Notes

Synthesis and Biological Activity of DL-3-(5-Benzimidazolyl)-2-methylalanine†

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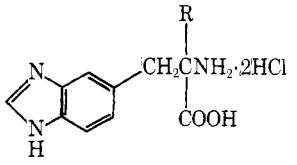
The synthesis and preliminary study of the *in vitro* activity of DL-3-(5-benzimidazolyl)alanine dihydrochloride (BA) have been reported.[1,2] BA (10^{-6} *M*) caused a 22% inhibition of bovine adrenal tyrosine hydroxylase and was nearly as effective an inhibitor of tyrosine hydroxylase obtained from a human adrenal neuroblastoma[3] as DL-α-methyltyrosine (αMT). When BA was administered to male Wistar rats at a dose of 1 mmol/kg, a decrease in brain serotonin (to 30% of control) was seen within 1 hr, and a decrease in heart norepinephrine (to 20% of control) and in brain norepinephrine (to 30% of control) was seen within 3 hr.[2] These decreases were shown[2] to be caused by *in vivo* inhibition of biosynthesis as well as release of biogenic amines from storage granules.

The synthesis of DL-3-(5-benzimidazolyl)-2-methylalanine dihydrochoride (MBA) was proposed because this compound was expected to exhibit a pattern of *in vitro* and *in vivo* activity significantly different from that observed for BA. *In vitro*, MBA was expected to be a more potent inhibitor of tyrosine hydroxylase than BA since the inhibitory activity of tyrosine analogs against tyrosine hydroxylase has been shown to be enhanced by the addition of a methyl group on the α carbon.[4] *In vivo*, α-methylamino acids cannot transaminate, racemize, or serve as substrates for amino acid oxidases; therefore, it was anticipated that MBA would exhibit greater metabolic stability and a greater duration of action than BA. The presence of an α-methyl group in MBA would forcibly retard the rate of decarboxylation of MBA as compared to BA[5,6] but channel most of the metabolism of MBA through DL-2-amino-1-(5-benzimidazolyl)propane (MBA-amine),[7] the decarboxylation product of MBA.

Since a decarboxylation product of BA was shown to have biological activity in its own right,[2] it was anticipated that the channeling of most MBA metabolism through MBA-amine would lead to metabolic products of significant biological activity. Finally, it was anticipated that MBA would be less effective than BA in lowering brain serotonin as *p*-chloro-α-methylphenylalanine had proved to be only a weak inhibitor of serotonin biosynthesis.[8]

The synthesis of MBA and investigation of its *in vitro* inhibition of bovine adrenal tyrosine hydroxylase and of its *in vivo* effects on heart norepinephrine and brain biogenic amines are described in this report.



DL-3-(5-benzimidazolyl)alanine dihydrochloride (BA), R = H
DL-3-(5-benzimidazolyl)-2-methylalanine dihydrochloride (MBA), R = CH_3

†Abstracted from a thesis submitted by Victor H. Morgenroth, III, to the Graduate School of the University of Maryland.

Results

Synthesis. *N*-Acetyl-2-methyl-3-phenyl-DL-alanine methyl ester (**1**) was nitrated, reduced, and acetylated to yield *N*-acetyl-3-(4-acetylaminophenyl)-2-methyl-DL-alanine methyl ester (**3**). Nitration of **3**, followed by hydrolysis, reduction, and cyclization, gave the title compound MBA (**5**).

Enzyme Studies. A Lineweaver–Burk plot of the reaction velocity of tyrosine hydroxylase showed that MBA is a competitive inhibitor of L-tyrosine. The K_i obtained for MBA was 2.3×10^{-5} when plotted according to Lineweaver and Burk and 2.1×10^{-5} plotted according to Dixon (Figure 1); BA has been reported[2] to be a competitive inhibitor of tyrosine hydroxylase with a K_i of 2×10^{-5}.

