Synthesis, Stability, and Pharmacological Evaluation of Nipecotic Acid Prodrugs

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Abstract □ Nipecotic acid (**1**), one of the most potent in vitro inhibitors of neuronal and glial γ-amino butyric acid (GABA) uptake, is inactive as an anticonvulsant when administered systemically. To obtain in vivo active prodrugs of (**1**), we synthesized four new nipecotic acid esters (**3–6**), which were obtained by chemical conjugation with glucose, galactose, and tyrosine. These compounds were assayed to evaluate their in vitro chemical and enzymatic hydrolysis. In addition, their anticonvulsant activity was evaluated in vivo in Diluted Brown Agouti (DBA)/2 mice, an excellent animal model for the study of new anticonvulsant drugs. Esters (**3–6**) appeared stable, at various temperatures, in a pH 7.4 buffered solution and showed susceptibility to undergoing in vitro enzymatic hydrolysis. Intraperitoneally injected nipecotic acid (**1**) and esters (**3–5)** did not protect mice against audiogenic seizures; conversely, nipecotic tyrosine ester (**6**) showed a significant dose-dependent anticonvulsant activity. The in vivo protective activity of the ester (**6**) and the inefficiency of nipecotic acid (**1**) in the same experimental conditions suggest that this ester prodrug could be actively transported intact across the blood–brain barrier, beyond which it could be hydrolyzed.

Introduction

Several potentially central nervous system (CNS)-active drugs have relatively unfavorable physicochemical characteristics that hinder their transport into the brain. In fact, the brain microvessel endothelium provides a barrier to the passive transport of hydrophilic drugs into the brain. Several strategies have been developed to overcome this problem.[1−3] The prodrug approach represents a very promising method to enhance drug delivery to the brain. In fact, prodrugs are normally inactive and must generate active drug at their target by enzymatically or chemically mediated cleavage of their promoiety. The development of CNS-active prodrugs has been generally aimed at obtaining an improvement in the lipophilic character of the drug by transiently masking ionized group(s) of the parent drug. However, the preferential delivery of drugs or their derivatives to brain may be improved by using endogenous facilitated transport systems present at the blood−brain interface. Thus, chemical conjugation of potentially CNS-active drugs with an amino acidic or glycoside moiety actively transported across the blood−brain barrier (BBB) represents a plausible means of improving their brain delivery by providing suitable substrates for active membrane transport. In particular, conjugation with tyrosine or glucose has recently been shown to be a successful means of selective drug delivery; for example, phosphonoformate-L-tyrosine conjugate is actively transported,[4] by means of active amino acid carriers, through monolayers of porcine brain microvessel endothelial cells, and a glycosyl phosphotriester prodrug of 3′-azido-3′-deoxythymidine (AZT) shows good delivery to the CNS.[5] Similarly, conjugating glucose or galactose to poorly absorbable drugs can improve their intestinal absorption by means of glucose transport carriers in the small intestine;[6−8] for example, tocopherol conjugation to a monocarboxylate or glycoside moiety has appeared to provide suitable substrates for active erythrocyte membrane transport.[9]

Nipecotic acid (**1**) is one of the most potent in vitro inhibitors of neuronal and glial γ-aminobutyric acid (GABA) uptake.[10] Disorders such as Parkinson's disease, Huntington's chorea, and epilepsy may result from abnormalities in the GABA system and, thus, enhancement of the activity of GABA may be a useful treatment for these disorders. Unfortunately, nipecotic acid is a highly polar compound that does not easily penetrate the BBB, and is therefore inactive as an anticonvulsant when administered systemically.[11,12]

Successful prodrugs of nipecotic acid should be readily transported into the CNS and hydrolyzed there to the parent drug. Several esters of nipecotic acid (e.g., alkyl-, substituted phenyl-, and triaryl-nipecotic esters) have been synthesized with the goal to increase drug lipophilicity and have been reported to have varying degrees of anticonvulsant activity and to inhibit in vitro GABA uptake after conversion to the parent drug.[13−15]

The purpose of our research was to synthesize new nipecotic acid esters (**3**−**6**) obtained by chemical conjugation with essential nutrients, such as glucose, galactose, or tyrosine, that are actively transported across the BBB. These compounds were assayed to evaluate their in vitro chemical stability and enzymatic hydrolysis. In addition, their anticonvulsant activity was evaluated in vivo in Diluted Brown Agouti (DBA)/2 mice, an excellent animal model for the study of certain kinds of epilepsy and for testing new anticonvulsant drugs.[16,17]

Methods and Materials

Materials—Nipecotic acid was obtained from Sigma Chemical Company, Boc-L-tyrosine was purchased from Fluka Chemical Company. All other reagent chemicals were from Aldrich Chemical Company.

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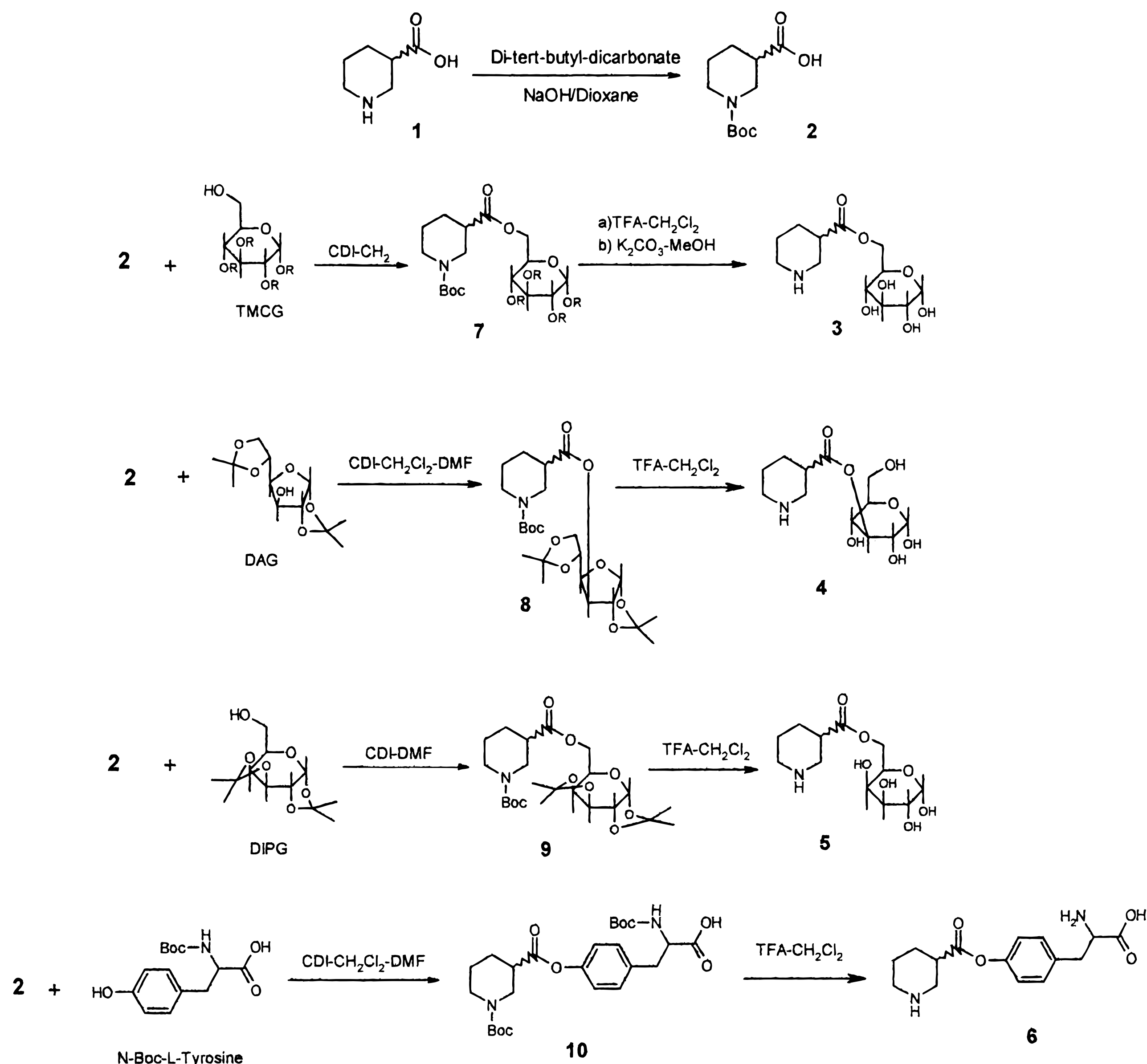

