Inactivation of Monoamine Oxidase B by Analogues of the Anticonvulsant Agent Milacemide (2-(*n*-Pentylamino)acetamide)

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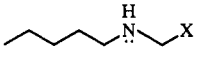
Analogues of the anticonvulsant agent milacemide (1, 2-(*n*-pentylamino)acetamide), in which the carboxamide group is changed to a nitrile (2), a carbethoxy group (3), a carboxylic acid (4), a cyanomethyl group (5), and a trifluoromethyl group (6), were synthesized and tested as substrates and inactivators of monoamine oxidase B (MAO B). The carboxylic acid was neither a substrate nor an inactivator. The trifluoromethyl compound was not soluble in buffer even when organic cosolvents were added, so it could not be tested. All of the other compounds were both substrates and time-dependent irreversible inactivators of MAO B. A plot of the logarithm of k_{cat}/k_{inact} (a measure of the efficiency of the inactivators) versus σ_I (Figure 1) shows a linear free energy relationship between the inactivator efficiency and the electron-withdrawing ability of the substituent. As the electron-withdrawing ability increases, the partition ratio decreases indicating that inactivation is becoming more efficient relative to substrate turnover to product. Milacemide was the least efficient of the compounds tested; the nitrile 2 was the most efficient.

Milacemide (1, 2-(*n*-pentylamino)acetamide) is a glycine prodrug with anticonvulsant activity in animals equivalent to that of the epilepsy drug valproate.[1,2] It was shown to be a substrate[3] and potent selective inhibitor[4] of brain monoamine oxidase B (MAO B; EC 1.4.3.4). In vivo studies indicated that MAO B activity was regenerated after milacemide inhibition, suggesting that a reversible inhibition was involved;[4] however, we have shown that milacemide forms a covalent bond to MAO B in vitro.[5] On the basis of our previous studies on the inactivation of MAO B by (aminomethyl)trimethylsilane[6] and 3-aryl-5-[(methylamino)methyl]-2-oxazolidinones[7] we proposed the inactivation mechanism shown in Scheme I.[5] The partitioning between the release of the imine (characterized by k_{cat}) and covalent attachment of the enzyme (characterized by k_{inact}) should be influenced by the electron-withdrawing ability of the substituent attached to the amino group. In order to obtain further support for this proposal a series of milacemide analogues containing different functional groups (2–6) was prepared and the effect of the electron-withdrawing ability of the modified groups on the effectiveness of the analogues to inactivate MAO B was measured.



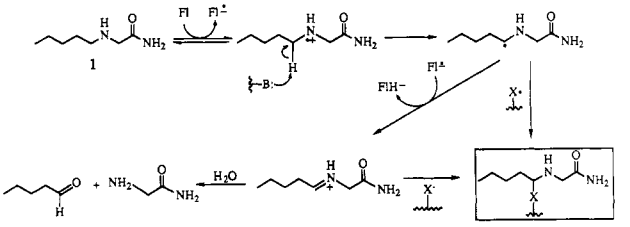
X

1, $CONH_2$ 4, CO_2H
2, CN 5, CH_2CN
3, CO_2Et 6, CF_3

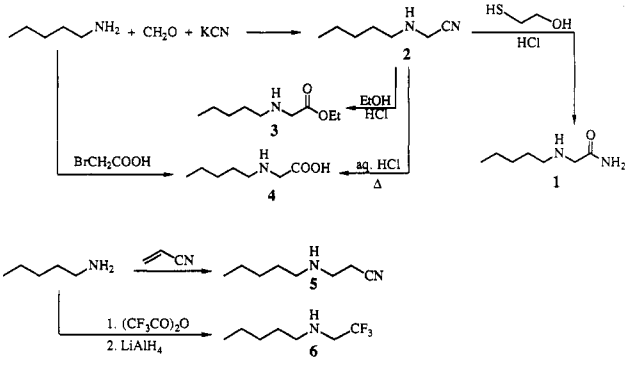
Results and Discussion

Chemistry. The syntheses of 1–4 were initiated by the reaction of pentylamine hydrochloride with formaldehyde and potassium cyanide (Scheme II). (*n*-Pentylamino)-acetonitrile (2) was converted by β-mercaptoethanol and dry HCl[8] into milacemide (1), by ethanolic HCl into the ester 3, and by concentrated HCl into the amino acid 4. The acid 4 also was synthesized by condensation of *n*-pentylamine and bromoacetic acid. The compound with an additional methylene in the substituent (5) was prepared by the Michael addition of *n*-pentylamine to acrylonitrile. The trifluoromethyl analogue was synthesized by trifluoroacetylation followed by lithium aluminum hydride reduction.

