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

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Abstract: The anticonvulsant agent milacemide (2-(*n*-pentylamino)acetamide) is known to inactivate monoamine oxidase-B (MAO-B). Various isotopically labeled analogues of milacemide are used to elucidate the mechanism of inactivation of MAO-B by this compound. The metabolites of the oxidation of milacemide by MAO-B (pentanoic acid, pentanal, and glycinamide) are shown not to be responsible for inactivation. MAO was inactivated with 2-(*n*-pentylamino)-acetamide (**1a**), 2-(*n*-pentylamino)[2,2-$^2H_2$]acetamide (**1b**), and 2-([1,1-$^2H_2$]-*n*-pentylamino)acetamide (**1c**). Compound **1b** exhibited little or no isotope effect on inactivation (k_{inact}/K_I) and **1c** showed an isotope effect of 4.55 on k_{inact}/K_I. These compounds also were found to be excellent substrates for MAO-B; **1b** showed no isotope effect, but **1c** exhibited an isotope effect of 4.53 on k_{cat}/K_m. Incubation of MAO with 2-(*n*-pentylamino)[2-^{14}C]acetamide followed by dialysis under denaturing conditions resulted in the incorporation of 0.7 equiv of radioactivity per enzyme molecule. The same treatment with 2-([1-^{14}C]-*n*-pentylamino)acetamide led to the incorporation of 4 equiv of radioactivity into the enzyme. The excess radioactivity bound presumably arises from the [^{14}C]pentanal that is generated during turnover. In order to test this, MAO-B was incubated with [1-^{14}C]pentylamine under similar conditions and 5.9 equiv of radioactivity was incorporated into the denatured enzyme. Therefore, the entire molecule becomes attached to the enzyme during inactivation. By following changes in the flavin absorption spectrum during inactivation with milacemide, it was shown that the flavin becomes reduced; however, denaturation of the inactivation enzyme causes flavin reoxidation under conditions where radioactivity for 2-(*n*-pentylamino)[2-^{14}C]acetamide remains bound. This suggests that milacemide is oxidized during inactivation and the adduct results from attachment of milacemide to an amino acid residue, not to the flavin cofactor. Inactivation with 2-([1-^{14}C]-*n*-pentylamino)acetamide produced [^{14}C]pentanoic acid and [^{14}C]-pentylamine in the ratio of 92:8. Inactivation of MAO with 2-(*n*-pentylamino)[2-^{14}C]acetamide gave [^{14}C]glycinamide and [^{14}C]oxamic acid, further supporting oxidation reactions at both the pentyl side chain and the acetamido methylene. All of these results indicate that milacemide is oxidized at both the pentyl methylene and the acetamido methylene. Pentyl oxidation leads to inactivation, but it is not clear if acetamido methylene oxidation also leads to inactivation (Scheme I).

Monoamine oxidase (MAO; E.C. 1.4.3.4) is a flavin-dependent enzyme that is important in the catabolism of various biogenic and xenobiotic amines.[1] In 1968 it was shown that MAO exists in two isozymic forms called MAO-A and MAO-B.[2] These forms have been shown to be two distinct enzymes on the basis of inhibitor and substrate studies and by the cloning of the cDNA of both

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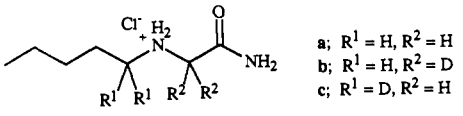
of these isozymes and showing that the primary sequence is different for the two forms of the enzyme.[3] It is now known that inhibitors of MAO-A, which has a substrate selectivity for serotonin and norepinephrine, exhibit antidepressant activity[4] whereas inhibitors of MAO-B, which metabolizes dopamine, are useful as adjuncts to the treatment of Parkinson's disease.[5]

Milacemide (2-(*n*-pentylamino)acetamide, **1a**), an effective anticonvulsant agent in numerous animal models,[6] does not produce a sedative effect but does exhibit antidepressant activity[7]



a; $R^1 = H, R^2 = H$
b; $R^1 = H, R^2 = D$
c; $R^1 = D, R^2 = H$

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and enhances cognition,[8] an important property for the treatment of the symptoms of Alzheimer's disease. In rodents and monkeys milacemide is essentially devoid of CNS side effects; many years of clinical studies also indicate that the compound is safe.[9] Although the anticonvulsant mechanism of action is still in debate, milacemide has been shown to cross the blood–brain barrier readily and is actively metabolized to glycine, an inhibitory neurotransmitter.[10] The anticonvulsant activity correlates with the increase in brain levels of glycine, which is generated upon oral administration.[10] Therefore, milacemide was thought to behave as a prodrug, and its oxidation in the brain to glycinamide was found to be produced by mitochondrial monoamine oxidase-B.[11] More recently, however, it was reported that α-methylmilacemide, which is not oxidized by MAO nor is glycine generated from it, also is an anticonvulsant agent.[12] Therefore, the anticonvulsant activity of milacemide may not be related to its oxidation by MAO. Milacemide also has been shown to be a selective time-dependent inhibitor of brain monoamine oxidase-B in vitro and in vivo,[13] but its mechanism of inactivation is unknown.