Scheme 1—Synthesis of nipecotic acid eters (**3**–**6**): synthetic routes and chemical structures of the obtained compounds (abbreviations: TMCG, 1,2,3,4-tetramethylcarbonate-D-glucose; DAG, diacetone-D-glucose; DIPG, 1,2,3,4-diisopropylidene-D-galactose).

Liquid Chromatographic (LC) grade acetonitrile and water, used in high-performance liquid chromatography (HPLC), were obtained from Merck and Carlo Erba, respectively.

Apparatus—Melting points were taken on Buchi 510 capillary melting point apparatus and are uncorrected.

The ^{1}H NMR and ^{13}C spectra were recorded on a Bruker WM 250 and on a Bruker AMX 500, respectively, using $CDCl_3$ as solvent. Chemical shifts are reported in ppm (δ) relative to tetramethylsilane as internal standard for ^{1}H NMR and in ppm relative to the solvent for ^{13}C NMR. Elemental analyses were performed on a Carlo Erba model 1108 elemental analyzer.

The flash chromatography was performed on Merck silica gel (0.040–0.063 mm) column. The HPLC apparatus consisted of a Varian 5000 system (Varian, Walnut Creek, CA) equipped with a 20-μL loop and an HP 1046A fluorescence detector (Hewlett-Packard, D-76337 Waldbrom, Germany). Integration of the chromatographic peaks was achieved with a 4290 integrator (Varian).

General Synthetic Method—All esters (**3**–**6**) were prepared as outlined in Scheme 1.

Initially we started from *N*-Boc nipecotic acid (**2**) obtained by treatment of (**1**) with di-*tert*-butyl-dicarbonate. Esterification of (**2**), in the presence of 1,1[1]-carbonyldiimidazole (CDI), with 1,2,3,4-*O*-tetramethyl carbonate-D-glucose (TMCG), diacetone-D-glucose (DAG), 1,2:3,4-di-*O*-isopropylidene-D-galactopyranose (DIPG), or *N*-Boc-L-tyrosine, afforded the corresponding esters (**7**–**10**). These esters, submitted to deprotection, allowed us to obtain compounds **3**–**6**.

*N-Boc-Nipecotic Acid (**2**)*—Nipecotic acid (**1**; 1 g, 7.75 mmol) was dissolved in dioxane (10.7 mL) and NaOH solution (1 N, 9.6 mL, 9.6 mmol). The resulting solution was stirred for 5 h, with the addition of the di-*tert*-butyl-dicarbonate (1.9 g, 10.0 mmol). The solvent was evaporated, and the resulting aqueous mixture was layered with ethyl acetate (EtOAc; 15 mL). Then, 1 N HCl solution was added with vigorous stirring until the pH of the aqueous phase was 2. The layers were separated, and the aqueous layer was extracted with EtOAc (3 × 10 mL). The EtOAc phases were combined, washed with brine (6 mL), dried over Na_2SO_4, filtered, and concentrated to give 1.42 g of (**2**) as a white solid (80% yield).

^{1}H NMR ($CDCl_3$): δ 4.1 + 3.85 (m, 2H, 2-H), 3.05 + 2.85 (m, 2H, 6-H), 2.50 (m, 1H, 3-H), 2.05 + 1.72 (m, 2H, 4-H), 1.70 + 1.46 (m, 2H, 5-H), 1.45 (m, 9H, -Boc).

*N-Boc-Nipecotic Acid (1′,2′,3′,4′–O-Tetramethyl carbonate)-D-glucopyranos-6′-yl Ester (**7**)*—To a stirred solution of *N*-Boc nipecotic acid (**2**; 1 g, 4.36 mmol) in CH_2Cl_2-dimethyl formamide (DMF) (9:1, 30 mL) was added CDI (706 mg, 4.36 mmol), and the mixture was stirred for 1 h at room temperature (RT). A solution of 1,2,3,4-tetramethyl carbonate-D-glucose (TMCG; 2.17 g, 5.26 mmol) in CH_2Cl_2/DMF (1:5, 30 mL) was added in a dropwise manner and

stirred for 24 h at RT. Most of the DMF was removed in vacuo, and the reaction mixture was taken up in EtOAc (30 mL), washed with water (20 mL) and brine (20 mL), and dried. Solvent was removed in vacuo and the crude was purified by flash chromatography eluting with hexane/EtOAc (6:4) to provide 1.90 g of (**7**) as a pale yellow oil (69.5% yield).

^{1}H NMR ($CDCl_3$): δ 6.00 (m, 1H, 1^1-H), 5.40 (m, 1H, 3^1-H), 4.92 (m, 1H, 2^1-H), 4.60 (m, 2H, 6^1-H), 4.15 (m, 2H, 4^1-H and 5^1-H), 4.05 + 3.85 (m, 2H, 2-H); 3.65 + 2.75 (m, 2H, 6-H), 3.55 (m, 12H, methyl carbonate groups), 2.55 (m, 1H, 3-H), 2.15 + 1.80 (m, 2H, 4-H), 1.45 + 1.35 (m, 2H, 5-H), 1.20(m, 9H, Boc).