The competitive inhibition of MBA and of BA *vs.* tyrosine necessitates their binding to the reduced form of the enzyme tyrosine hydroxylase[9] but does not preclude the additional possibility of their interference with the binding of the reduced pteridine cofactor (2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine, $DMPH_4$) to the oxidized form of the enzyme. Since BA is a structural analog of 3,4-dihydroxyphenylalanine (DOPA), which is a competitive antagonist of the binding of $DMPH_4$ to oxidized tyrosine hydroxylase,[10] the possibility that benzimidazoleala-nines (BA and MBA) might interfere with the binding of $DMPH_4$ was explored. If MBA interferes with $DMPH_4$ binding, the inhibition it produces should be greater at lower $DMPH_4$ concentrations. The degree of inhibition produced by MBA at varying concentrations of $DMPH_4$, shown in Table I, indicates that there is no apparent relationship between the concentration of $DMPH_4$ and the inhibition of the enzyme by MBA. Similar results were obtained for BA and are not shown.

Pyrroloisoxazoles, which are somewhat similar to benzimidazoles, inhibit tyrosine hydroxylase by competing with ferrous ion.[11] Inhibition by such compounds can be completely reversed by addition of ferrous ion. The inhibition of tyrosine hydroxylase by BA and MBA was unaffected by the addition of ferrous ion at 10^{-2} *M*; the data are not shown.

The results reported here are consistent with the *in vitro* action of BA and MBA being limited to competition with the substrate L-tyrosine.

The *in vitro* results indicate also that the K_i of MBA is not markedly different from that of BA; this does not conform to α-methylated 3-substituted tyrosines, which are more potent *in vitro* inhibitors of tyrosine hydroxylase than their nonmethylated counterparts.[4] The K_i of MBA as inhibitor of tyrosine hydroxylase is essentially the same as that of αMT, the inhibitor of norepinephrine synthesis used most frequently in experimentation and clinical practice.

Effects on Biogenic Amines. Time Course of Action. To ascertain the effect of MBA on biogenic amine levels, the compound was injected intraperitoneally at various times into male Wistar rats at a dose of 0.3 mmol/kg, the animals were sacrificed at the same set time (11:00 a.m. ± 45 min), and biogenic amines were analyzed according to the procedures described in the Experimental Section.

The analysis of brain serotonin (Figure 2) showed that the serotonin level had not changed 2 hr after MBA administration, was depressed to 80% of normal at 8 and 12

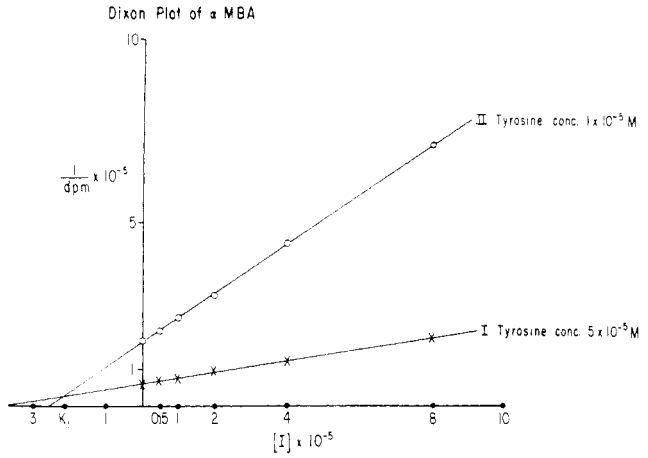


Figure 1. Inhibition of tyrosine hydroxylase by MBA. Inhibition of bovine adrenal tyrosine hydroxylase was determined as a function of inhibitor concentration and plotted according to the method of Dixon,[16] and K_i, found on the abcissa point corresponding to the intersection of line I and line II, was 2.1×10^{-5} *M*.