We have been interested in the mechanisms of inactivation of monoamine oxidase by various pharmacologically active agents for many years[14] and, therefore, were intrigued by the reported inactivation of MAO by milacemide. Here we report our research directed at the elucidation of this inactivation mechanism.

Results

Effect of Pentanoic Acid, Butanal, and Glycinamide on MAO Activity. It has been shown that milacemide is metabolized in vivo to glycinamide and pentanoic acid (presumably the result of secondary oxidation of pentanal).[11] In order to determine if MAO inactivation is caused by any of these metabolites, the effect of these compounds on MAO activity was assessed. Unfortunately, pentanal was not sufficiently soluble in buffer to get a meaningful result; however, butanal was soluble. Neither pentanoic acid, butanal, glycinamide, nor the combination of butanal and glycinamide at 50 mM concentration caused inactivation of the enzyme, suggesting that they are not responsible for the inactivation of MAO by milacemide.

Effect of 2-(*n*-Pentylamino)acetamide and 2-(*n*-Butylamino)-acetamide on MAO Activity. Because pentanal could not be tested as an inactivator of MAO (see above) and butanal had to be used in its place, it was necessary to determine if the butyl analogue of milacemide, namely, 2-(*n*-butylamino)acetamide, is an inactivator of MAO. 2-(*n*-Butylamino)acetamide was found to be a substrate and an inactivator of MAO. Its K_m and k_{cat} were 0.172 mM and 25 min^{-1}, respectively, compared with 0.149 mM and 178 min^{-1} for milacemide. Binding to the active site, therefore, is comparable, but the rate of turnover of the butyl analogue is only $^1/_7$th that of milacemide. The K_I and k_{inact} values for inactivation of MAO, however, show considerable differences for the two compounds. Whereas the K_I value for 2-(*n*-butylamino)acetamide is 20 mM, that for milacemide is 1.0 mM, and k_{inact} for 2-(*n*-butylamino)acetamide is only 0.0026 min^{-1}, but for milacemide is 0.02 min^{-1}. However, the partition ratio for 2-(*n*-butylamino)acetamide (9600) is comparable to that for milacemide (8900).

Deuterium Isotope Effects during Inactivation of MAO by Milacemide. MAO was inactivated with 2-(*n*-pentylamino)-acetamide (**1a**), 2-(*n*-pentylamino)[2,2-$^2H_2$]acetamide (**1b**), and 2-([1,1-$^2H_2$]-*n*-pentylamino)acetamide (**1c**). The results of these inactivation experiments are summarized in Table I. Compound **1b** exhibited a negligible isotope effect (1.25) on k_{inact}/K_I, and **1c** showed a normal isotope effect (4.55) on k_{inact}/K_I.

Deuterium Isotope Effects during Oxidation of Milacemide by MAO. Milacemide (**1a**) and its deuterated analogues (**1b** and **1c**) were found to be excellent substrates for MAO-B (Figure 1). The kinetic constants for oxidation are summarized in Table II. Compound **1b** showed no isotope effect, but **1c** exhibited a normal

Table I. Kinetic Constants for the Inactivation of MAO by **1a–c**

compound	K_I (mM)	k_{inact} (min^{-1})	k_{inact}/K_I	$(k_{inact}/K_I)_H/(k_{inact}/K_I)_D$
1a	1.0 ± 0.1	0.020 ± 0.004	0.020	
1b	1.16 ± 0.5	0.018 ± 0.005	0.016	1.25
1c	0.72	0.0019	0.00264	4.55

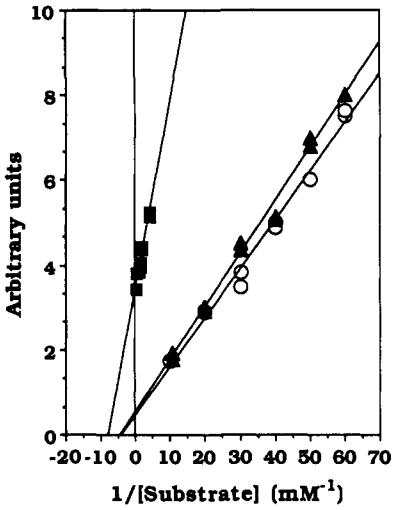

Figure 1. Rate of oxidation of milacemide and its isotopomers versus the inverse of the concentration of **1a** (○), **1b** (▲), and **1c** (■).

