Synthesis, Brain Uptake, and Pharmacological Properties of a Glyceryl Lipid Containing GABA and the GABA-T Inhibitor γ-Vinyl-GABA

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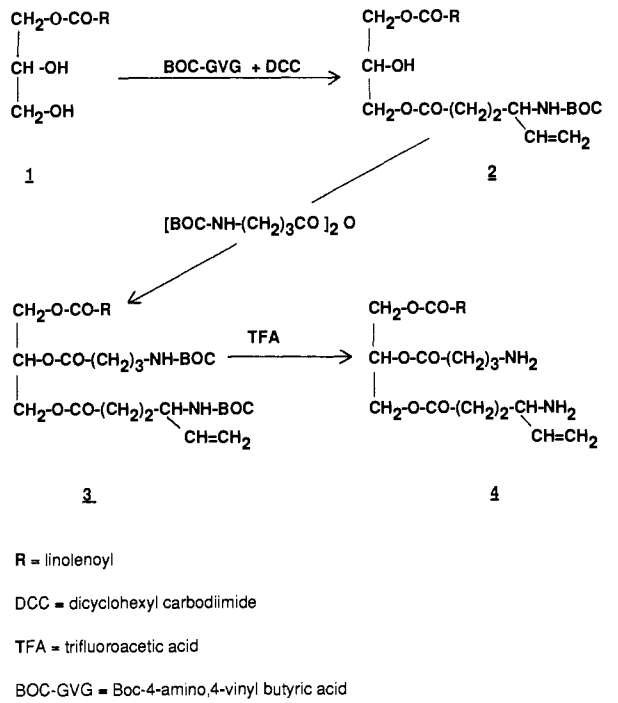
1-*O*-Linolenoyl-2-*O*-(4-aminobutyryl)-3-*O*-(4-vinyl-4-aminobutyryl)glycerol (LGV) was synthesized as an example of a prodrug which readily penetrates the blood–brain barrier (brain penetration index 97% ± 15%) and releases two active substances in the central nervous system (CNS): GABA (4-aminobutanoic acid) and the GABA transaminase inhibitor (GABA-T) of GABA breakdown. In vitro studies showed that the compound can inhibit GABA-T after hydrolysis by CNS esterases and that it enhanced GABAergic inhibition when applied to rat hippocampus slices. In vivo studies indicate that LGV depresses the spontaneous locomotor activity of mice. Its activity on a molar basis was some 300 times greater than that of γ-vinyl-GABA.

Several current hypotheses about the mechanisms of epilepsy suggest that enhancing the function of inhibitory GABA (4-aminobutanoic acid) neurons may be an important factor in controlling many seizures.[1,2] This might be accomplished by increasing the level of GABA available in the central nervous system (CNS), either by supplying GABA in a form which penetrates the blood-brain barrier[3-7] or by slowing the normal enzymatic degradation of the neurotransmitter by inhibiting the enzyme GABA-T.[8]

We have previously reported the synthesis of a series of steroid and glyceryl lipid esters which effectively transport GABA through the blood–brain barrier into the central nervous system.[3,6,7] These prodrug compounds have been shown to release GABA after enzymatic hydrolysis by CNS esterases and to have pharmacological and neurophysiological[9,10] effects consistent with GABAergic agents. Inhibiting the enzymatic hydrolysis of these GABA prodrugs reduces or eliminates their GABAergic neurophysiological effects.[9] These compounds appear to be prodrugs which move readily into the CNS but are inactive until the active moiety (GABA in this case) is released by enzymatic hydrolysis.

