Synthesis and Pharmacological Evaluation of a Major Metabolite of Ameltolide, a Potent Anticonvulsant

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The 4-aminobenzamides have provided several anticonvulsants that have been extensively investigated. Ameltolide, 4-amino-*N*-(2,6-dimethylphenyl)benzamide (compound **2**, LY201116), is the most potent analogue studied to date. This drug is inactivated in vivo by metabolic N-acetylation and addition of a hydroxy moiety to one of the methyl substituents, resulting in compound **7**, *N*-[4-[[[2-(hydroxymethyl)-6-methylphenyl]amino]carbonyl]phenyl]acetamide. This metabolite was prepared in five steps from a readily available starting material. Compound **7** and its nonacetylated analogue **6** were compared to ameltolide as anticonvulsants. After oral administration to mice, the MES ED_{50} values of ameltolide, **6**, and **7** were 1.4, 10.9, and >100 mg/kg, respectively, demonstrating that hydroxylation and acetylation dramatically decrease the anticonvulsant potency of ameltolide. This rank order of MES anticonvulsant potency was also seen after iv administration to mice, suggesting that these data reflect intrinsic pharmacological activities. After oral administration of 2.0 mg/kg of ameltolide to mice, parent drug, *N*-acetyl metabolite **3**, and the hydroxy metabolite **7** were detected in plasma; the C_{max} values were 572, 387, and 73 ng/mL, respectively. Compound **7** was the primary metabolite excreted in urine. These data indicate that **7** is a major metabolite of ameltolide, but does not contribute significantly to the pharmacological effects seen after administration of ameltolide to mice.

Introduction

Because of major deficiencies in the efficacies and side-effect profiles of existing antiepileptic medications, there has been a resurgence of interest in development of new classes of antiepileptic drugs.[1] The 4-aminobenzamides are a chemically novel series of potential antiepileptic drugs, and several representatives have undergone extensive evaluation. The original member of the series, **1** (Chart I), was a potent maximal electroshock (MES) selective anticonvulsant in mice and rats, but untoward toxicological findings precluded development of this compound or either of its enantiomers.[2,3] Ameltolide, 4-amino-*N*-(2,6-dimethylphenyl)benzamide (compound **2**, LY201116), is the most potent benzamide anticonvulsant studied to date. This compound potently inhibited MES-induced seizures in mice, but was ineffective against a variety of chemically induced seizures. This phenytoin-like profile, coupled with a high protective index, suggests that the compound may be suitable for treatment of generalized tonic–clonic and partial seizures in man, and clinical studies are in progress.[4-6] Recent studies have revealed that ameltolide is inactivated by metabolism to the *N*-acetyl analogue **3** [LY201979, 4-(acetylamino)-*N*-(2,6-dimethylphenyl)benzamide], and this metabolic pathway appears to be reversible in a variety of species.[7,8]

Efforts to limit this metabolic inactivation led to synthesis of **4** [LY201409, 4-amino-*N*-(2,6-dimethylphenyl)-3,5-dimethylbenzamide] a potent MES-selective anticonvulsant in mice and rats that is not metabolized by *N*-acetylation.[9,10] Moreover, a series of dialkylglycinamide derivatives of ameltolide were prepared, and diethyl analogue **5** was the optimal compound.[11-13] This compound was found to serve as a prodrug of ameltolide which possesses little, if any, intrinsic activity.[12]

Metabolism studies in rats revealed that hydroxylation of ameltolide occurred in addition to metabolic N-acetylation.[8] One expected position for hydroxylation