Scheme I



Scheme II



Enzymology. Compound 6 was not soluble in buffer at pH 7.0 or at 9.0, even in the presence of 20% DMSO or ethanol. The electron-withdrawing effect of the trifluoromethyl group lowers the pK_a of the amine to about 6; consequently, the amino group is not protonated in the buffer, and the compound is too hydrophobic to dissolve. As a result of its insolubility, no enzymology with 6 could be carried out. Compound 4 showed no substrate or

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Table I. Kinetic Constants for the Milacemide Analogues

compd	K_i (mM)	K_I (mM)	k_{inact} (min^{-1})	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/k_{inact}
1	1.08	0.94	0.97×10^{-2}	0.29	255	26289
2	4.94	13.99	3.3×10^{-2}	0.021	112	3394
3	2.0	14.22	2.3×10^{-2}	0.26	102	4435
4	–	–	–	–	–	–
5	4.94	5.79	1.1×10^{-2}	0.21	100	9091

inhibitory activity with MAO. Amino acids are not substrates for this enzyme.

All of the other milacemide analogues were competitive inhibitors of the monoamine oxidase B-catalyzed oxidation of benzylamine. Their K_i values are listed in Table I. In addition to being competitive inhibitors, all of the analogues except for **4** and **6** also were time-dependent inactivators of MAO B. From replots of the half-lives of inactivation versus the reciprocal of the inactivator concentration[9] the K_I and k_{inact} values for each compound were obtained and are shown in Table I. Although milacemide (**1**) has the lowest K_I value, it is the acetonitrile analogue **2** that has the largest rate constant for inactivation at saturation (k_{inact}). All of the inactivations were irreversible as measured by the inability of dialysis against Tris-HCl buffer, pH 9.0 for 24 h to restore any enzyme activity. All of the compounds except for **4** and **6** also were substrates of MAO B. The K_m and k_{cat} values were obtained from Hanes plots[10] and are given in Table I. The Michaelis constant representing the tightest binding substrate (smallest dissociation constant) was observed for **2**, which was 0.07 times that for milacemide and 0.08 times that for the ethyl ester **3**. There was little difference in the rate constants for substrate turnover. The partition ratio for inactivation, k_{cat}/k_{inact}, a measure of the efficiency of the inactivator,[11] was highest (least efficient) for milacemide and lowest (most efficient) for the acetonitrile analogue **2**. A plot of the logarithm of k_{cat}/k_{inact} versus σ_I[12] (Figure 1) shows a linear free energy relationship between the inactivator efficiency and the electron-withdrawing ability of the substituent with a correlation coefficient (r) of 0.93. As the electron-withdrawing ability increases, the partition ratio decreases, indicating that inactivation is becoming more efficient relative to the turnover of substrate to product. The increased electron-withdrawing ability of the substituents should increase the reactivity of the intermediate (either the iminium ion or the radical) for reaction with the enzyme which would decrease the partition ratio, as is observed.[13]

The results of this study indicate that several other analogues of milacemide are more efficient mechanism-based inactivators of MAO than milacemide itself, although the K_I values are not as low as that for milacemide. Furthermore, it appears that the efficiency of inactivation, as measured by the partition ratio, is a function of the electron-withdrawing ability of the substituent attached to the pentylamino backbone. This is consistent with the proposed mechanism for inactivation (Scheme I) which involves attachment of the enzyme to the C-1 of the pentyl chain of milacemide.[5]

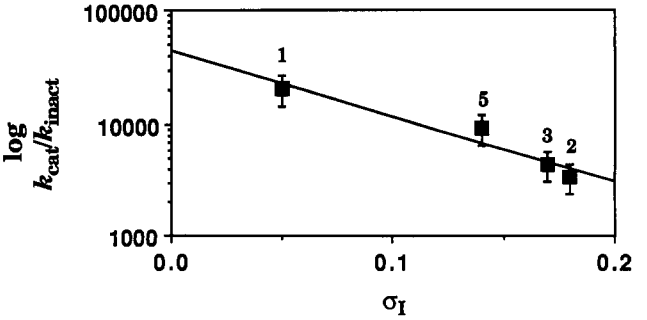

Figure 1. Plot of the log of the partition ratio vs the σ_I values for the milacemide analogues.