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Table II. Kinetic Constants for Oxidation of **1a–c** by MAO

compound	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m	$(k_{cat}/K_m)_H/(k_{cat}/K_m)_D$	partition ratio (k_{cat}/k_{inact})
1a	0.149 ± 0.028[a]	178 ± 24	1195		8 900
1b	0.182 ± 0.025	212 ± 22	1165	1.03	11 800
1c	0.129 ± 0.009	24 ± 0.4	264	4.53	12 600

[a] Numbers were derived from a nonlinear regression analysis of the data.[28]

isotope effect (4.53) on k_{cat}/K_m. The partition ratios (a measure of the turnover to product per inactivation event) were very high and were similar for the three compounds.

Inactivation of MAO by 2-(*n*-Pentylamino)[2-^{14}C]acetamide and 2-([1-^{14}C]-*n*-Pentylamino)acetamide. Incubation of MAO with 2-(*n*-pentylamino)[2-^{14}C]acetamide followed by dialysis under denaturing conditions resulted in the incorporation of 0.7 equiv of radioactivity per enzyme molecule. The same treatment with 2-([1-^{14}C]-*n*-pentylamino)acetamide led to the incorporation of 4 equiv of radioactivity into the enzyme.

Incubation of MAO with [1-^{14}C]Pentylamine. In order to determine if pentanoic acid is an oxidation product of the expected milacemide oxidation product, namely, pentanal, MAO was incubated with a good substrate, [1-^{14}C]pentylamine, known to produce pentanal. Using the extended incubation times of the milacemide experiments for this experiment, no pentanal was detected; only pentanoic acid. When catalase was added prior to the MAO and this experiment was repeated, then no pentanoic acid was produced. This suggests that it is the hydrogen peroxide generated during enzyme turnover (from the oxidation of reduced flavin to oxidized flavin) that is oxidizing the pentanal to pentanoic acid. Dialysis and denaturation of the MAO after [1-^{14}C]-pentylamine treatment resulted in the incorporation of 5.9 equiv of radioactivity into the enzyme, apparently at peripheral sites, because the enzyme was still active. This experiment establishes the origin of the excess radioactivity attached to MAO during incubation with 2-([1-^{14}C]-*n*-pentylamino)acetamide.

Identification of the Metabolites Generated during Inactivation of MAO by 2-([1-^{14}C]-*n*-Pentylamino)acetamide. MAO was inactivated with 2-([1-^{14}C]-*n*-pentylamino)acetamide, and then the small molecules were isolated. The HPLC (0.5 mL/min) chromatograms of the metabolites obtained from method A (see Experimental Section) show that there is a radioactive peak at T_R = 32 min (pentanoic acid by comparison with the standard) and no radioactive peak at T_R = 42 min (the (2,4-dinitrophenyl)-hydrazone of pentanal). When the flow rate was decreased to 0.25 mL/min, the T_R of the metabolite was extended to 66 min (pentanoic acid), and the T_R of the (2,4-dinitrophenyl)hydrazone of pentanal was 84 min. Pentanoic acid, but not pentanal, also was detected in the HPLC chromatograms of the metabolites obtained by method B. A small amount of pentylamine was found as a metabolite obtained from method C (and much radioactive milacemide). The ratio of pentanoic acid to pentylamine was 92:8.

Identification of the Metabolites Generated during Inactivation of MAO by 2-(*n*-Pentylamino)[2-^{14}C]acetamide. When 2-(*n*-pentylamino)[2-^{14}C]acetamide was used as the inactivator, no pentylamine was detected, but instead radioactive glycinamide and oxamic acid were observed; however, because the retention time was similar to that of milacemide, quantitation of the oxamic acid was not possible.

Changes in the Flavin Absorption Spectrum during Inactivation of MAO by Milacemide. Treatment of MAO with milacemide produced a bleaching of the absorbance in the flavin visible spectrum; when the enzyme was completely inactivated, the flavin was reduced. Denaturation of the inactivated enzyme under conditions where 2-(*n*-pentylamino)[2-^{14}C]acetamide remained bound to the enzyme, however, resulted in a return of the flavin to its oxidized form. This indicates that the milacemide is not bound to the flavin.