Nipecotic Acid D-glucos-6′-yl Ester (**3**)—To a solution of **7** (1.90 g, 3.04 mmol) in CH_2Cl_2 (5 mL), 5 mL of trifluoroacetic acid (TFA) was added, and the mixture was stirred at RT for 2 h. Evaporation of the solvent gave a residue that was poured into a saturated solution of $NaHCO_3$ (20 mL) and extracted with $CHCl_3$ (3 × 15 mL). The organic layers were dried over Na_2SO_4 and concentrated in vacuo. This material (1.5 g) was taken directly into the next reaction without further purification. To a solution of crude (1.5 g) in MeOH (10 mL) was added K_2CO_3 (100 mg), and the mixture was stirred at RT for 12 h. Evaporation of the solvent gave a residue that was taken up in water and neutralized with 6 N aqueous HCl, diluted with more water, and washed with $CHCl_3$.The aqueous layers were concentrated in vacuo, and the residue was submitted to cation-exchange chromatography, eluting with 10% pyridine in water to give 610 mg of (**3**) (69% yield) as a pale yellow solid (mp 145−146 °C).

^{1}H NMR (D_2O): δ 4.65 (m, 1H, 1^1-H), 4.60 (m, 1H, 3^1-H), 3.70 (m, 1H, 2^1-H), 3.50 (m, 3H, 6^1-H and 5^1-C), 3.15 (m, 3H, 3^1-C and 2-H), 2.95 + 2.75 (m, 2H, 6H), 2.45 (m, 1H, 3-H), 1.90 + 1.65 (m, 2H, 4-H), + 1.55 (m, 2H, 5-H).

^{13}C NMR (D_2O + MeOD): δ 172 (−COOR), 96.84 (C-1^1), 76.86−76.66 (C-3^1), 75.71 (C-2^1), 75.31−75.08 (C-5^1), 70.56 (C-4^1), 63.57−63.33 (C-6^1), 48.78 (C-2), 45.02−44.91 (C-6), 39.57 (C-3), 26.63 (C-5), 21.98 (C-4).

*N-Boc Nipecotic Acid Diacetone-α-D-glucofuranos-3′-yl Ester (**8**)*—To a stirred solution of *N*-Boc-nipecotic acid (**2**; 1 g, 4.36 mmol) in CH_2Cl_2/DMF (9:1, 30 mL) was added CDI (706.97 mg, 4.36 mmol), and the mixture was stirred for 1 h at RT. A solution of DAG (1.36 g, 5.23 mmol) in CH_2Cl_2/DMF (9:1, 30 mL) was added in a dropwise manner and stirred for 24 h at RT. Most of the DMF was removed in vacuo, and the reaction mixture was taken up in EtOAc (30 mL), washed with water (20 mL) and brine (20 mL), and dried. Solvent was removed in vacuo, and the crude was purified by flash chromatography, eluting with hexane/Et_2O (8:2) to provide 1.34 g of **8** as a white oil (65% yield).

^{1}H NMR ($CDCl_3$): δ 5.85 (m, 1H, 1^1-H), 5.25 (m, 1H, 3^1-H), 4.41 (m, 1H, 2^1-H), 4.15 (m, 3H, 6^1-H and 5^1-H), 4.05 (m, 1H, 4^1-H), 3.95 (m, 2H, 2-H), 2.85 + 2.72 (m, 2H, 6-H), 2.48 (m, 1H, 3-H), 2.00 + 1.70 (m, 2H, 4-H), 1.70 + 1.55 (m, 2H, 5-H), 1.40 (m, 21H, -Boc and ketals).

*Nipecotic Acid D-Glucos-3′-yl Ester (**4**)*—To a solution of (**8**; 1.34 g, 2.84 mmol) in CH_2Cl_2 (5 mL) was added TFA (5 mL), and the mixture was stirred at RT for 12 h. Evaporation of the solvent gave a residue that was taken up in water (20 mL) and neutralized with 10% aqueous NH_4OH, diluted with more water, and washed with $CHCl_3$ (3 × 15 mL). The aqueous layers were concentrated in vacuo, and the residue was submitted to cation-exchange chromatography, eluting with 10% pyridine in water to give 690 mg of **4** (84% yield) as a pale yellow solid (mp 135−136 °C).

^{1}H NMR (D_2O): δ (m, 1H, 1^1-H), 4.41 (m, 1H, 3^1-H), 3.65 (m, 1H, 2^1-H), 3.50 (m, 2H, 6^1-H), 3.21 (m, 1H, 5^1-H), 3.15 (m, 2H, 4^1-H), 3.15 + 3.03 (m, 2H, 2-H), 2.90 + 2.81 (m, 2H, 6-H), 2.42 (m, 1H, 3-H); 1.81 + 1.67 (m, 2H, 4-H), 1.51 (m, 2H, 5-H).

^{13}C NMR (D_2O + MeOD): δ 180.03 (−COOR), 97.17−93.30 (C-1^1), 77.96 (C-3^1), 77.06 (C-2^1), 75.44 (C-5^1), 70.85 (C-4^1), 61.87 (C-6^1), 48.53 (C-2), 46.78 (C-6), 39.44 (C-3), 26.60 (C-5), 22.01 (C-4).

*N-Boc-Nipecotic Acid (1′,2′:3′,4′-diisopropylidene)-α-D-galactopyranos-6′-yl Ester (**9**)*—To a stirred solution of *N*-Boc-nipecotic acid (**2**; 1 g, 4.36 mmol) in CH_2Cl_2/DMF (9:1, 30 mL) was added CDI (706 mg, 4.36 mmol), and the mixture was stirred for 1 h at RT. A solution of DIPG (1.36 g, 5.23 mmol) in CH_2Cl_2/DMF (1:5, 30 mL) was added in a dropwise manner and stirred for 24 h at RT. Most of the DMF was removed in vacuo, and the reaction mixture was taken up in EtOAc (30 mL), washed with water (20 mL) and brine (20 mL), and dried. Solvent was removed in vacuo, and the crude was purified by flash chromatography eluting with hexane/Et_2O (8:2) to provide 1.75 g of **9** (85% yield) as a white oil.

^{1}H NMR ($CDCl_3$): δ 5.50 (m, 1H, 1^1-H), 4.60 (m, 1H, 3^1-H), 4.32 (m, 1H, 2^1-H), 4.20 (m, 3H, 6^1-H and 5^1-H), 4.00 (m, 1H, 4^1-H), 2.95 (m, 2H, 2-H), 2.75 (m, 1H, 3-H), 2.75 + 2.50 (m, 2H, 6-H), 2.05 + 1.70 (m, 2H, 4-H), 1.55 + 1.40 (m, 2H, 5-H), 1.40 (m, 21H, Boc and ketals).

*Nipecotic Acid D-Galactos-6′-yl Ester (**5**)*—To a solution of **9** (1.75 g, 3.71 mmol) in CH_2Cl_2 (5 mL), TFA (5 mL) was added, and the mixture was stirred at RT for 2 h. Evaporation of the solvent gave a residue that was taken up in water (20 mL), neutralized with 10% aqueous NH_4OH, diluted with more water, and washed with $CHCl_3$ (3 × 15 mL). The aqueous layers were concentrated in vacuo, and the crude was submitted to cation-exchange chromatography, eluting with 10% pyridine in water to give 915 mg of **5** (84% yield) as pale yellow solid (mp 138−139 °C).