Experimental Section

Analytical Methods. Optical spectra and MAO assays were recorded on either a Perkin-Elmer Lambda 1 or Beckman DU-40 UV/vis spectrophotometer. NMR spectra were recorded on a Varian EM-390 90-MHz spectrometer or a Varian XLA-400 MHz spectrometer. An Orion Research Model 601 pH meter was used for pH measurements. Amine hydrochlorides were visualized on TLC plates by spraying with a solution of ninhydrin (300 mg) with pyridine (2 mL) in acetone (100 mL) and then heating.

Reagents. Pentylamine, chloroform-*d*, deuterium oxide (>99.96 atom% D and 99.8 atom% D), hydrogen chloride, anhydrous diethyl ether, trifluoroacetic anhydride, leuco crystal violet, ninhydrin, tetrahydrofuran, and tetramethylsilane were purchased from Aldrich Chemical Co. Benzene, chloroform, anhydrous magnesium sulfate, anhydrous potassium carbonate, and potassium cyanide were obtained from Mallinckrodt. Absolute ethanol was purchased from Midwest Grain Co. of Illinois. Horseradish peroxidase (type II) and tris(hydroxymethyl)-aminomethane (Tris) were purchased from Sigma. Diethyl ether and tetrahydrofuran for reactions were distilled from sodium with benzophenone ketyl indicator under nitrogen immediately prior to use. All other chemicals were used without further purification. Distilled water was deionized or deionized and redistilled.

2-(*n*-Pentylamino)acetonitrile Tosylate (2). Formaldehyde (37%, 838 μL, 10 mmol) was added dropwise with stirring over 30 min to pentylamine (1.16 mL, 10 mmol) and potassium cyanide (661 mg, 10 mmol) in an ice bath. The reaction mixture was allowed to stir at 0 °C for 1.5 h, and then 5 N HCl (18.6 mL, 93 mmol) was added dropwise over 2 h maintaining the temperature between 5 and 10 °C. The solution was allowed to stir at 0 °C for 2 h, and then the reaction mixture was extracted with diethyl ether three times. The combined organic layers were dried over anhydrous K_2CO_3 and the solvent was removed by rotory evaporation in vacuo to yield a yellow oil (0.68 g, 54%), bp 92–93 °C/10 mmHg. The product was purified by silica gel column chromatography (2:1 hexane/ethyl acetate), NMR (DMSO-d_6) δ 0.90 (t, 3 H), 1.29–1.58 (m, 6 H), 2.54 (t, 2 H), 3.20 (s, 1 H), 3.58 (s, 2 H).

p-Toluenesulfonic acid monohydrate was dehydrated by heating at 105 °C under reduced pressure for 4 h. Anhydrous *p*-toluenesulfonic acid (1.35 g, 7.9 mmol) in 30 mL of ether was added to 2-(*n*-pentylamino)acetonitrile (1.0 g, 7.9 mmol) in 20 mL of ether. The ether was removed and the residue was recrystallized from benzene to give **2** as white crystals (0.61 g, 45%): mp 138–139 °C; NMR (D_2O) δ 0.67 (t, 3 H), 1.14 (m, 4 H) 1.50 (m, 2 H), 2.18 (s, 3 H), 2.98 (t, 2 H), 4.07 (s, 2 H), 7.16 (d, 2 H), 7.48 (d, 2 H); IR (KBr) 2250 cm^{-1} (CN). Anal. ($C_{14}H_{22}N_2O_3S$) C, H, N, S.