Chart I

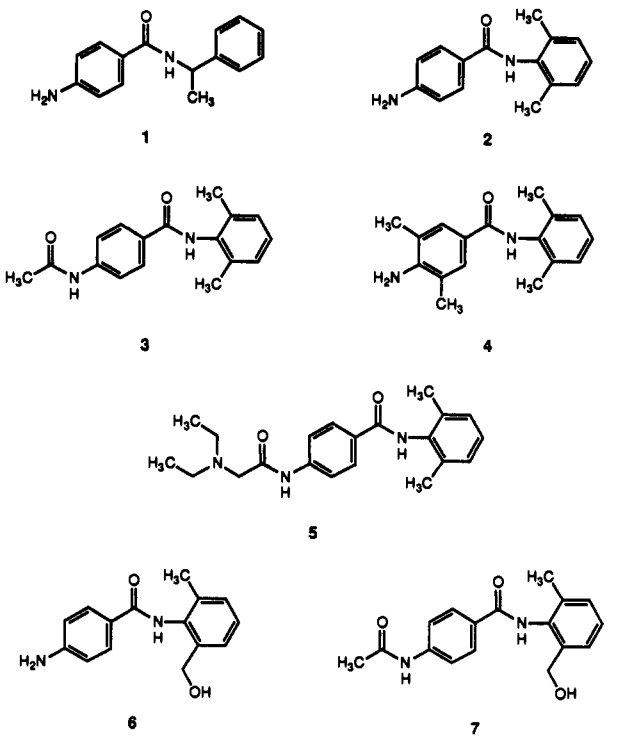

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Table I. Biological Activities of Benzamide Anticonvulsants after Administration to Mice

compound	TPE,[a] h	MES[b] ED_{50}, mg/kg, po	HS[c] ED_{50}, mg/kg, po	PI[d]	MES ED_{50}, mg/kg, iv[e]
ameltolide (2)	0.5	1.4 (1.2–1.6)[f]	23.0 (20.6–25.7)	13	0.59 (0.50–0.66)
3	2.0	4.7 (4.0–5.6)	96.4 (80.5–138)	21	19.9 (17.4–23.0)
6	1.0	10.9 (9.6–12.3)	66.0 (58.5–75.2)	6	3.9 (3.4–4.5)
7	1.0	>100	NT[g]		>80
phenytoin	2.0	8.1 (6.7–9.5)	83.7 (72.6–96.4)	10	NT

[a] TPE = time to peak anticonvulsant effect after oral dosing. [b] MES = maximal electroshock assay. [c] HS = horizontal screen assay. [d] PI = protective index after oral dosing (HS ED_{50}/MES ED_{50}). [e] Compounds were injected into the tail vein 5 min before the MES test was conducted, as described in the Experimental Section. [f] Values in parentheses represent 95% confidence limits. [g] NT = not tested.

would be on the two aromatic methyl substituents, a well-precedented metabolic pathway.[14] The objectives of the present studies were to prepare authentic derivatives of compounds 6 and 7, the hydroxy derivatives of ameltolide and 3, respectively, and to determine whether they contribute to the pharmacological effects produced by ameltolide in mice. The present study confirmed that 7 is indeed a major metabolite of ameltolide in mice. Moreover, pharmacological studies demonstrated that N-acetylation and hydroxylation of one of the methyl substituents leads to a substantial decline in the potency of ameltolide as an anticonvulsant in mice.