^{1}H NMR (D_2O): δ 4.95 (m, 1H, 1^1-H), 4.28 (m, 1H, 3^1-H), 3.60 (m, 1H, 2^1-H), 3.42 (m, 2H, 6^1-H), 3.35 (m, 1H, 5^1-H), 3.25 (m, 1H, 4^1-H), 3.10 + 2.95 (m, 2H, 2-H), 2.80 (m, 2H, 6-H), 2.33 (m, 1H, 3-H), 1.75 + 1.65 (m, 2H, 4-H), 1.45 (m, 2H, 5-H).

^{13}C NMR (D_2O + MeOD): δ 179.41 (−COOR), 96.72 (C-1^1), 75.15 (C-3^1), 72.04 (C-2^1), 70.40 (C-5^1), 68.82 (C-4^1), 60.97 (C-6^1), 46.01 (C-2), 43.99 (C-6), 40.76 (C-3), 25.73 (C-4), 21.01 (C-5).

*N-Boc-Nipecotic Acid N-Boc-L-Tyrosyl Ester (**10**)*—CDI (0.71 g, 4.37 mmol) was added to a solution of **2** (1 g, 4.37 mmol) in CH_2Cl_2/DMF (3:1, 50 mL), and the resulting mixture was stirred at RT. After disappearance of the starting materials (determined by thin-layer chromatography), *N*-Boc-L-tyrosine (1.11 g, 4.15 mmol) in CH_2Cl_2/DMF (3:1, 150 mL) was added in a dropwise manner to the mixture over 120 min, and stirring was then continued for 12 h. After evaporation of the solvent, the residue was taken up in EtOAc (60 mL) and washed with water (2 × 30 mL). The organic layer was dried on Na_2SO_4 and evaporated to give a residue (3 g) that was submitted to flash chromatography, eluting with $CHCl_3$/MeOH/AcOH (95:4.5:0.5) to afford the title compound (**10**) as a pale yellow oil (1.5 g, 71%).

^{1}H NMR ($CDCl_3$): δ 6.85 (d, 2H J = 7 Hz, 2^1-H and 6^1-H), 6.64 (d, 2H J = 7 Hz, 3^1-H and 5^1-H), 4.91 (br, 1H, −NH−Boc), 4.45 (m, 1H, 8^1-H), 4.15 + 3.80 (m, 2H, 2-H), 2.85 (m, 2H, 7^1-H), 2.84 + 2.75 (m, 2H, 6-H), 2.40 (m, 1H, 3-H), 2.00 + 1.43 (m, 2H, 4-H), 1.40 (m, 20H, 5-H and -Boc).

*Nipecotic Acid L-Tyrosyl Ester (**6**)*—To a solution of **10** (1.75 g, 3.71 mmol) in CH_2Cl_2 (5 mL), TFA (5 mL) was added, and the mixture was stirred at RT for 2 h. Evaporation of the solvent gave a residue that was taken up in water (20 mL), neutralized with 10% aqueous NH_4OH, diluted with more water, and washed with $CHCl_3$ (3 × 15 mL). The aqueous layers were concentrated in vacuo, and the crude was submitted to cation-exchange chromatography, eluting with 10% pyridine in water to give 760 mg of **6** (73% yield) as a white solid (mp 134−135 °C).

^{1}H NMR (DMSO): δ 7.30 (d, 2H J = 7 Hz, 2^1-H and 6^1-H), 7.20 (d, 2H J = 7 Hz, 3^1-H and 5^1-H), 4.00 (m, 1H, 7^1-H), 3.50 + 3.30 (m, 2H, 2-H), 3.19 (m, 2H, 6^1-H), 3.05 (m, 2H, 6-H), 2.95 + 1.82 (m, 2H, 4-H), 1.82 + 1.70 (m, 2H, 5-H).

^{13}C (DMSO): δ 173.50 (−COOH); 172.84 (−COO−), 156.35 (C-4^1), 134.64 (C-1^1), 131.75−131.63 (C-2^1 and C-6^1), 116.79 (C-3^1 and C-5^1), 55.87 (C-7^1), 44.96 (C-2), 44.84 (C-6), 39.20 (C-3), 36.31−36.06 (C-6^1), 25.52 (C-5), 21.73 (C-4).

Chemical Stability—Nipecotic ester solutions were prepared by dissolving an aliquot of **3**−**6** in pH 7.4 phosphate buffer to give a final concentration of $\approx 10^{-5}$ M. The solution was maintained at 37 °C, and aliquots were withdrawn every 2 h for the initial 12 h of incubation and successively every 12 h for 7 days. The disappearance of the nipecotic esters was followed by HPLC analysis using the method reported later. Pseudo-first-order rate constants for chemical hydrolysis were determined from slopes of linear plots obtained by reporting the logarithm of residual nipecotic ester against time. All experiments were carried out in triplicate.

Enzymatic Stability—Enzymatic hydrolysis of nipecotic esters (**3**−**6**) was determined using the procedure described in the literature.[18] Porcine liver esterase (obtained from Sigma) was diluted 10 times with phosphate buffer and used to hydrolyze nipecotic esters. Nipecotic ester solutions were prepared by dissolving an aliquot of **3**−**6** in phosphate buffer to give a final concentration of $\approx 10^{-5}$ M. The solution was maintained at 37 °C, and 325 μL of porcine esterase were added to achieve a concentration of 1.3 U/mL. Aliquots of 300 μL were withdrawn every hour for 16 h and combined with 600 μL of 0.01 N HCl in methanol. After centrifugation at 5000 × g for 5 min, an aliquot of supernatant was derivatized by the method reported later and

then monitored by HPLC. Pseudo-first-order rate constants for enzymatic hydrolysis were determined from the slopes of linear plots obtained by reporting the logarithm of residual nipecotic ester against time. All experiments were carried out in triplicate.

Analytical Procedures—For dansyl derivatization of nipecotic acid (**1**) and of **3**−**5**, 100 μL of sample or of stock standard solutions (prepared in pH 7.4 phosphate buffer) were combined with 9 μL of dansyl chloride (1.5 mg/mL in acetonitrile) and 100 μL of 80 mM Li_2CO_3, pH 9.5. The mixture was kept at room temperature for 2 h in darkness. The reaction was stopped by adding 10 μL of 2% methylamine hydrochloride solution. For derivatization of nipecotic tyrosine ester (**6**), 125 μL of sample or of stock standard solutions (prepared in pH 7.4 phosphate buffer) were combined with 55 μL of fluorescamine (2 mg/mL in acetone) and 125 μL of 120 mM phosphate buffer, pH 8.0; then, the mixture was mixed by vortex for a few minutes. An aliquot of derivatized drug solutions (20 μL) was analyzed by the chromatographic method described next.

HPLC analysis was performed with an ODS Hypersil column (particle size, 5 μm; 125 × 4 mm i.d.; Hewlett-Packard, D-76337 Waldbrom, Germany). The mobile phase consisted of sodium acetate buffer (100 mM)/acetonitrile (75:25). The flow rate was set at 1.0 mL/min. Each sample was filtered prior to injection with a Millex HV13 filter (Waters-Millipore Corporation, Milford, MA), and an aliquot (20 μL) was injected into the HPLC apparatus. Dansyl derivatives of nipecotic acid (**1**) and **3**−**5** were monitored at 330 nm (excitation wavelength) and 510 nm (emission wavelength), and the fluorescamine derivative of the nipecotic tyrosine ester (**6**) was monitored at 260 nm (excitation wavelength) and 470 nm (emission wavelength). The retention times of dansyl derivatives of **1** and **3**−**5** were 6.29, 6.7, 6.9, and 7.37 min, respectively. The retention time of the fluorescamine derivative of ester **6** was 4.65 min. The limit of sensitivity was <2 μM for detection of dansyl derivatives of **1** and **3**−**5** and <3 μM for detection of the fluorescamine derivative of the ester **6**.