Ethyl 2-(*n*-Pentylamino)acetate Hydrochloride (3). 2-(*n*-Pentylamino)acetonitrile was dissolved in 95% ethanol and HCl gas was bubbled in. Ammonium chloride was filtered and the solvent was evaporated. The residue was dissolved in saturated $NaHCO_3$, and then the solution was extracted with ether. The ether was removed in vacuo and the residue was purified by silica gel chromatography (2:1 hexane/ethyl acetate). The isolated product was dissolved in anhydrous ether and HCl gas was bubbled in. The solvent was removed and the product was recrystallized from ethanol several times: mp 176–177 °C; NMR ($CDCl_3$) δ 0.87 (t, 3 H), 1.1–1.4 (m, 7 H), 1.5–1.8 (m, 2 H), 2.9 (t, 2 H), 3.90 (s, 2 H) 4.20 (q, 2 H), 9.1–9.8 (br, 1 H); IR (KBr) 1752 cm^{-1} (C=O). Anal. ($C_9H_{20}ClNO_2$) C, H, Cl, N.

2-(*n*-Pentylamino)acetic Acid Hydrochloride (4). To a stirred solution of pentylamine (4.35 g, 50 mmol) in ether (10 mL) was slowly added bromoacetic acid (1.39 g, 10 mmol) at 0 °C. The reaction mixture was stirred for 10 h at 0 °C, and then the pH was raised to 13 with 30% potassium hydroxide solution, followed by extraction with ether to remove the amine. Hy-

drochloric acid (2 N) was added to the aqueous layer to pH 2, and then the water was evaporated. Methanol (10 mL) was added to the residue and the solid was filtered. The methanol was evaporated, and the residue was recrystallized from methanol–ethyl acetate to give **4** as a white solid (1.53 g, 84%): mp 188–189 °C; NMR (D_2O) δ 0.68 (t, 3 H), 1.14 (m, 4 H), 1.50 (m, 2 H), 2.98 (t, 2 H), 3.68 (s, 2 H); IR (KBr) 1759 cm^{-1} (C=O). Anal. ($C_7H_{16}ClNO_2$) C, H, Cl, N.

This compound also was prepared from 2-(*n*-pentylamino)-acetonitrile (3 g, 24 mmol) which was dissolved in concentrated HCl (20 mL) and was stirred at room temperature for 3 h and then at reflux for 2 h. After allowing to cool, the aqueous solution was washed with $CHCl_3$. The water was removed in vacuo, then ethanol was added to the residue. The precipitate (NH_4Cl) was removed by filtration and the filtrate was applied to a Dowex 50 X 8-400 ion-exchange column. The amino acid-positive fractions were combined, the solvent was removed in vacuo, and the residue was recrystallized twice from methanol–ethyl acetate.

3-(*n*-Pentylamino)propionitrile Hydrochloride (5). The procedure of Tarbell et al.[14] was followed, and the product was purified by distillation to give a colorless oil (75% yield). This oil (1.0 g, 7.1 mmol) was dissolved in ether and the HCl salt was formed by bubbling in dry HCl. The solution was rotary evaporated in vacuo to dryness and the remaining white solid was recrystallized from methanol to give **5** (1.01 g, 81%): mp 183–184 °C; NMR (DMSO-d_6) δ 0.88 (t, 3 H), 1.2–1.38 (m, 4 H), 1.55–1.68 (m, 2 H), 2.88 (t, 2 H), 3.01 (t, 2 H), 3.23 (t, 2 H), 9.2–9.4 (br, 1 H); IR (KBr) 2247 cm^{-1} (CN). Anal. ($C_8H_{17}ClN_2$) C, H, N.