In this paper, we have extended this method of delivery compounds to the CNS to the synthesis and pharmacological properties of a triacyl glyceryl lipid which has the capacity to release both GABA and an inhibitor of its enzymatic breakdown. γ-vinyl-GABA (GVG), a specific irreversible inhibitor of the enzyme GABA-2 oxoglutarate:aminotransferase (GABA-T),[11] and GABA were linked to the 3- and 2-positions of linolenoylglycerol by ester bonds to give 4 (see Scheme I). The objective of this approach was to deliver both GABA and a GABA-T inhibitor to the same cell in equimolar quantities. Thus, following hydrolysis of the primary and secondary ester linkages, a GABAergic neuron could increase its GABA content as well as accumulate GVG to block local GABA breakdown. One possible advantage of such a multifunctional prodrug may be a capacity to have a rapid "activity onset" time (provided by the higher GABA levels) and a prolonged duration of activity as a result of GVG effects. Our studies of the in vivo pharmacological potency of the conjugated glyceride indicate that 4 was active at over a 300-fold lower molar concentration level than GVG, presumably due to its increased ability to penetrate the blood–brain barrier. In addition, electrophysiological data, using rat brain hippocampal slices as an in vitro test system, indicate that 4 has a more rapid "activity onset" time than GVG, a result consistent with the expected effects of an enhanced GABA release.

Scheme I



Chemistry

Scheme I illustrates the synthesis of the triglyceride (LGV) containing GABA and γ-vinyl-GABA. The amino groups of γ-vinyl-GABA and GABA were protected by

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Table I. Brain Penetration Index (BPI)[a] of LGV

compound	dose, mg/kg	dose, (μM/kg)	[brain], nmol/g of tissue	[liver], nmol/g of tissue	BPI	partition coefficient[b]
GABA[c]	3-39	(30-379)	0.04 - 0.17	4.17 - 65.05	1	0.004
LGV	33	(60)	8.34 ± 1.42	8.94 ± 1.06	97 ± 15	1.76

[a] BPI = ([brain]/[liver]) × 100 at 5 min after sc administration of ^{14}C-labeled test compound in mice (n = 3). [b] Octanol/water partition coefficient. [c] Data from ref 3.

conversion to their *tert*-butoxy (boc) derivatives. 1-*o*-Linolenoylglycerol[6] (1) was treated first with γ-vinyl-Boc-GABA in the presence of dicyclohexylcarbodiimide to obtain diglyceride 2. This was then reacted with Boc-GABA anhydride to obtain Boc-LGV (3). The final triglyceride (LGV, 4) was obtained by removing the amino protecting groups by treatment with either dilute HCl or trifluoroacetic acid. The lipid was used as the hydrochloride or as the free amine for brain-uptake studies. The final compound may have a small percent of γ-vinyl-GABA at the 2-position through ester exchange; we have not attempted to separate the two isomers since it was not critical in the present study.

Results and Discussion

Brain Uptake Studies. GABA is virtually excluded from entering the central nervous system. The brain penetration index (BPI) for GABA was found to be 1% regardless of the mode of administration and the dose.[3] In contrast, there is little or no blood–brain barrier to the relatively nonpolar lipid LGV. It had a BPI value of 97% ± 15%, indicating that its uptake into brain is at about equal to that in liver. This is consistent with the observed enhanced octanol/water partition coefficient for the compound as compared to GABA (see Table I).

Evidence for metabolism of the compound in the CNS was obtained by a thin-layer chromatographic (TLC) analysis of brain homogenates at 1 h after injection of labeled LGV with all its radioactivity present as ^{14}C in the GABA moiety of the compound. The results indicated that the majority (97%) of the radioactivity comigrated with acetic acid and was located in the high-R_f, non-ninhydrin-positive regin of the chromatogram; 2% corresponded to the region representing the original compound, and about 1% of the radioactivity corresponded to the region representing GABA on the TLC plate. This finding is consistent with the rapid metabolic transformation of GABA after it is released from the LGV molecule in the brain.