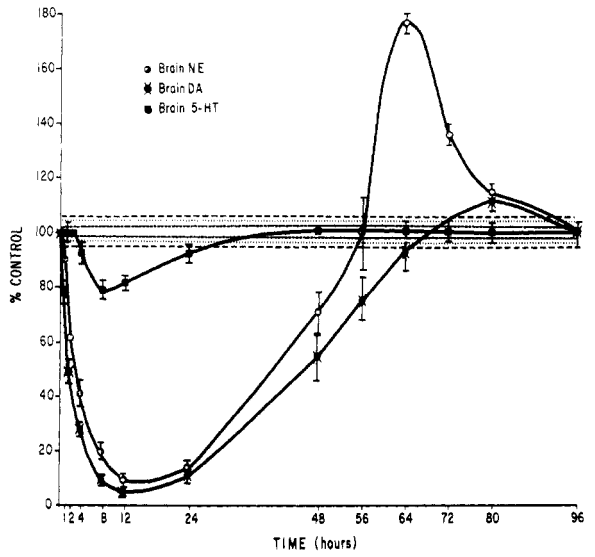


Figure 2. Time course of depletion of brain biogenic amines by MBA. Male Wistar rats were administered one dose (0.3 mmol/kg) of MBA at various times before sacrifice. All animals were sacrificed within 45 min of 11:00 a.m. Results are expressed as per cent control ± the standard error of the mean. The standard error of the means of the controls is represented by large dashes (dopamine), dots (norepinephrine), and small dashes (serotonin).

hr, and had returned to control levels by 48 hr. Brain norepinephrine and dopamine concentrations were maximally depressed (to 9 and 5% of control, respectively) at 12 hr and remained depressed for at least 24 hr (Figure 2).

The brain concentration level of norepinephrine rose to 173% of control at 64 hr and that of dopamine to 111% at 80 hr. The brain concentrations of both amines returned to control levels within 96 hr. The administration of MBA led to changes in heart norepinephrine levels which are represented in Figure 3. Heart norepinephrine concentrations were maximally depressed at 12 hr, rose above control levels at 72 hr, and returned to control levels within 96 hr.

The *in vivo* results demonstrate that the *in vivo* activity of MBA differs significantly from that of BA; the depletion of brain serotonin by MBA was more delayed and more moderate while the depression of brain and heart catecholamine levels was more pronounced and more prolonged than observed with BA.[2] Since BA and MBA have the same affinity for tyrosine hydroxylase, the *in vivo* effects on catecholamines can be explained in terms of an α-methyl group altering the metabolism and duration of action of MBA. The time course study reveals that MBA administration produces a biphasic effect on heart and brain catecholamines: a rapid depletion followed by a delayed rise. The mechanisms involved in these effects are currently under investigation.[12]

Table I. Inhibition of Tyrosine Hydroxylase by MBA[a] at Varying Concentrations of $DMPH_4$

$DMPH_4$ concn, *M*	μCi of ^3H_2O produced	% inhibition[b]
10^{-2}	0.060	46 ± 1[c]
10^{-3}	0.048	48 ± 2
10^{-4}	0.035	46 ± 1
10^{-5}	0.027	50 ± 1

[a] MBA concentration 2×10^{-5} *M*. [b] Tyrosine concentration 5×10^{-5} *M* L-tyrosine. [c] Values are the mean of nine samples.

Experimental Section

Melting points were determined on a Thomas-Hoover melting point apparatus and are uncorrected. Ir spectra were obtained on a Perkin-Elmer Model 247 grating infrared spectrophotometer and nmr spectra on a Jeolco C-60HC high-resolution instrument (60 MH). Elemental analyses were performed on a Hewlett-Packard (F & M) Model 185 analyzer by Aldrich Associates & Co., Washington, D. C., or by Galbraith Laboratories, Nashville, Tenn.

***N*-Acetyl-2-methyl-3-(4-nitrophenyl)-DL-alanine Methyl Ester (2).** *N*-Acetyl-2-methyl-3-phenyl-DL-alanine methyl ester (**1**,[13] 5 g, 0.02 mol) was added slowly to 20 ml of stirring HNO_3 (red, fuming) at −15°. After stirring for an additional hour at −15°, the mixture was added slowly to a vigorously stirred, ice-cold, saturated $NaHCO_3$ solution. The resulting precipitate was filtered, washed with H_2O, and crystallized twice from C_6H_6 yielding 3.1 g (52%) of **2**: mp 173–174°; nmr ($CDCl_3$) δ 7.55 (d, 2 H, arom, J_{ab} = 8 Hz) and 6.85 (d, 2 H, arom, J_{bc} = 8 Hz) indicating para substitution. *Anal.* ($C_{13}H_{16}N_2O_5$) C, H, N.