Anticonvulsivant Activity—DBA/2 mice, purchased from Charles River (Calco, Como, Italy), were used to evaluate the anticonvulsant activity of **1** and **3**−**6**. The DBA strain of the house mouse, *Mus musculus*, inbred since 1909, has been known since 1947 to be susceptible to sound-induced seizures. Nearly 100% of the males and females of the DBA/2 strain undergo an age-dependent, often fatal, sequence of convulsions (a wild running phase, followed by clonic convulsions and tonic extensions, ending in respiratory arrest or full recovery) when initially exposed to a loud mixed-frequency sound (12−16 kHz; 90−120 dB) such as a door bell. The age of maximum susceptibility of the DBA/2 strain to the sound-induced seizures has been reported as 20−39 days, 21−28 days, or 16−26 days. Other strains of audiogenic mice often require acoustic priming in the neonatal period to attain a similar degree of seizure susceptibility.[19]

In our study, male DBA/2 mice (10 for each experimental group), 21−28 days old, were exposed to auditory stimulation (109 dB for 60 s or until tonic extension occurred), 30 min following injection of the drugs tested **1** and **3**−**6** or of a same volume of their vehicle. Compounds **1** and **3**−**5** were dissolved in isotonic phosphate buffer, pH 7.4; **6** was dissolved in 20% EtOH/H_2O (v/v). The injection volume (both for drug solutions and the vehicle alone) was 0.1 mL/10 g body weight.

The intensity of seizure response (SR) was assessed on the following scale: 0 = no response, 1 = wild running, 2 = clonus, 3 = tonus, and 4 = respiratory arrest. The maximum response was recorded for each animal. Rectal temperature was recorded immediately prior to auditory testing using an Elektrolaboratoriet thermometer type T.E.3. Behavioral changes (spontaneous activity, explorative behavior, tremor, spontaneous convulsions) were observed during the period between drug administration and auditory testing.

Statistical comparisons between controls and drug-treated groups were carried out with Fisher's exact probability test (incidence of seizure phases) and the Mann−Whitney U test. The percentage of incidence of each seizure phase was calculated for the different doses of the drugs administered. Fifty percent efficient doses (ED_{50}s) with 95% confidence limits (95% CL) of the nipecotic tyrosine ester (**6**) were estimated, concerning tonus and clonus phases, by the method of Litchfield and Wilcoxon.

Table 1—Chemical and Enzymatic Stability of Esters 3–6

drug	$t_{1/2}$ (h)[a]	
	pH 7.4 buffer	esterase (1.3 U/mL)
3	150.00	8.30
4	169.42	10.70
5	197.48	14.0
6	27.37	2.5

[a] $t_{1/2}$ was calculated from the equation: $t_{1/2} = (\ln 0.5)/K^1$, where K^1 is the pseudo-first-order rate constant.

Results and Discussion

Data concerning chemical and enzymatic stability of ester prodrugs **3**−**6** are reported in Table 1. Figure 1 and Figure 2 show the times courses of disappearance of nipecotic esters (**3**−**6**) in pH 7.4 buffer solution and in the presence of porcine esterase. When we evaluated their chemical stability, the compounds appeared stable in a pH 7.4 buffered solution; however, prodrugs **3**−**5** disappeared more slowly than the ester **6**.

To confirm that the tested prodrugs can be enzymatically hydrolyzed, we evaluated their stability in the presence of porcine esterase. The findings indicate that each of the four esters **3**−**6** is capable of being cleaved in vitro by esterase.

Half-life times concerning enzymatic stability were notably lower than those obtained in buffer solution. Moreover, we found a good correlation between chemical and enzymatic decomposition rates of the prodrugs examined.

Table 2 shows the results obtained in in vivo experiments with a genetically seizure-prone strain (DBA/2) of mice. DBA/2 mice are a useful model to test compounds acting on the GABAergic system;[20] furthermore, this model avoids the problem of possible effects of tested substances on the absorption or metabolism of the convulsant compound that arises with drug-induced seizures.[21,22] According to data reported by other authors, we confirmed that nipecotic acid (**1**; 0.75 mmol/kg), given systemically 30 min before sound exposure, possesses no anticonvulsant effect.[11,12] Also, **3**−**5**, intraperitoneally injected in the same experimental schedule at dose levels up to 0.75 mmol/kg, did not protect mice against all phases of audiogenic seizures. Conversely, when the ester prodrug **6** was administered systemically, a significant dose-dependent anticonvulsant activity was observed. In fact, a marked reduction in the incidence of each phase (wild running, clonus, tonus, respiratory arrest) of audiogenic seizures was evident at the dose levels studied (0.125, 0.17, 0.21, 0.25, 0.5, and 0.75 mmol/kg). Furthermore, no behavior alteration or change in rectal temperature in comparison with control animals were recorded after administration of **3**−**6** (data not shown).

The in vivo protective activity of systemically administered nipecotic tyrosine ester **6** against audiogenic seizures and the inefficiency of nipecotic acid (**1**) under the same experimental conditions suggest that this ester prodrug is transported intact across the BBB, beyond which it could be hydrolyzed. Tyrosine enters the brain through the large neutral amino acid transporter system. This system, present in the brain microvessel endothelial cells, has been extensively characterized and is of particular interest in the transport of amino acid drugs into the brain.[23,24] Moreover, Walker and co-workers[4] have recently demonstrated that a phosphonoformate−L-tyrosine conjugate possesses a high affinity for large amino acid transporters in monolayers of porcine brain microvessel endothelial cells and so might be a substrate for facilitated transport at the BBB. As to our findings, one tempting hypothesis is that the tyrosine moiety acts as a vector, which should allow

