quaternary piperidinol derivatives **IIIb**, **IIIc**, **IIId** and **IIIe** were 2.72, 1.52, 1.21 and 2.05 times more toxic than the reference compound 5-fluorouracil, respectively. The toxicity of bis Mannich base **IIe** was approximately equal to that of the reference compound 5-fluorouracil. Toxicities of the other bis Mannich bases (**IIa**, **IIb**, **IId**) and piperidinol derivative **IIIa** were 2.34–6.82 times less than that of the 5-fluorouracil in the brine shrimp bioassay.

Conversion of bis Mannich bases to their corresponding piperidinols increased the toxicity in all cases (see Table 2). Piperidinol derivatives **IIIa**, **IIIb**, **IIId** and **IIIe** were 1.67, 18.54, 6.98 and 2.08 times more toxic than the corresponding bis derivatives **IIa**, **IIb**, **IId** and **IIe**, respectively. Quaternization increased the toxicity in piperidine derivatives. Quaternary piperidine derivative **IIIf**, where the phenyl ring was non-substituted, was 15 times more toxic than the corresponding non-quaternary derivative **IIIa**. Quaternary piperidine derivative **IIIg**, where the phenyl ring was methyl substituted, was 1.73 times more toxic than the corresponding non-quaternary derivative

Table 2—Molecular weights of the compounds, and reference compound 5-fluorouracil, and their toxicity results in the brine shrimp bioassay

Compounds	Molecular weight	LD_{50} (μM)	95% confidence intervals
IIa	345.5	90.04	21.19–397.25
IIb	373.5	156.87	42.51–343.07
IId	414.5	132.26	69.35–236.28
IIe	357.5	23.36	5.08–59.93
IIIa	345.5	53.86	25.37–88.51
IIIb	373.5	8.46	1.42–20.46
IIIc	405.5	15.09	4.40–32.02
IIId	414.5	18.94	4.59–48.78
IIIe	357.5	11.22	3.74–22.43
IIIf	451.0	3.59	1.44–4.49
IIIg	479.0	4.89	1.22–9.77
5-Fluorouracil	130.0	23.00	13.38–294.85

Three vials have been prepared at each concentration to obtain the get LD_{50} values of the compounds (see Materials and Methods).

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IIIb. The increased toxicity due to quaternization in this study agrees with the findings of our previous studies in brine shrimp nauplii (Gul *et al*., 2002c) and Jurkat cells (Gul *et al*., 2000). The increased toxicity in quaternary derivatives may be attributed to α,β-unsaturated ketone intermediates having a marked affinity to nucleophiles, which are formed by fast elimination of the compounds via the Hoffmann reaction (March, 1985). Replacement of the phenyl ring with thienyl increased the toxicity in the bis Mannich bases (3.85 times) and the piperidinol derivatives (4.8 times).

In terms of cytotoxicity in Jurkat cells when the results are given as micromolar concentrations, all compounds were more potent than 5-fluorouracil (Gul *et al*., 2002b). In parallel with our finding in the brine shrimp bioassay, the quaternization procedure also increased the cytoxicity in Jurkat cells. Replacement of the phenyl ring with thienyl in bis Mannich bases also increased the cytotoxicity in Jurkat cells (Gul *et al*., 2002b). It has been reported that cytoxicity determined by means of the brine shrimp bioassay correlates well with the results obtained by cell lines KB and P-388 (Meyer *et al*., 1982), and L5178Y and Ll210 (Crispino *et al*., 1989). In accordance with these studies, the results of the toxicity obtained by the brine shrimp bioassay in this study and by the cell culture test using Jurkat cells in our previous study (Gul *et al*., 2002b) correlated well. However, the effect of conversion of bis Mannich bases to their corresponding piperidinol derivatives on cytotoxicity was inconsistent in Jurkat cells (Gul *et al*., 2002b). This difference may result from the different toxicity tests using cells and whole organisms and also the different test media, culture medium and seawater, which may affect the pharmacokinetics of the compounds.

The correlation between partition coefficients and the toxicity of the compounds was not significant (see Fig. 3).

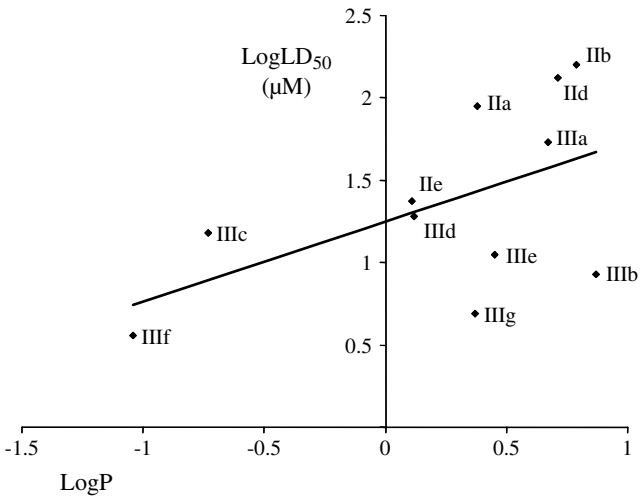

Figure 3. Relationship between log P and log LD_{50} values of the compounds (Pearson's correlation coefficient = 0.532, $P = 0.092$).

This may mean that the changes in toxicity of the compounds were not affected directly by their solubility in lipids.

It is concluded that of the Mannich bases synthesized, the quaternary piperidine derivatives **IIIf** and **IIIg** and also the non-quaternary piperidine derivatives **IIIb**, **IIIe**, **IIIc** and **IIId** were more toxic than 5-fluorouracil in the brine shrimp bioassay. Quaternization and conversion of the bis Mannich bases to their corresponding piperidines improved the toxicity. The lipid solubility of the compounds may not affect the toxicity. In particular, quaternary piperidine derivatives **IIIf** and **IIIg** can be used in further drug development and also *in vivo* experiments.

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