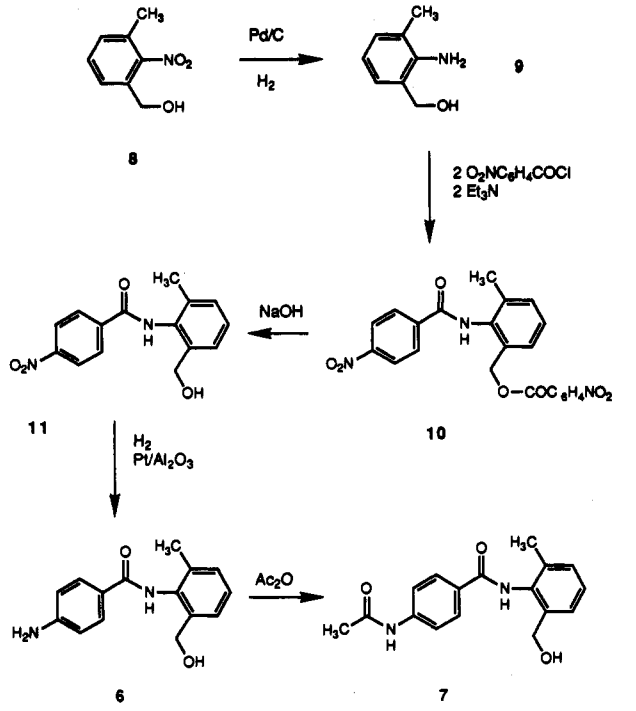
Results and Discussion

Chemistry. The key intermediate in the synthesis of 6 was 2-amino-3-methylbenzyl alcohol (9), which was generated by catalytic reduction of the commercially available nitro compound 8 (Scheme I). Attempts to monoacylate this sterically hindered aniline with 1 equiv of 4-nitrobenzoyl chloride failed, apparently due to competing ester formation with the benzylic alcohol. Attempts to protect this alcohol by reaction of 3-methyl-2-nitrobenzyl alcohol (8) with acetic anhydride, followed by catalytic reduction of the nitro group, led to a facile hydrogenolysis of the benzylic ester moiety to generate 2,6-dimethylaniline. Because of these synthetic difficulties, amino alcohol 9 was treated with 2 equiv of 4-nitrobenzoyl chloride and 2 equiv of triethylamine to generate the doubly acylated product 10. Gentle hydrolysis with sodium hydroxide selectively removed the ester, and catalytic hydrogenation provided 6, the hydroxylated congener of ameltolide. Reaction of 6 with neat acetic anhydride led selectively to monoacylated compound 7 due to its insolubility and rapid precipitation from acetic anhydride.

Pharmacology. To determine whether 7 could contribute to the anticonvulsant effects of ameltolide, the activities of ameltolide, 3, 6, and 7 were compared after oral administration to mice. As previously reported, ameltolide produces a dose-dependent anticonvulsant effect in mice, and an ED_{50} of 1.4 mg/kg was calculated (Table I). Its hydroxy congener 6 was substantially less active and an ED_{50} of 10.9 mg/kg was obtained. N-Acetylated compound 3 had an ED_{50} of 4.7 mg/kg. Hydroxy metabolite 7 was found to be considerably less active than any of the other compounds included in this study. This compound was inactive at 100 mg/kg po, and only 75% of the animals were protected after administration of 300 mg/kg po (n = 4). For comparative purposes, the anti-MES activity of the prototypic anticonvulsant phenytoin was also measured, and the ED_{50} was 8.1 mg/kg (Table I).

These compounds were also studied in the horizontal screen test (HS) to determine their propensities to produce neurological deficit or ataxia (Table I). The ED_{50} values of ameltolide, 3, 6, and phenytoin were 23.0, 96.4, 66.0, and 83.7 mg/kg, respectively, suggesting that all of these compounds are fairly selective regarding their effects on the central nervous system. Compound 3 had the largest protective index (HS ED_{50}/MES ED_{50}).

Scheme I



To ensure that the differences in potency seen in this oral study were not due to differences in absorption or pharmacokinetics, the anticonvulsant activities of these compounds were also studied after iv administration to mice, and the data are summarized in Table I. Ameltolide was again the most potent anticonvulsant, and an ED_{50} of 0.59 mg/kg was obtained 5 min postadministration. N-Acetyl metabolite 3 was substantially less potent after iv administration than after po dosing (ED_{50} values were 19.9 and 4.7 mg/kg, respectively). The iv ED_{50} of 6 was 3.9 mg/kg, confirming that a hydroxy group on one of the aromatic methyl substituents leads to a decrease in activity. Finally, metabolite 7 was virtually inactive after iv administration. A 20 mg/kg dose protected only 17% of the animals, and increasing the dose to 40 and 80 mg/kg failed to increase the efficacy of the drug.