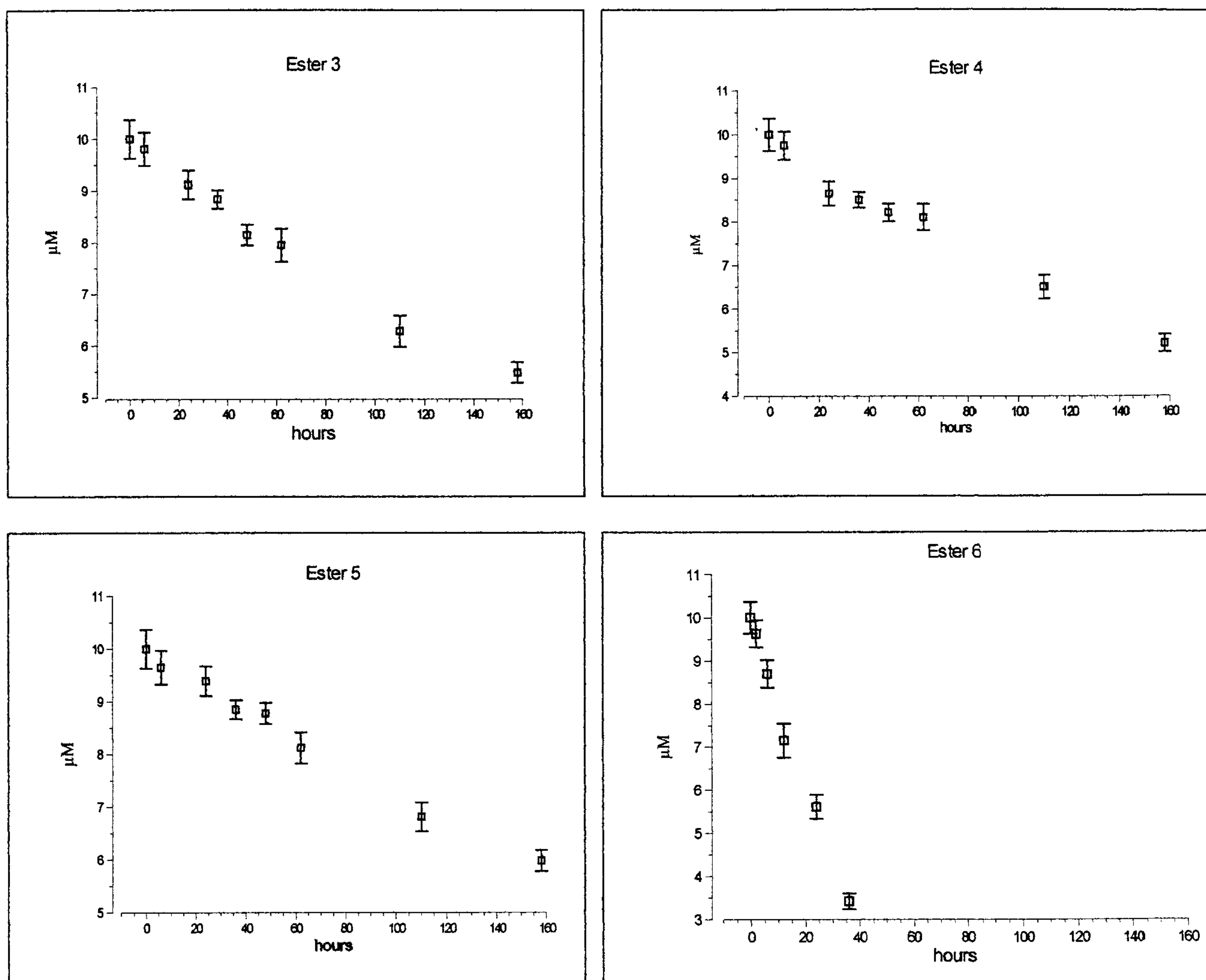

Figure 1—Time courses of disappearance of nipecotic esters (**3–6**) in pH 7.4 buffer solution obtained by plotting time (h) versus drug concentration (μM). Each point represents the mean ± SD of three experiments.

the transport of the nipecotic tyrosine ester (**6**) across the BBB; as the drug appears to achieve pharmacologically active concentrations in the brain, it could be supposed to be endowed with a high affinity for the amino acid carriers. Unfortunately, the almost absolute insolubility of nipecotic tyrosine ester (**6**) in water made it impossible to calculate its water/oil partition coefficient. Of course, further studies are needed to clarify if the ability of **6** to cross the BBB is dependent also on its lipophilicity. For example, Stark et al.[25] have demonstrated that des-tyrosine1-D-phenylalanine3-beta-casomorphin is able to cross the BBB by paracellular transport without using a carrier system. One could to point out that the nipecotic tyrosine ester (**6**) seems more potent than other lipophilic nipecotic acid esters, such as (+)ethyl nipecotate[12] or pivaloyloxymethyl ester;[11] however, its ED_{50}s calculated for tonus (ED_{50}, 0.130 mmol/kg; 95% CL, 0.115−0.146 mmol/kg) and clonus (ED_{50}, 0.173 mmol/kg, 95% CL, 0.162−0.186 mmol/kg) are very higher than those of tiagabine (a GABA uptake inhibitor, structurally related to nipecotic acid), as measured in the same experimental models[26] (tonus: ED_{50}, 1 μmol/kg; clonus: ED_{50}, 5 μmol/kg).

Furthermore, the present findings do not allow us to establish exactly whether the observed anticonvulsant activity of **6** could be due to nipecotic acid derived from its enzymatic hydrolysis or, partially at least, to the intact compound. However, several papers have provided information that the anticonvulsant effect and GABA uptake inhibition, induced by various esters of nipecotic acid, are mostly due to free nipecotic acid generated after hydrolysis in the brain.[27,28]

Similar results have also been reported for esters of isoguvacine, a GABA uptake inhibitor, structurally related to nipecotic acid.[29] Finally we can exclude that the observed anticonvulsant effect of **6** is due to the free promoiety (tyrosine) generated in the brain; in fact, tyrosine, systemically injected under the same experimental conditions at a dose level of 0.75 mmol/kg, elicited no protective effect against audiogenic seizures (data not shown).

Concerning prodrugs **3**−**5**, several possible explanations can be offered for their lack of anticonvulsant activity. First, β−D-glucose and galactose enter the brain through the transporter GLUT-1.[30] A high transporter affinity is required, so that a drug may be efficiently transported by the carrier across the BBB. Prodrugs **3**−**5** might have insufficient affinity for this carrier to achieve significant brain uptake and/or their transport through the BBB might be limited by competition with the endogenous substrate. In addition, the hydrophilic character of esters **3**−**5** would preclude their passive transport through the BBB.

Second, carbohydrate receptor-mediated targeting of drugs has been proposed as a potential method for site-specific delivery of drugs to cells possessing carbohydrate receptors on their surface. Several types of galactosylated drugs are reported to be interesting candidates for successful delivery to liver cells.[31] So, nipecotic derivatives