Discussion

Milacemide is known to undergo oxidation to pentanoic acid (presumably an oxidation product of the actual metabolite, pentanal) and glycinamide in vivo.[11] Consequently, we first determined whether milacemide or one of these metabolites was responsible for the inactivation of MAO. Pentanal, however, was not sufficiently soluble in aqueous buffer to test as an inactivator, but butanal was. No inactivation of MAO by pentanoic acid, butanal, glycinamide, or a combination of butanal and glycinamide at concentrations as high as 50 mM for several hours was observed. In order to show the relevance of butanal as a substitute for pentanal, 2-(*n*-butylamino)acetamide was synthesized and was shown to be a substrate and time-dependent inactivator of MAO-B. The K_m values for milacemide and the butyl analogue were comparable, but the k_{cat} for the butyl analogue was $^1/_7$th that of milacemide. The K_I value for the butyl analogue was 20 times larger than that for milacemide, and the k_{inact} value was 0.13 times that for milacemide. Nonetheless, since 2-(*n*-butylamino)acetamide behaves similarly to milacemide, the use of butanal as a substitute for pentanal is valid. Therefore, these results indicate that the metabolites of milacemide are not responsible for inactivation of MAO.

On the basis of our earlier studies of the inactivation of MAO by (aminomethyl)trimethylsilane[15] and 3-aryl-5-((methylamino)-methyl)-2-oxazolidinones,[14] the mechanism for inactivation of MAO by milacemide is proposed to be that shown in Scheme I. Single-electron transfer from the amino group of milacemide to the flavin of MAO would produce the amino radical cation **2**. This could partition between loss of the α-proton of the pentyl side chain (pathway a) to give the corresponding radical **3** followed either by second electron transfer (pathway c) to the imine **4**, which would hydrolyze to the expected metabolites pentanal and glycinamide, or by combination with an active-site radical (pathway e) to give inactivated enzyme **5**. Removal of the acetamide proton (pathway b) would give radical **6**, followed either by transfer of a second electron (pathway f) to give the imine **7**, which would hydrolyze to pentylamine and 2-oxoacetamide, or by combination with an active-site radical (pathway h) to produce inactivated enzyme **8**. Because of the greater kinetic acidity of the acetamide methylene proton, in solution it would be expected to be removed more easily than the pentyl methylene proton. However, the enzyme, presumably, is set up to remove the proton on the α-carbon of the pentyl chain, so the less kinetically acidic proton (on the pentyl chain) may be the one removed more often. Adducts **5** and **8** also could be derived from similar mechanisms except that, instead of radical combination occurring (pathways d and h), addition of an active-site nucleophile (X^-) to **4** and **7** could give **5** and **8**, respectively, as well.

In order to test whether oxidation and/or inactivation proceeds by pathway a or b, the corresponding dideuterated analogues, 2-(*n*-pentylamino)[2,2-$^2H_2$]acetamide (**1b**) and 2-([1,1-$^2H_2$]-*n*-pentylamino)acetamide (**1c**), were used as substrates/inactivators of MAO-B. Oxidation of **1b** took place with no isotope effect on k_{cat}/K_m (1.03), which suggests that either cleavage of the acetamide C–H bond does not occur or it is not a rate-determining step. The increased ease of acetamide methylene cleavage would be consistent with the greater kinetic acidity of the acetamide methylene proton. Oxidation of **1c**, however, occurs with an isotope effect on k_{cat}/K_m of 4.53, which is similar to the isotope effect for the oxidation of a variety of substrates for MAO such as kynuramine,[16] tyramine,[16] dopamine,[17] and serotonin.[17] This is consistent with turnover requiring cleavage of this C–H bond,

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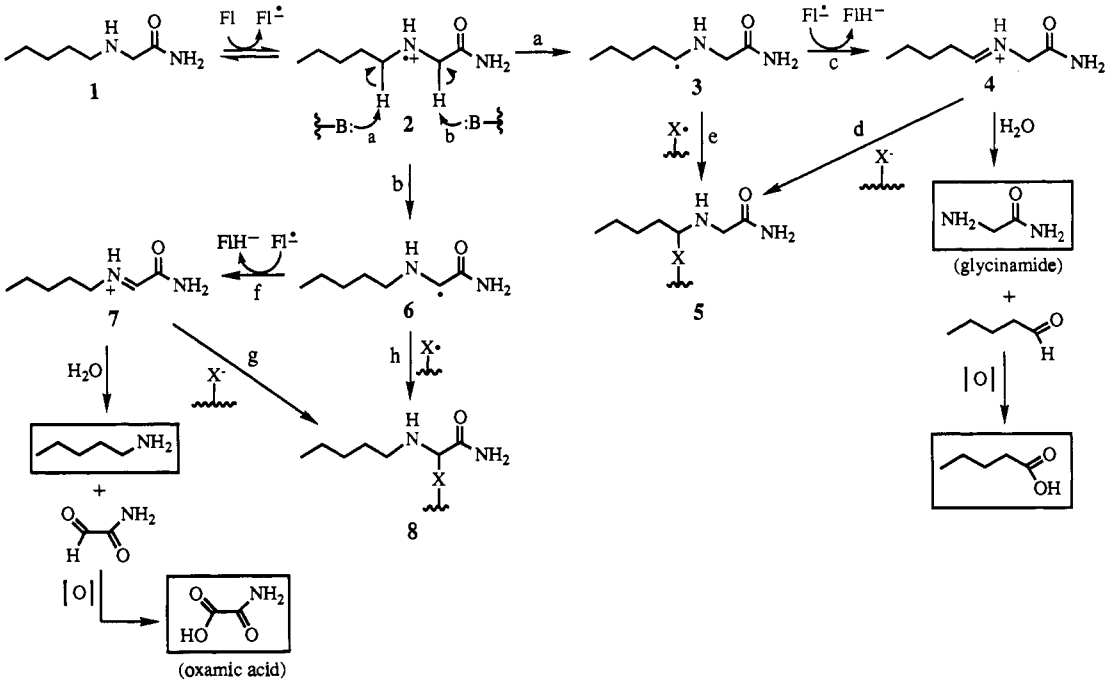
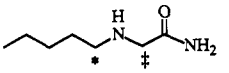
Scheme I