Although 3 was one-third as potent as ameltolide after oral administration, it was 35 times less potent after iv administration. The iv ED_{50} values were generated 5 min postadministration, but the po ED_{50} values were generated at the time of maximal anticonvulsant effects and were individualized for each compound (Table I). Ameltolide appears to be rapidly absorbed, and the time to peak anticonvulsant effect (TPE) was 0.5 h. In contrast, the onset of action of 3 was relatively slow, and the TPE was 2 h. One plausible explanation for the discrepancy between the oral and iv results would be time-dependent, metabolically

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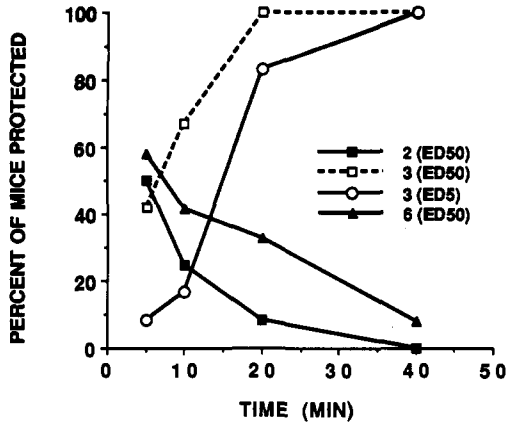

Figure 1. Duration of effect data after iv administration of ameltolide, **3**, and **6** to mice. The compounds were given at doses corresponding to their calculated ED_{50} values (Table I) and the percentage of animals protected from MES seizures was determined at the indicated times. For comparative purposes, compound **3** was also given at its calculated ED_5 dose. Each point represents data from 12 mice.

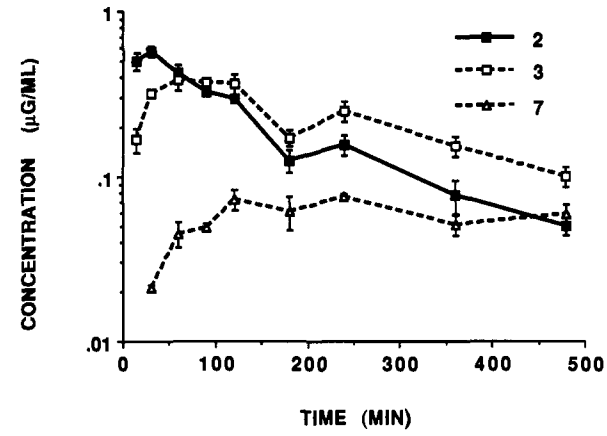

Figure 2. Plasma concentrations of ameltolide and metabolites after oral administration of ameltolide (2 mg/kg) to mice. At the indicated time intervals, animals were sacrificed and plasma concentrations of ameltolide-derived substances were determined as indicated in the Experimental Section. Each point represents the mean ± SEM of three mice.

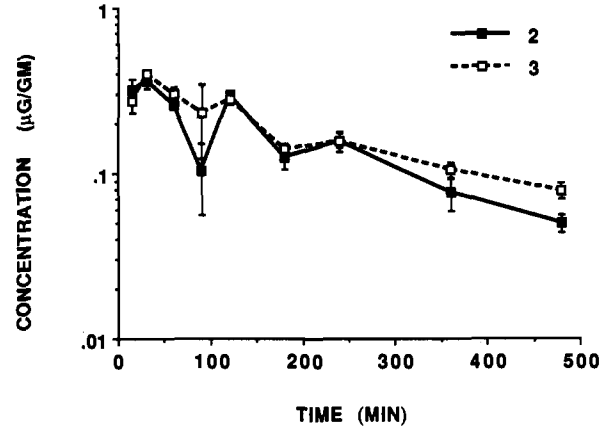

Figure 3. Brain concentrations of ameltolide and metabolites after oral administration of ameltolide (2 mg/kg) to mice. At the indicated time intervals, animals were sacrificed and brain concentrations of ameltolide-derived substances were determined as indicated in the Experimental Section. No hydroxy metabolite **7** could be detected in the brain. Each point represents the mean ± SEM of three mice.

mediated liberation of the potent anticonvulsant ameltolide from **3**. Ample precedent demonstrates that N-acylated aromatic amines such as procainamide can be deacylated in vivo.[15]