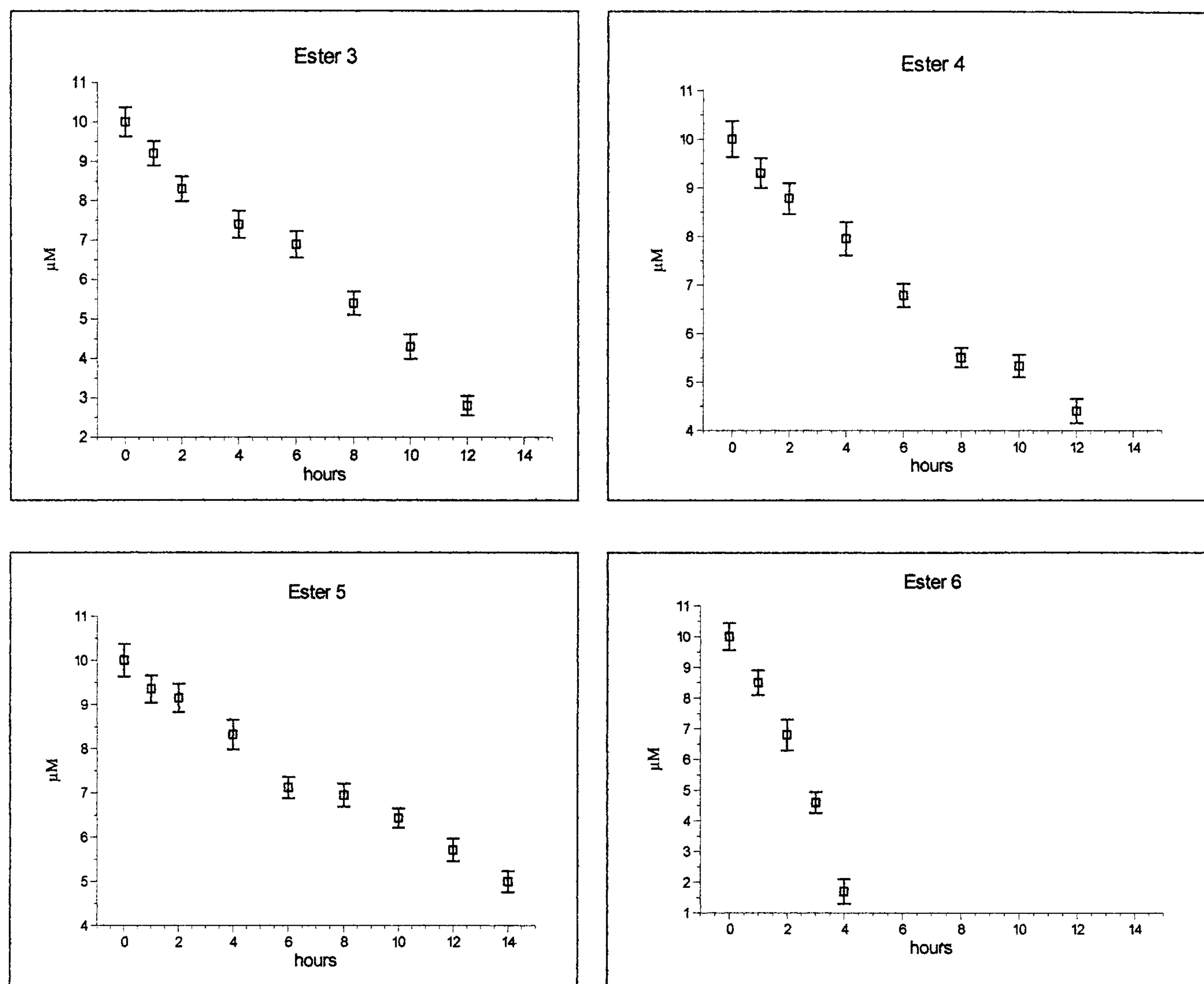

Figure 2—Time courses of disappearance of nipecotic esters (**3**–**6**) in the presence of porcine esterase obtained by plotting time (h) versus drug concentration (μM). Each point represents the mean ± SD of three experiments.

Table 2—Effects of Intraperitoneal Injection of 1, 3, 4, 5, and 6 on Audiogenic Seizures in DBA/2 Mice[a]

		% response					
drug	dose, mmol/kg	W.R.[b]	clonus	tonus	R.A.[b]	SR[b]	n[b]
controls A[c]	vehicle (0)	100	100	80	60	3.40	10
1	0.750	100	100	88.8	55.5	3.44	9
3	0.750	100	100	88.8	55.5	3.44	9
4	0.750	100	100	88.8	55.5	3.44	9
5	0.750	100	100	88.8	55.5	3.44	9
controls B[d]	vehicle (0)	100	100	87.5	50	3.37	8
6	0.125	75	75	50	25	2.25	8
6	0.170	60	50	40	10	1.6[e]	10
6	0.210	40	40	30	0	1.1[e]	10
6	0.250	20	20[d]	20[d]	0[d]	0.60[e]	10
6	0.500	20[d]	10[d]	0[d]	0[d]	0.30[e]	10
6	0.750	20[d]	20[d]	0[d]	0[d]	0.40[e]	10

[a] The incidence of each seizure phase is expressed as the percentage of mice in each group displaying that phase. [b] W.R. = wild running; R.A. = respiratory arrest; SR = seizure response expressed as arithmetic mean of the maximum individual response in each group; n = number of mice. [c] Mice given buffer, pH 7.4, 10 mL/kg, ip. [d] Mice given 20% EtOH/H_2O solution, 10 mL/kg, ip. [e] $p < 0.05$ from controls B, as assessed by Fisher's exact probability test. [f] $p < 0.05$ from controls B, as assessed by Mann–Whitney U test.

3–**5**, obtained by the conjugation with glucose and galactose, could be rapidly taken up and degraded by the liver after systemic injection. Therefore, these derivatives are hindered in the achievement of pharmacologically active concentrations in the brain.

Finally, a correlation between in vitro rates of enzymatic hydrolysis and its pharmacologic activity is generally expected for CNS-active prodrugs.[29] In fact, the rate of hydrolysis in plasma would have to be sufficiently slow to allow the prodrug to gain access to the brain, but not so slow as to remain inactive within the brain. Compounds **3**–**5** appeared much more resistant to enzyme-catalyzed hydrolysis ($t_{1/2}$ = 8.30, 10.70, and 14.0 h, respectively, for **3**, **4**, and **5**) than the prodrug **6** ($t_{1/2}$ = 2.5 h), but unlike **6** do not give protection against audiogenic seizures 30 min after administration. Moreover, no anticonvulsant effect was observed when audiogenic seizures were induced 1, 2, or 3 h after injection of **3**–**5** (data not shown). Further investigations would clarify if a significant pharmacological effect may be detectable at longer times after administration of the very slowly hydrolyzed esters **3**–**5**.

In conclusion, the present findings indicate that nipecotic tyrosine ester **6** is an excellent prodrug for potential application in the treatment of epilepsy and of other disorders resulting from abnormalities in the GABA system. Further studies are in progress to investigate the bioavailability of this compound and to clarify its mechanism of action.