with cleavage of this bond being rate-limiting, and with the formation of the amino radical introducing a forward commitment to catalysis. Cavalier et al.[18] reported earlier that **1c** exhibited an isotope effect of 5 on the V_{max} for the brain MAO-B-catalyzed formation of glycinamide from milacemide.

Isotope effects during inactivation also were measured. Compound **1b** produced a negligible isotope effect on k_{inact}/K_I (because of the relatively large experimental error in these experiments, 1.25 may be zero), and **1c** exhibited an isotope effect on k_{inact}/K_I of 4.55, which is identical to the isotope effect on k_{cat}/K_m and which is similar to the isotope effect for inactivation of MAO by (aminomethyl)trimethylsilane.[15] As in the case of the isotope effects on oxidation of the milacemide isotopomers, it is apparent that cleavage of the C–H bond of the pentyl methylene is involved in the inactivation process, but it is not clear if cleavage of the C–H bond of the acetamide methylene is involved or if it is just not a rate-determining step, presumably because of the greater kinetic acidity of that proton. This question is answered below with the use of radioactively labeled analogues. The partition ratio, the amount of turnover to product per inactivation event, is very high for milacemide and both of its isotopomers (Table II), but there is little difference in the partition ratios of the three compounds. This is consistent with the mechanism shown in Scheme I, in which partitioning between metabolite formation and inactivation occurs subsequent to the C–H bond cleavage step.[19]

In order to determine if cleavage of the acetamide C–H bond occurs during oxidation of milacemide, the metabolites generated were examined. Incubation of MAO with 2-([1-^{14}C]-*n*-pentylamino)acetamide (**9b**) gave [^{14}C]pentanoic acid (derived from oxidation of the pentyl side chain to give pentanal, which, apparently (vide infra), is oxidized to pentanoic acid under the conditions of the experiment) and [^{14}C]pentylamine (from oxidation of the acetamide methylene) in a ratio of 92:8. The amount of pentylamine obtained represents a lower limit because



9

a, * = ^{12}C; ‡ = ^{14}C b, * = ^{14}C; ‡ = ^{12}C

it is an excellent substrate for MAO and may be partially degraded during the experiment. Incubation of MAO with [^{14}C]pentylamine, an excellent substrate, under the conditions of the experiment with **9b** produced [^{14}C]pentanoic acid, the H_2O_2 oxidation product of [^{14}C]pentanal. In the presence of catalase, to consume the H_2O_2 generated by the enzyme, no pentanoic acid was detected. Therefore, the [^{14}C]pentanoic acid is a nonenzymatic oxidation product of [^{14}C]pentanal, the actual product of the oxidation.

Incubation of MAO with 2-(*n*-pentylamino)[2-^{14}C]acetamide (**9a**) produced [^{14}C]glycinamide ($NH_2{}^{14}CH_2CONH_2$) from oxidation of the pentyl methylene of milacemide and [^{14}C]oxamic acid ($HOO^{14}CCONH_2$), the H_2O_2 oxidation product of 2-oxoacetamide, formed as a result of oxidation of the acetamido methylene of milacemide. Because of partial overlap of the peaks in the HPLC, it was not possible to get an accurate ratio of the two metabolites. Nonetheless, the experiments with both **9a** and **9b** indicate that MAO catalyzes the oxidation of both the pentyl methylene and acetamido methylene groups of milacemide.