***N*-Acetyl-3-(4-acetylaminophenyl)-2-methyl-DL-alanine Methyl Ester (3).** Compound **2** (3 g, 0.011 mol) in MeOH (150 ml) was hydrogenated at 3.16 kg/cm² over Pd/C 5%. Filtration through Celite and removal of solvent gave an oil to which was added C_6H_6 (100 ml) and Ac_2O (12 g, 0.12 mol). This mixture was refluxed for 30 min; the resulting precipitate was filtered and crystallized from MeOH to yield 2.3 g (73.5%) of **3**, mp 211–213°. *Anal.* ($C_{15}H_{20}N_2O_4$) C, H, N.

***N*-Acetyl-3-(4-acetylamino-3-nitrophenyl)-2-methyl-DL-alanine Methyl Ester (4).** Compound **3** (2.00 g, 0.077 mol) was nitrated and isolated as in the preparation of **2**. Crystallization from MeOH yielded 1.56 g (68%) of **4**: mp 167–169°; ir (KBr) λ_{max} 800 cm^{-1} (1,2,4-substituted benzene); nmr ($CDCl_3$) δ 8.70 (d, 1 H, J_{ab} = 8 Hz), 7.75 (d, 1 H, J = 3 Hz), 7.25 (q, 1 H, J_{ab} = 8 Hz, J_{ax} = 2 Hz). *Anal.* ($C_{15}H_{19}N_3O_6$) C, H, N.

DL-3-(5-Benzimidazolyl)-2-methylalanine Dihydrochloride (5). Compound **4** (1.3 g, 0.004 mol) was refluxed in 5 *N* HCl (20 ml) for 2 hr. Formic acid (25 ml) was added to the solution and the mixture hydrogenated at 3.16 kg/cm² over 5% Pd/C in a steam jacketed flask. After 2 hr the H_2 was displaced with N_2 and the mixture was steam heated for 3 hr. After filtration of the cooled solution (Celite), the solvent was evaporated off and the resulting solid crystallized from EtOH–Et_2O (40:60) yielding 940 mg (83.5%) of a grey white amorphous solid: mp 310° (darkens above 295°); nmr (D_2O) δ 9.30 (s, 1 H, benzimidazole C_2 proton indicating ring closure), 8.0 (d, arom, 1 H, J_{ab} = 8 Hz), 7.80 (s, arom, 1 H), 7.60 (q, arom, 1 H, J_{ab} = 10 Hz, J_{ax} = 2 Hz). Signals at δ 3.75 (q, J = 7.0 Hz) and 1.30 (t, J = 7.0 Hz) gave an integration which indicated the presence of 0.5 mol of EtOH of crystallization. Tlc showed one ninhydrin-positive spot in each of two systems (silica gel–BuOH–AcOH–H_2O–MeOH, 80:20:10:10, and silica gel–MeOH). *Anal.* C, H; N: calcd, 13.33; found, 13.86. **5** (0.5 g) was derivatized by conversion to its ethyl ester dihydrochloride **5a** by refluxing **5** for 4 hr in EtOH (75 ml) saturated with HCl.

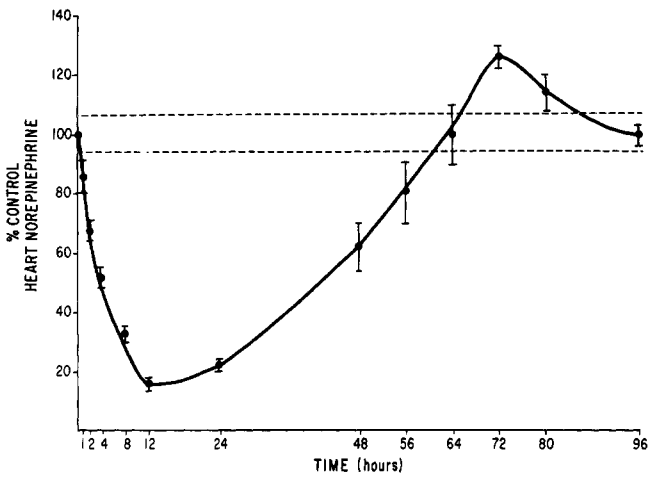


Figure 3. Time course of depletion of heart norepinephrine by MBA. Male Wistar rats were administered one dose (0.3 mmol/kg of MBA at various times before sacrifice. All animals were sacrificed within 45 min of 11:00 a.m. Results are represented as per cent control ± the standard error of the mean.