References and Notes

1. Greig, N. H. Drug entry into the brain and its pharmacological manipulation. In: *Physiology and Pharmacology of the Blood-Brain Barrier*. Bradbury, M. W. B., Ed.; Springer-Verlag: New York, 1992; pp 487–524.
2. Robinson, P. J.; Rapoport, S. I. Transport of drugs. In: *Physiology and Pharmacology of the Blood-Brain Barrier*. Bradbury, M. W. B., Ed.; Springer-Verlag: New York, 1992; pp 279–300.
3. Greenwood, J. Experimental manipulation of the blood-brain and blood-retinal barriers. In: *Physiology and Pharmacology of the Blood-Brain Barrier*. Bradbury, M. W. B., Ed.; Springer-Verlag: New York, 1992; pp 459–486.
4. Walker, I.; Nicholls, D.; Irwin, W. J.; Freeman, S. Drug delivery via active transport at the blood-brain barrier: affinity of a prodrug of phosphonoformate for the large amino acid transporter. *Int. J. Pharm*. **1994**, *104*, 157–167.
5. Namane, A.; Gouyette, C.; Fillion, M.; Fillion, G.; Huynh-Dinh, T. Improved brain delivery of AZT using a glycosyl phosphotriester prodrug, *J. Med. Chem.* **1992**, *35*, 3039–3044.
6. Mizuma, T.; Ohta, K.; Hayashi, M.; Awazu, S. Intestinal active absorption of sugar-conjugated compounds by glucose transport system: implication of improvement of poorly adsorbable drugs, *Biochem. Pharmacol.* **1992**, *43*, 2037–2039.
7. Mizuma, T.; Ohta, K.; Hayashi, M.; Awazu, S. Comparative study of active absorption by the intestine and disposition of anomers of sugar-conjugated compounds. *Biochem. Pharmacol.* **1993**, *45*, 1520–1523.
8. Mizuma, T.; Ohta, K.; Awazu, S. The beta-anomeric and glucose preferences of glucose transport carrier for intestinal active absorption of monosaccharide conjugates. *Biochim. Biophys. Acta* **1994**, *1200*, 117–122.
9. Bonina, F. P.; Lanza, M.; Montenegro, L.; Salerno, L.; Smeriglio, P.; Trombetta, D.; Saija, A. Transport of alpha-tocopherol and its derivatives through erytrocyte membranes. *Pharm. Res*. **1996**, *13*, 1342–1346.
10. Krogsgaard-Larsen, P.; Johnston, G. A. R. Inhibition of GABA uptake in rat brain slices by nipecotic acid, various isoxazoles and related compounds. *J. Neurochem.* **1975**, *25*, 797–802.
11. Meldrum, B. S.; Croucher, M. J.; Krogsgaard-Larsen, P. GABA-uptake inhibitors as anticonvulsant agents. In: *Problems in GABA Research*; Okada, Y.; Roberts, E., Eds.; Exerpta Medica: Amsterdam, 1982; pp 182–191.
12. Horton, R. W.; Collins, J. F.; Anlezark, G. M.; Meldrum, B. S. Convulsant and anticonvulsant actions in DBA/2 mice of compounds blocking the reuptake of GABA. *Eur. J. Pharmacol.* **1979**, *59*, 75–83.
13. Hinko, C. N.; Seibert, K.; Crider, A. M. Anticonvulsant activity of the nipecotic acid ester, (±)-*m*-nitrophenyl-3-piperidinecarboxylate, *Neuropharmacology* **1984**, *23*, 1009–1014.
14. Hinko, C. N.; Crider, A. M.; Wood, J. D. A comparison of prodrug esters of nipecotic acid. *Neuropharmacology* **1988**, *27(5)*, 475–483.
15. Dhar, T. G.; Borden, L. A.; Tyagarajan, S.; Smith, K. E.; Branchek, T. A.; Weinshank, R. L.; Gluchowski, C. Design, synthesis and evaluation of substituted triaryl nipecptic acid derivatives as GABA uptake inhibitors: identification of a ligand with moderate affinity and selectivity for the cloned human Gaba transporter GAT.3. *J. Med. Chem.* **1994**, *37-(15)*, 2334–2342.
16. Chapman, A. G.; Croucher, M. J.; Meldrum, B. S. Evaluation of anticonvulsant drugs in DBA/2 mice with sound-induced seizures. *Arzneim.–Forsch./Drug Res*. **1984**, *34*, 1261–1264.
17. Seyfried, T. N.; Glaser, G. H. A review of mouse mutants as genetic models of epilepsy. *Epilepsia* **1985**, *26*, 143–150.
18. Bonina, F. P.; Montenegro, L.; Guerrera, F. Naproxen 1-alkylazacicloalkan-2-one esters as dermal prodrugs: in vitro evaluation. *Int. J. Pharm*. **1993**, *100*, 99–105.
19. Fischer, R. S. Animal models of the epilepsies. *Brain Res. Rev.* **1989**, *14*, 245–278.
20. Horton, R. W.; Prestwich, S. A.; Meldrum, B. S. Gamma-aminobutyric acid and benzodiazepine binding sites in audiogenic seizure-susceptible mice. *J. Neurochem*. **1982**, *39*, 864–870.
21. De Sarro, G. B.; Chimirri, A.; De Sarro, A.; Gitto, R.; Grasso, S.; Giusti, P.; Chapman. A. G. GYKI 52466 and related 2,3-benzodiazepines as anticonvulsant agents in DBA/2 mice. *Eur. J. Pharmacol*. **1995**, *294*, 411–422.
22. Suzdak, P. D.; Jansen, J. A. A review of the preclinical pharmacology of tiagabine: a potent and selective anticonvulsant GABA uptake inhibitor. *Epilepsia* **1995**, *36*, 612–626.
23. Smith, Q. R.; Momma, S.; Aoyagi, M.; Rapoport, S. I. Kinetics of neutral amino acid transport across the blood-brain barrier. *J. Neurochem.* **1987**, *49*, 1651–1658.
24. Reichel, A.; Begley, D. C.; Ermisch, A. Arginine vasopressin reduces the blood-brain transfer of L-tyrosine and L-valine: further evidence of the effect of the peptide on the L-system transporter at the blood-brain barrier. *Brain Res*. **1996**, *713*, 232–239.
25. Stark, H.; Van Bree, J. B.; de Boer, A. G.; Jaehde, U.; Breimer, D. D. In vitro penetration of des-tyrosine 1-D-phenylalanina-3-beta-casomorphin across the blood-brain barrier. *Peptides* **1992**, *13*, P47–51.
26. Dalby, N. O.; Nielsen, E. B. Comparison of the preclinical anticonvulsant profiles of tiagabine, lamotrigine, gabapentin and vigabatrin. *Epilepsy Res.* **1997**, *28*, 63–72.
27. Crider, A. M.; Tita, T. T.; Wood, J. D.; Hinko, C. N. Esters of nipecotic and isonipecotic acids as potential anticonvulsants. *J. Pharm. Sci*. **1982**, *71*, 1214–1219.
28. Nassereddine-Sebaei, M.; Crider, A. M.; Carrol, R. T.; Hinko, C. N. Determination of *m*-nitrophenol and nipecotic acid in mouse tissues by high-performance liquid chromatography after administration of the anticonvulsivant *m*-nitrophenyl-3-piperidinecarboxylate hydro-chloride. *J. Pharm. Sci*. **1993**, *82*, 39–43.
29. Falch, E.; Krogsgaard-Larsen, P. Esters of isoguvacine as potential prodrugs. *J. Med. Chem.* **1981**, *24*, 285–289.
30. Fuglsang, A.; Lomholt, M.; Gjedde, A. Blood-brain transfer of glucose and glucose analogues in newborn rats. *J. Neurochem.* **1986**, *46*, 1417–1428.
31. Takakura, Y.; Hashida, M. Macromolecular carrier systems for targeted drug delivery: pharmacokinetics considerations on biodistribution. *Pharm. Res*. **1996**, *13*, 820–831.

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