Inactivation of MAO with radioactively labeled milacemide analogues **9a** and **9b** also was carried out in order to determine if the entire molecule becomes attached to MAO upon inactivation and to measure the number of equivalents of milacemide that are bound per enzyme molecule. Radioactivity remains attached to the enzyme after inactivation and urea denaturation by both derivatives; an average of 0.7 equiv of ^{14}C from **9a** is attached per MAO molecule and 4 equiv of ^{14}C is derived from **9b** inactivation. In order to determine if the origin of the excess amount of radioactivity bound to the enzyme by inactivation with **9b** was derived from the [^{14}C]pentanal that is produced, MAO was incubated with [1-^{14}C]pentylamine, an excellent substrate for MAO-B. This compound led to the incorporation of 5.9 equiv of radioactivity into the enzyme even after denaturation. This accounts for the excess radioactivity incorporated during inactivation of MAO by **9b** which is not observed with **9a**. The amount of [^{14}C] incorporation by [1-^{14}C]pentylamine

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depended upon the length of incubation time. These results suggest that the entire milacemide molecule is attached to the enzyme.

The fact that the flavin becomes reduced during inactivation indicates that transfer of electrons from milacemide to the flavin occurs. However, denaturation of the inactivated enzyme, under conditions where the radioactivity from **9a** and **9b** remains bound, results in reoxidation of the flavin. This suggests that attachment of milacemide occurs to an active-site amino acid, not to the flavin cofactor, as was observed with the 3-aryl-5-((methylamino)-methyl)-2-oxazolidinones[14] and with (aminomethyl)trimethylsilane.[15]

The deuterium isotope effect results are consistent with pathways a and e (or a, c, and d) in Scheme I as leading to inactivation, but partial inactivation by pathways b and h (or b, f, and g) cannot be ruled out. The stability of adduct **5** (X = amino acid residue) could be derived from two factors. It was previously proposed that inactivation of MAO by (aminomethyl)-trimethylsilane[15] and 3-aryl-5-((methylamino)methyl)-2-oxazolidinones[14] was the result of adducts related to **5** and that their stabilities were derived from electron-withdrawing groups attached nearby which stabilize the sp^3 character of the carbon to which the enzyme group is attached. As a consequence of the hypothesis, the corresponding 5-(aminomethyl)-3-aryldihydrofuran-2(3*H*)-ones[20] and 4-(aminomethyl)-1-aryl-2-pyrrolidinones[21] were designed and shown to be effective time-dependent inhibitors of MAO. These compounds also have vicinal electron-withdrawing groups. In the case of milacemide, the acetamido group may serve this electron-withdrawing function. However, we have recently found that *N*-substitution is another important factor in producing an inhibitory effect.[22] Therefore, the acetamido group also may be acting by a stereoelectronic block of the decomposition of the enzyme adduct.

Conclusions

The mechanism of inactivation of MAO-B by milacemide appears to involve the oxidation of the pentyl side chain and attachment of the oxidized drug to an active-site amino acid. The acetamido methylene also is oxidized, but it is not clear if this oxidation also leads to inactivation. This suggests that analogues of milacemide in which the acetamido group is replaced by other carbonyl derivatives also should be effective inactivators. We have recently found this to be the case.[23]

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 XLA-400 spectrometer. An Orion Research Model 601 pH meter was used for pH measurements. Elemental analyses were done by G. D. Searle Co. (Skokie, IL) or Oneida Research Servies (Whitesboro, NY). HPLC was performed on Econosil C_{18} 10 μ 4.6 × 250 mm columns using Beckman 110B pumps in series with a Beckman 163 variable-wavelength detector for ultraviolet detection and a Radiomatic Instruments Flo-One/Beta Model CR radioactivity detector utilizing Packard Radiomatic FLO-SCINT II or RPI 3a70B scintillation cocktail for radioactivity detection. Liquid scintillation counting was done in a Beckman LS-3133T scintillation counter using 10 mL of RPI 3a70B scintillation cocktail. [^{14}C]Toluene (4×10^5 dpm/mL) from New England Nuclear was used as an internal standard. Radiopurity of radioactive compound was assessed by TLC (Merck Kieselgel 60 plates without fluorescent indicator) followed by cutting the plates into strips and counting each strip with scintillation cocktail in a scintillation counter. 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. Aldehydes were converted to the corresponding (2,4-dinitrophenyl)hydrazone using the standard reagent[24] and recrystallized from absolute ethanol.

Reagents. *n*-Pentylamine, *n*-butylamine, bromobutane, chloroform-*d*, deuterium oxide (>99.96 atom % D and 99.8 atom % D), hydrogen chloride, anhydrous diethyl ether, leucocrystal violet, ninhydrin, tetrahydrofuran, tetramethylsilane, and 2-mercaptoethanol were purchased from Aldrich. Anhydrous potassium carbonate, potassium cyanide, and HPLC-grade acetonitrile, ethyl acetate, hexane, and water were obtained from Mallinckrodt and were filtered prior to use. Absolute ethanol was purchased from Midwest Grain Co. of Illinois. Horseradish peroxidase (type II), tris(hydroxymethyl)aminomethane (Tris), and [^{14}C]potassium cyanide (21.6 mCi/mmol) were purchased from Sigma. 2-(*n*-Pentylamino)[2-^{14}C]acetamide (**9a**) (specific activity, 8.14×10^6 dpm/μmol), 2-(*n*-pentylamino)[2,2-$^2H_2$]acetamide (**1b**), and 2-([1,1-$^2H_2$]-*n*-pentylamino)acetamide (**1c**) were generous gifts of G. D. Searle & Co. (Mont-Saint-Guibert, Belgium); NMR spectroscopy of the deuterated analogues indicated complete deuteration. Diethyl ether and tetrahydrofuran used 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.