***N*-(2,2,2-Trifluoroethyl)pentylamine Hydrochloride (6).** Trifluoroacetic anhydride (5.0 g, 24 mmol) in 10 mL of ether was slowly added to a stirred solution of pentylamine (2.1 g, 2.8 mL, 24 mmol) and triethylamine (3.0 g, 4.2 mL, 30 mmol) in 10 mL of ether. After 1 h the solvent was removed in vacuo and ether was added to the residue. The organic layer was washed two times each with water, saturated $NaHCO_3$, and saturated NH_4Cl and was dried over Na_2SO_4. The solvent was removed under reduced pressure, and the residue was dried under vacuum at 100 °C for 1 h. Dry ether (150 mL) was added follow by lithium aluminum hydride (1.83 g, 48 mmol) in small portions until no more hydrogen was liberated, and then the mixture was stirred overnight. The reaction mixture was quenched with saturated Na_2SO_4, the solution was filtered through a pad of Celite, and the solvent was removed under reduced pressure. The residue was taken up in hexane and HCl gas was bubbled in. The white solid that formed was filtered, washed with hexane, and recrystallized from ethanol–ether to give 1.04 g (21%) of **6**: mp 178–180 °C; NMR (D_2O) δ 0.87 (t, 3 H), 1.34 (m, 4 H), 1.71 (m, 2 H), 3.17 (t, 2 H), 3.95 (q, 2 H); ^{19}F NMR (D_2O, 282 MHz; CF_3COOH used as external standard, δ 76.8 ppm) δ 68.878 (t); IR (KBr) 847 and 1431 cm^{-1} (C–F). Anal. ($C_7H_{15}ClF_3N$) C, H, Cl, N.

Enzymes and Assays. Bovine layer MAO B was isolated according to the published method.[15] MAO activity was assayed by the method Tabor et al.[16] Protein assays were done with either Pierce BCA protein assay reagent or Pierce Coomassie protein assay reagent using bovine serum albumin for standard curves. All buffers and enzyme solutions were prepared with doubly distilled deionized water.

General Procedure for Substrate Activity Assay. All glassware was thoroughly washed with 50% nitric acid, and then thoroughly rinsed with doubly distilled and deionized water. The general method of Mottola et al.[17] for the assay of H_2O_2 was used. Solutions of inactivators were prepared at several concentrations in 100 mM Tris-HCl buffer. Solutions of leuco crystal violet (0.050 g diluted to 100 mL with 1:195 concentrated hydrochloric acid/water), horseradish peroxidase, (1.0 mg/mL in water), and sodium acetate (2.0 M, pH 4.5) also were prepared. Aliquots of the inactivator solutions (2 × 490 mL) and aliquots of buffer as a control (1 × 490 mL) were preincubated at 25 °C. A stock solution of MAO was prepared by diluting 245 μM MAO (10 μL) with 100 mM Tris-HCl buffer pH 9.00 (1035 μL). Aliquots (10 μL) of the MAO stock solution were added to each of the above inactivator and control solutions, and then the solutions were allowed to incubate for 90 s (1 min for **1**; 2 min for **6**) before adding 110 μL of leuco crystal violet solution, 56 μL of horseradish peroxidase solution, and 445 μL of sodium acetate buffer, vortexing briefly. The absorbance at 596 nm was read on an instrument zeroed on a mixture of the control MAO solution with 110 μL of leuco crystal violet solution, 56 μL of horseradish peroxidase solution, and 445 μL of sodium acetate buffer. Absorbance values were converted to molarity using the reported extinction coefficient[17] corrected for pH difference.

General Procedure for Inhibition of the MAO-Catalyzed Oxidation of Benzylamine. Solutions of inhibitors in Tris-HCl buffer (100 mM), benzylamine (1.5 mM) in Tris-HCl buffer (100 mM), and Tris-HCl buffer (100 mM) were prepared; the pH was adjusted in each solution to 9.00 with potassium hydroxide. Solutions of inactivator and benzylamine (1.0, 0.5, 0.33, 0.25, 0.20, 0.167, 0.125, 0.1, and 0.0 mM final concentrations) were prepared. Mixtures (20 μL:470 μL) of each of the inhibitor solutions were prepared with each of the benzylamine solutions. Stock solutions of MAO (60 μL, 215 μM) in Tris-HCl buffer (100 mM, pH 9.00, 1140 μL) also were prepared. The rate of change in the optical density at 250 nm was monitored for the above 20 μL:470 μL inhibitor/benzylamine mixtures plus 10 μL of stock MAO solution.

General Procedure for Time-Dependent Inactivation of MAO by Milacemide Analogues. Solutions of various concentrations of inactivator in Tris-HCl buffer (10 mM, pH 9.00) were preincubated at 25 °C. A MAO stock solution was prepared by mixing 5 μL of 215 μM MAO solution and 350 μL of 100 mM Tris-HCl buffer, pH 9.00. The solutions of inactivators (380 μL) were incubated with MAO (20 μL) and aliquots were removed and assayed periodically. A control with no inactivator also was run.

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