To explore possible in vivo liberation of the more potent anticonvulsant ameltolide from **3**, these compounds were injected iv into mice, and their anticonvulsant activities vs time were determined. Compound **6** was also included for comparative purposes and the data are displayed in Figure 1. Administration of either ameltolide or **6** at their calculated ED_{50} values (Table I) led to maximal anticonvulsant effects at the earliest time point measured (5 min), and the percentage of mice protected declined with time, as expected; 40 min postadministration, the anticonvulsant activities of both drugs had virtually disappeared. In contrast, iv administration of 19.9 mg/kg, the calculated ED_{50} of **3**, led to protection of 42% of the mice after 5 min and the percent of mice protected *increased* with time; 20 and 40 min postadministration 100% of the mice were protected from MES-induced seizures. Administration of 9.9 mg/kg, the calculated ED_5 of **3**, protected 8% of the animals 5 min after administration, but even this low dose led to 100% abolition of MES-induced seizures 40 min after administration. These data suggest time-dependent, metabolically mediated liberation of ameltolide from **3**. Subsequent studies have unambiguously confirmed this hypothesis by monitoring plasma concentrations of ameltolide after administration of **3**.

Metabolism Studies. After oral administration of [^{14}C]ameltolide to mice, approximately equivalent amounts of radioactivity were excreted in the urine and feces (49% and 41% of total radioactivity, respectively). A major metabolite, representing 30.6% of the radioactivity, was excreted in mouse urine; all other metabolites individually represented 7% or less of the radioactivity in the urine. By comparison of its HPLC and mass spectral characteristics to those of a synthetic standard, the major metabolite was unambiguously determined to be **7**. Compound **6** could not be detected as a metabolite, suggesting that either N-acetylation occurs before oxidation of the methyl substituent or N-acetylation is very rapid following methyl hydroxylation.

The quantities of metabolite **7** in plasma and brain were examined, and the data are summarized in Figures 2 and 3. Mice were given 2 mg/kg of ameltolide orally, and at various times plasma and brain samples were obtained and concentrations of ameltolide, *N*-acetyl metabolite **3**, and hydroxy-*N*-acetyl metabolite **7** were determined. Plasma concentrations of ameltolide and **3** rose rapidly (Figure 2). Peak plasma concentrations of 572 ng/mL of ameltolide were achieved 30 min after dosing, whereas peak concentrations of **3** were observed 60 min after dosing. Plasma concentrations of ameltolide and its *N*-acetyl metabolite decayed in unison, but the parent compound appeared to have a slightly shorter terminal half-life than **3**. Hydroxy metabolite **7** achieved its peak concentrations (73 ng/mL) at the 2-h time point, and plasma concentrations were relatively constant for the remainder of the experiment (detection limit = 20 ng/mL of plasma).

Peak brain concentrations of ameltolide (365 ng/g) were achieved 30 min after dosing and the concentrations of **2** and **3** were essentially equivalent throughout the experiment (Figure 3). No hydroxy metabolite **7** could be detected in the brain (detection limit = 50 ng/g of brain).

One possible interpretation of these data is that in mice little, if any, of hydroxy-*N*-acetyl metabolite **7** penetrates the blood–brain barrier, perhaps due to its increased hydrophilicity relative to ameltolide or **3**. However, the ob-

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servation that plasma concentrations of this metabolite are less than or equal to those of ameltolide and 3, whereas in urine 7 is the major ameltolide-derived substance, suggests that 7 is rapidly and preferentially excreted via the mouse kidney into urine. Moreover, the assay for 7 in brain tissue has relatively low sensitivity. The combination of rapid elimination of 7 and low sensitivity of the assay represent the most likely explanations for our inability to detect this metabolite in brain tissue of mice treated with ameltolide.

Summary and Conclusions

In this paper we have documented an expeditious synthesis of 7 and demonstrated that it is a major metabolite of ameltolide in mice. The metabolite can be detected in plasma and urine following oral administration of ameltolide to mice, but cannot be detected in the brain. After both iv and oral administration to mice, metabolite 7 is demonstrably less potent as an anticonvulsant than either ameltolide or *N*-acetyl metabolite 3. These data indicate that N-acetylation and hydroxylation of one of the benzylic methyl groups results in virtually complete metabolic inactivation of ameltolide.

Experimental Section

Methods. Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are not corrected. Microanalytical data were provided by the Physical Chemistry Department of Lilly Research Laboratories; only symbols of elements analyzed are given, and they were within 0.4% of theoretical values unless indicated otherwise.