[1-^{14}C]Pentylamine Hydrochloride and 2-([1-^{14}C]-*n*-Pentylamino)-acetamide Hydrochloride. Potassium cyanide (100 mg, 1.5 mmol) containing 500 μCi of [^{14}C]potassium cyanide in water (0.2 mL) was stirred for 2 min, and then bromobutane (430 μL, 4 mmol) was added followed by the addition of ethanol (1 mL). The mixture was heated to reflux overnight and cooled; then the reaction mixture was extracted with hexane three times, and the combined organic layers were washed with water twice and dried over anhydrous $MgSO_4$. After removal of the drying agent, the mixture was diluted with ether (50 mL), and solid lithium aluminum hydride was added in small portions until no further hydrogen was liberated. Stirring continued overnight; then the reaction was quenched with saturated sodium sulfate and was filtered through a pad of Celite. Hydrogen chloride gas was bubbled through the filtrate; then the solvent was removed in vacuo to give 142 mg of [1-^{14}C]-pentylamine hydrochloride. To [1-^{14}C]pentylamine hydrochloride (104 mg, 0.84 mmol) was added formaldehyde (37% in water, 68 μL, 0.93 mmol) and water (68 μL). While the reaction mixture was being stirred in an ice bath, potassium cyanide (55 mg, 0.93 mmol) was added. After 30 min ether (1 mL) was added and stirring was continued for 2 h. Water was added, and the solution was extracted with diethyl ether three times. The combined organic layers were washed with water twice and dried over anhydrous K_2CO_3. After removal of the solvent in vacuo, freshly distilled THF (1.5 mL) and 2-mercaptoethanol (70 μL, 1.0 mmol) were added.[25] HCl gas was bubbled through the solution until a white precipitate appeared; then the mixture was stirred overnight under nitrogen. The white precipitate was filtered, washed with THF, and recrystallized from water–acetone to give 2-([1-^{14}C]-*n*-pentylamino)-acetamide (23 mg); radiopurity was determined to be 98% by TLC (2:1 methanol–water); specific activity 5.2×10^5 dpm/μmol.

2-(*n*-Butylamino)acetamide. Butylamine hydrochloride (1.1 g, 10 mmol) was added to a stirred solution of formaldehyde (37% aqueous, 0.81 mL, 10 mmol) in water (0.4 mL) followed by the addition of potassium cyanide (0.65 g, 10 mmol) in water (0.5 mL). The reaction was stirred in an ice bath for 4 h; then the reaction solution was extracted with ether, washed with water (10 mL), and dried over anhydrous sodium sulfate. The organic solvent was removed by rotary evaporation, and the residue was distilled under vacuum to give a colorless liquid (0.65 g, 58%); bp 75–78 °C/10 mm Hg; ^{1}H NMR ($CDCl_3$) δ 0.93 (t, 3 H), 1.36 (m, 2 H), 1.47 (m, 2 H), 2.71 (t, 2 H), 3.22 (s, 1 H), 3.59 (s, 1 H). Freshly distilled anhydrous THF (10 mL) and 2-mercaptoethanol (0.49 mL, 7 mmol) were added to the product; then dry HCl gas was bubbled in until a white precipitate appeared. The reaction mixture was stirred overnight under nitrogen, and the product was collected by filtration. Recrystallization from methanol–ether afforded the product as a white solid; mp 193–194 °C; ^{1}H NMR (D_2O) δ 0.88 (t, 3 H), 1.38 (m, 2 H), 1.67 (m, 2 H), 3.08 (t, 2 H), 3.88 (s, 1 H); IR (KBr) 1693 cm^{-1} (C=O). Anal. Calcd for $C_6H_{15}ClN_2O$: C, 43.24; H, 9.01; N, 16.82. Found: C, 43.14; H, 8.91; N, 16.71.