GABA-T Inhibition. When GVG was incubated with brain homogenate for 10 min prior to assaying for GABA-T activity, there was a concentration-dependent decrease in enzyme activity (Figure 1). When the same procedure was used with LGV there was little or no change in GABA-T activity. However, when LGV was subjected to hydrolysis prior to the enzyme assay, it inhibited GABA-T activity (Figure 2). The degree of GABA-T inhibition increased with increasing time of hydrolysis of LGV. Under the conditions of these assays, a hydrolysis time of about 12 min was sufficient to convert LGV to a product which had the same GABA-T inhibiting activity as GVG (Figure 2). Additional exposure to the esterase activity of brain homogenates beyond 12 min produced no increase in GABA-T-inhibiting capability. We infer from these results that LGV in its native state has no GABA-T-inhibiting activity, but upon hydrolysis by brain esterases its inhibitory effect is the same as that of GVG. This property would suggest that the compound is most likely acting as a prodrug in vivo.

Electrophysiological Studies. Investigations of the electrophysiological effects of GVG and LGV using rat

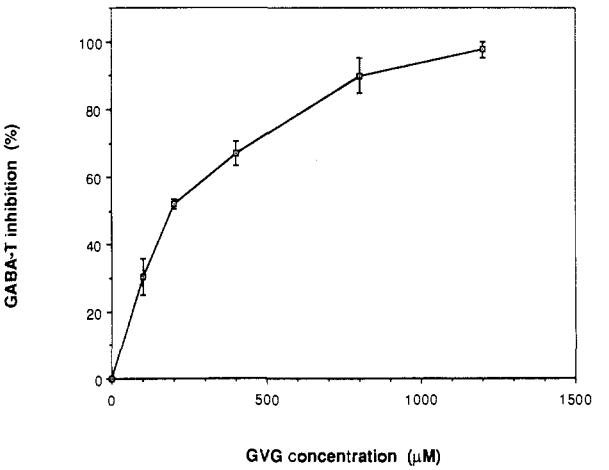

Figure 1. Inhibition of GABA-T by γ-vinyl-GABA in a brain homogenate preparation. The GABA-T assay preparation was incubated with various concentrations of GVG for 10 min at 21 °C prior to the GABA-T assay. Data were normalized to the results obtained from preparations with no GVG added.

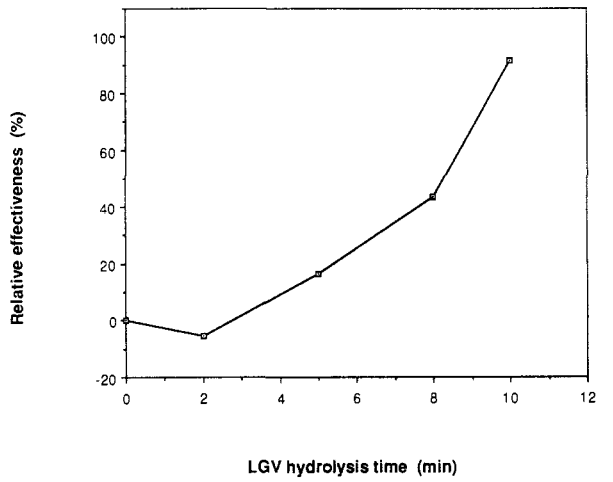

Figure 2. Inhibition of GABA-T by LGV in a brain homogenate preparation as a function of hydrolysis time. The results were normalized to the effect produced by an equal concentration (333 μM) of GVG in this assay (see Figure 1).

hippocampus slices as a model system show that both suppress the CA1 population spike as would be expected of compounds which enhance GABAergic neurotransmission.[9,10] The inhibitory effect was dose-dependent (Figure 3), and both compounds had essentially the same inhibitory effect. For LGV we estimate the concentration for a 50% reduction in the CA1 population spike to be about 800 μM. For both compounds the inhibitory effect was long-lasting (Figure 4). Recovery to base line required greater than 60 min for the doses used. The effect of both compounds was considerably different from that of GABA, which has a rapid onset and recovery.[9] In the same procedure, GABA produces a 50% reduction in the CA1 population spike at a concentration of about 3.6 mM; the maximum inhibitory effect occurs within a few seconds after the droplet is ejected from a micropipette, and re-