Except where noted, a standard procedure was used for product isolation. This involved quenching by addition to water, filtration, or exhaustive extraction with a solvent (washing of extract with aqueous solutions, on occasion), drying over an anhydrous salt, and evaporation of solvent under reduced pressure. Particular solvents, aqueous washes (if needed), and drying agents are mentioned in parentheses after the phrase "product isolation".

***N*-[6-[(4-Nitrobenzoyl)methyl]-2-methylphenyl]-4-nitrobenzamide (10).** A mixture of 3-methyl-2-nitrobenzyl alcohol (50 g, 300 mmol) and 5.0 g of 5% Pd/C in 545 mL of THF was stirred under 60 psi of hydrogen until the theoretical amount had been consumed (4 h). The reaction mixture was filtered through diatomaceous earth. Removal of solvent under reduced pressure yielded 40 g (98%) of essentially homogeneous 2-amino-3-methylbenzyl alcohol (9) that was carried on without purification.

A solution of 4-nitrobenzoyl chloride (21.9 g, 118 mmol) in THF was added in one portion to a solution of 2-amino-3-methylbenzyl alcohol (8.1 g, 59 mmol) and triethylamine (16.5 mL, 118 mmol) in THF at room temperature. The reaction was then heated at reflux overnight. After cooling to room temperature, water and chloroform were added, and the insoluble product was removed by filtration. Recrystallization from THF provided 9 g (35.0%) of product as light yellow powder with mp 210–211 °C. Anal. ($C_{22}H_{19}N_3O_7$) C, H, N.

***N*-[2-(Hydroxymethyl)-6-methylphenyl]-4-nitrobenzamide (11).** Sodium hydroxide (5 N, 11.7 mL, 58.5 mmol) was added to a mixture of *N*-[6-[(4-nitrobenzoyl)methyl]-2-methylphenyl]-4-nitrobenzamide (10.2 g, 23.4 mmol) in ethanol/THF (1:1) at room temperature. Within 10 min the reaction became homogeneous and orange, and TLC analysis revealed complete disappearance of starting material. Solvents were removed under reduced pressure, and product isolation (water, chloroform, water, brine, Na_3SO_4) provided homogeneous product as a solid. This material was combined with the product that had been insoluble in the initial water/chloroform extraction, and the material was recrystallized from methanol to provide 5.3 g (79.1%) of yellow crystals with mp 174–175 °C. Anal. ($C_{15}H_{14}N_2O_4$) C, H, N.

4-Amino-*N*-[2-(hydroxymethyl)-6-methylphenyl]benzamide (6). A mixture of *N*-[2-(hydroxymethyl)-6-methylphenyl]-4-nitrobenzamide (5 g, 17.5 mmol) and 0.4 g of 5% Pt/Al_2O_3 in 95 mL of THF was stirred under 60 psi of hydrogen until the theoretical amount had been consumed. The reaction mixture was filtered through diatomaceous earth. Removal of solvent under reduced pressure and flash chromatography (silica gel eluted with 5% methanol in methylene chloride) provided 3.7 g (82.2%) of homogeneous 6 as a white solid. The analytical sample was provided by recrystallization of a small portion from methanol/water: mp 192–193 °C. Anal. ($C_{15}H_{16}N_2O_2$) C, H, N.

***N*-[4-[[[2-(Hydroxymethyl)-6-methylphenyl]amino]carbonyl]phenyl]acetamide (7).** Acetic anhydride (100 mL, 1.06 mol) was added to 4-amino-*N*-[2-methyl-6-(hydroxymethyl)phenyl]benzamide (3 g, 11.7 mmol) with stirring at room temperature. The reaction became homogeneous and in ca. 10 min product 7 precipitated from solution. TLC analysis revealed a single homogeneous product that was more polar than starting material. Excess reagent was removed under reduced pressure, toluene was added, and the solution was taken to dryness again. Recrystallization from methanol provided 2.5 g (62.8%) of product 7 as a white powder with mp 204 °C, resolidification, and a second mp 240–245 °C dec. ^1H NMR and MS analyses confirmed that mono-N-acetylation occurred in this reaction. Anal. ($C_{17}H_{18}N_2O_3$) C, H, N.