Removal of the solvent and crystallization from EtOH gave **5a**: mp 240-241°; nmr (D_2O) δ 4.25 (q, 2 H, J = 7.0 Hz) and 1.25 (t, 3 H, J = 7.0 Hz, ethyl ester protons). *Anal.* ($C_{13}H_9N_3Cl_2O_2$) C, H, N, Cl.

Tyrosine Hydroxylase Inhibition Studies. The materials and methods used in tyrosine hydroxylase purification and assay were the same as previously reported.[14,15] Tyrosine hydroxylase kinetics were determined by the method of Lineweaver-Burk[16] with substrate concentrations varying from 6×10^{-6} to 10^{-4} M and by the method of Dixon[17] with substrate concentrations set at 5×10^{-5} and at 1×10^{-5} M. $DMPH_4$ concentration was constant in the inhibition studies at 10^{-3} M but was later varied between 1×10^{-2} and 1×10^{-5} M.

Biogenic Amine Studies. Male Wistar rats (90-120 g) were sacrificed with a guillotine and a glass and Teflon homogenizer was used in all homogenization procedures.

Biogenic amines were determined by the modification of the procedures of Chang, *et al.*[18] (norepinephrine and dopamine), and of Maickel, *et al.*[19] (serotonin), as described by Johnson, *et al.*[2] The control values obtained for biogenic amines were, in μg/g ± S.E., 0.57 ± 0.02 for heart norepinephrine, 0.46 ± 0.01 for brain norepinephrine, 0.50 ± 0.01 for brain serotonin, and 0.45 ± 0.02 for brain dopamine.

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Synthesis of α-Heterosubstituted Nitrosamines. Novel Test Substances for Cancer and Mutagenesis Research?†

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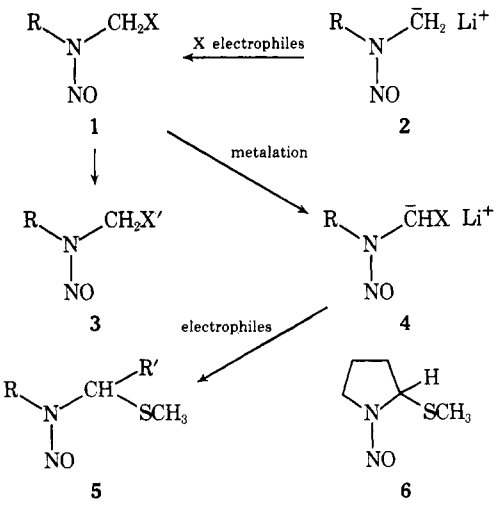
Because of their environmental occurrence, high activity, and often application-independent, organospecific effects, nitrosamines have gained broad application and worldwide interest for practical and mechanistic studies in cancer and mutagenesis research; the alkylation hypothesis of carcinogeneity requires that they are hydroxylated enzymatically to give, for instance, 1 (X = OH) which is a potential precursor of alkylating diazonium ions and/or diazo compounds.[1-4] In order to further test this theory, new methods of preparation of α-heteronitrosamines were desirable. Hitherto, only ethers of type 1 (X = OR) were readily available.‡[,5-9] We describe here a general route to and first examples of the preparation of α-sulfur-, α-selenium-, α-silicon-, and α-tin-substituted nitrosamines **1**, **3**, **5**, and **6** (see Table I). The method rests upon the availability of lithiated nitrosamines such as **2**[10-12] whose reactions with the heteroelectrophiles dimethyl and diphenyl disulfide, diphenyl diselenide, trimethylchlorosilane, and trimethylchlorotin lead to the derivatives **1**. These were amenable to further structural modifications either by transformation of X in **1** into X′ in **3** (*cf.* $SCH_3 \rightarrow SOCH_3$) or by metalation of **1** to give **4** which was derivatized to higher heteronitrosamines **5**. Cyclic compounds such as **6** were also available by our route. The yields were good to excellent; data of the products prepared are listed in Table I. The new compounds can be stored for months in a refrigerator (0°). The low-molecu-



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‡ Most of the numerous examples known and easiest to prepare are cyclic derivatives. One α-acyloxy derivative[7] is also known.