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Enzymes and Assays. Bovine liver MAO-B was isolated and assayed as described previously.[15] 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 of 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.[26] for the assay of H_2O_2 was used. Solutions of inactivator were prepared at several concentrations (98, 50, 33, 25, 20, 17 μM were final concentrations for **1a** and **1b**; 2.0, 1.0, 0.67, 0.50, 0.33, 0.25 mM were used for **1c**) in 100 mM Tris-HCl buffer. Solutions of leucocrystal 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 μL) and aliquots of buffer as a control (1 × 490 μL) were preincubated at 25 °C. A stock solution of MAO was prepared by diluting 268 μM MAO (40 μL) with 100 mM Tris-HCl buffer, pH 9.0 (760 μL; 13.4 μM final concentration). Aliquots (10 μL) of the MAO stock solution were added to each of the above inactivator and control solutions; then the solutions were allowed to incubate for 1 min before the addition of 110 μL of leucocrystal violet solution, 56 μL of horseradish peroxidase solution, and 445 μL of sodium acetate buffer. After the solution was vortexed briefly, the absorbance at 596 nm was read on an instrument zeroed on a mixture of the control MAO solution with 110 μL of leucocrystal 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[26] corrected for pH differences.

General Procedure for Time-Dependent Inactivation of MAO. MAO (2.5 μM final concentration) was preincubated with various concentrations of inactivators (0, 0.4, 0.7, 1.0, 2.5, 7.5 mM final concentration for milacemides) in Tris-HCl buffer (50 mM, pH 9.0) at 25 °C in a total volume of 400 μL. Periodically aliquots (40 μL) were removed and assayed.

Covalent Labeling of MAO by 2-([1-^{14}C]-*n*-Pentyl)acetamide, 2-*n*-Pentyl[1-^{14}C]acetamide, and [1-^{14}C]Pentylamine. A solution of labeled milacemide (5 mM) in 100 mM Tris-HCl buffer at pH 9.0 was incubated at 25 °C with MAO (4.1 μM final concentration) in a total volume of 620 μL until no activity remained (ca. 40 h). The inactivated enzyme was separated from excess inactivator by either of two methods: (1) microdialysis in 3 mL of buffer followed by dialysis in Tris buffer (2 × 500 mL) and then denaturation in 6 M urea; (2) the method of Penefsky.[27] The enzyme was assayed, the protein concentration was determined, and the amount of radioactivity bound was measured by scintillation counting. This experiment was repeated with [1-^{14}C]pentylamine instead of milacemide for times varying from 40 to 72 h.

Metabolite Isolation and Identification. Four procedures were used to isolate and characterize the metabolites formed during the enzyme-catalyzed reactions with labeled milacemides and pentylamine. *Method A*: To the microdialysate from the experiment described above (method 1) was added (2,4-dinitrophenyl)hydrazine reagent (500 μL), and the solutions were allowed to react overnight at room temperature. The microdialysates were extracted with chloroform (3 × 5 mL); then the combined organic extracts were washed with water (2 × 5 mL) and evaporated to a residue which was redissolved in 60% acetonitrile in water and analyzed using analytical reversed-phase HPLC (60:40 acetonitrile–water, 0.5 or 0.25 mL/min; FLO-SCINT II scintillation fluid 0.75 mL/min) monitoring with both the Radiomatic detector and the UV detector set at 254 or 238 nm. *Method B*: The inactivated enzyme was directly extracted with ether (3 × 5 mL), and the combined extracts were washed with water (2 × 5 mL) and evaporated. HPLC analysis was as described in method A. *Method C*: The inactivated enzyme was directly extracted with ether (3 × 5 mL), the combined extracts were extracted with 4 N HCl (3 × 5 mL), and the acidic extracts were evaporated. HPLC analysis was as described in method A. *Method D*: An aliquot of the inactivated enzyme was injected directly into a Hewlett Packard 5730 gas chromatograph equipped with a flame ionization detector and a 5 m × 0.53 mm fused silica capillary column (methyl silica gum, chromosorb HP-1, 2.65 μm film thickness). Injections were made in the split mode with the injector port and the detector port at 250 °C. The column temperature was maintained isothermally at 80 °C and then increased at a rate of 16 °C/min to 250 °C, which was maintained for 3 min; T_R for glycinamide is 6.7 min, and T_R for milacemide is 8.0 min (oxamic acid is not eluted because it is in the salt form). Another aliquot was injected directly into the HPLC and analyzed as decribed in method A.

Changes in the Flavin Absorption Spectrum. MAO (2.0 μM) was treated with milacemide (45 mM) at 25 °C in 100 mM Tris-HCl buffer, pH 9.0, until no enzyme activity remained (12 h). A difference absorption spectrum of the inactivated enzyme and a control having the same amount of enzyme but which was incubated with buffer containing no inactivator was taken. After dialysis (3 × 500 mL) in the same buffer, there was no change in the absorption spectrum. To each of the solutions was added urea to a concentration of 8 M, and each solution was incubated for 18 h. Another difference spectrum was taken.

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