Pharmacological Methods. Male mice (CF-1, Charles River Breeding Laboratories, Portage, MI) weighing 18-26 g were used. The animals were maintained on Purina chow and water in a colony room for several days before use. For oral studies, compounds were suspended in 5% acacia/water and given to mice (1.5 h food and water deprived) in a volume of 0.01 mL/g of body weight. For intravenous studies, the compounds were dissolved in 2.5% ethanol–2.5% emulphor/water solution[16] and injected into the tail vein at various times before the maximal electric shock test was conducted. Control experiments indicated that the vehicle had no anticonvulsant activity.

To determine potencies for anticonvulsant and neurological impairment, groups of 12 mice were tested with various doses of compounds. The ED_{50} doses (effect in 50% of the animals) and the 95% confidence intervals were calculated by computer from the dose–effect data. The anticonvulsant test used was the maximal electric shock (MES) assay.[17] This test measures the ability of the test compound to abolish the tonic extension of hind limbs during MES-induced seizures. In the MES test, a shock of 50 mA of ac current was delivered for 0.1 s through corneal electrodes. Mice that did not exhibit a tonic extensor response after the MES were considered to be protected by the test compound. The horizontal screen test was utilized as a measure of neurological impairment.[18] Previously trained mice were injected with the compound and then placed individually on top of a square (13 × 13 cm) wire screen (no. 4 mesh) which was mounted on a metal rod. The rod was rotated 180° and the number of mice that returned to the top of the screen within 1 min was determined.

Benzamide Plasma and Brain Assays. One milliliter CN (end capped) Bond Elut columns (Analytichem Int.) were prewashed with 1 mL of MeOH and 1 mL of H_2O prior to the addition of 200 μL of plasma and 50 μL of a 10 μg/mL solution of the internal standard, 4-(acetylamino)-*N*-(1-phenethyl)benzamide (12, LY197777), which was synthesized as previously described.[19] Then a Vac-Elut apparatus (Analytichem Int.; 10–15 mmHg) was used to pull the entire mixture through a column filled with distilled water. After the column was washed with 2 column volumes of H_2O, the drug, metabolites, and internal standard were eluted from the column with 0.5 mL of 0.1 M ammonium acetate/methanol/acetonitrile/acetic acid/diethylamine (DEA) (50:25:25:2:0.5). A Varian 9090 autoinjector was used to inject 94 μL of the eluate into a Varian 5000 HPLC system containing a C-18 (25 cm × 4.6 mm, 5 μm) Alltech cartridge column. The flow rate of the mobile phase, 0.1 M ammonium acetate/methanol/acetonitrile/acetic acid/DEA (467:382:150:0.6), pH 6.0, was 0.7 mL/min. A Kratos Spectraflow 783 absorbance detector, set at a wavelength of 270 nm, was used. Under these conditions the

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retention times were as follows: **7**, 410 s; **2**, 580 s; **3**, 690 s; and **12**, 780 s. Brain tissue was homogenized in 5 mL of 0.1 M trichloroacetic acid/g of tissue. One-hundred microliters of the internal standard solution was added to 1.0 mL of the homogenate. The mixture was vortexed and centrifuged at 1000*g* for 10 min. The supernatant (500 μL) and 500 μL of distilled water were added to a conditioned CN extraction column and analyzed as outlined for plasma. The standard curves were prepared by diluting the compounds in 10% methanol and adding them to drug-free plasma or brain homogenate to achieve concentrations of 50–2000 ng/mL. The lease-squares regression lines for all of the standard curves gave correlation coefficients of greater than 0.995. The assay is sensitive to at least 20 ng/mL for plasma and 50 ng/g for brain tissue.

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Registry No. **2**, 787-93-9; **3**, 794-98-9; **6**, 131545-97-6; **7**, 125558-43-2; **8**, 80866-76-8; **9**, 57772-50-6; **10**, 131545-98-7; **11**, 131545-99-8; *p*-$O_2NC_6H_4CH_2OH$, 122-04-3; phenytoin, 57-41-0.