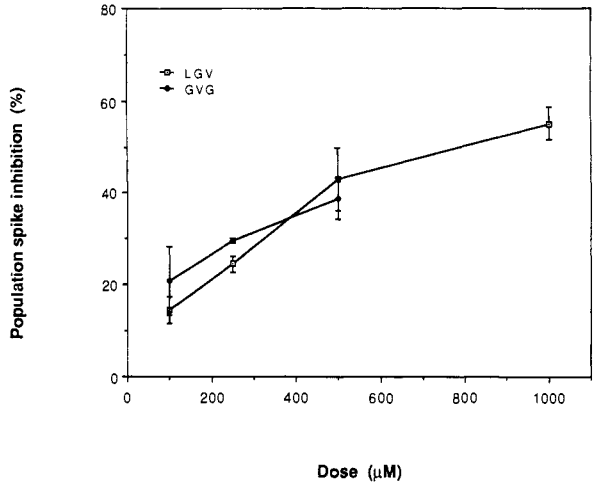

Figure 3. Inhibition of the stimulus-evoked CA1 population spike in hippocampal slices by LGV and GVG. Each point is the mean (±SEM) of the maximum inhibition observed in each of four to eight slices. The maximum inhibitory effect occurred at 4–8 min after application of a microdroplet of one of the compounds to the slice. The horizontal axis indicates the concentration of LGV or GVG in the micropipettes used to apply the drugs to the slices.

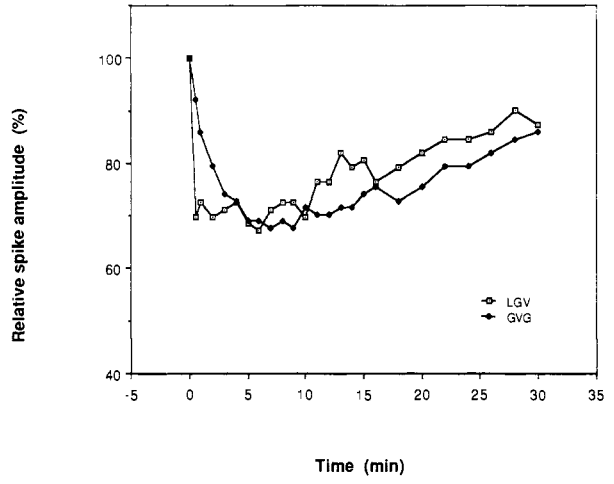

Figure 4. Time course of the inhibitory effect of LGV and GVG in hippocampal slices. The drugs were applied to the slices at "0" time by pressure ejection of a microdroplet (volume about 300 pL) from a glass micropipette. The drug concentration was 500 μM.

covery to base line is complete within 1–2 min.[9] In addition, comparison of the properties of LGV with those of GVG (see Figure 4) shows the LGV effect has a more rapid onset. This is consistent with the expected capacity of the compound to release GABA as well as GVG. Thus, released GABA is immediately active, while the GABA-T inhibitor GVG required about 5 min to produce its indirect effect via inhibition of GABA-T.

General Motor Activity. GVG injections significantly reduced the spontaneous locomotor activity of mice (Figure 5) at doses in the range that have anticonvulsant activity.[8] The dose required to produce a 50% reduction in general motor activity is estimated to be about 9000 μmol/kg. LGV also reduced general motor activity (Figure 5), but at molar doses of less than 0.3% of comparably effective GVG doses. The LGV dose for a 50% reduction in general motor activity is estimated to be about 25 μmol/kg.

Conclusion

LGV appears to act as a prodrug. It is as effective as GVG in inhibiting GABA-T in vitro, but only *after* enzymatic hydrolysis releases GVG. It shows activity comparable to that of GVG in the neurophysiological assay when applied directly to slices of rat brain hippocampus. In vivo it is some 300 times more potent than GVG on a molar-dose basis. It seems most probable then that the large difference between the in vivo activities of LGV and GVG is due to increased passage into the brain of LGV relative to GVG.

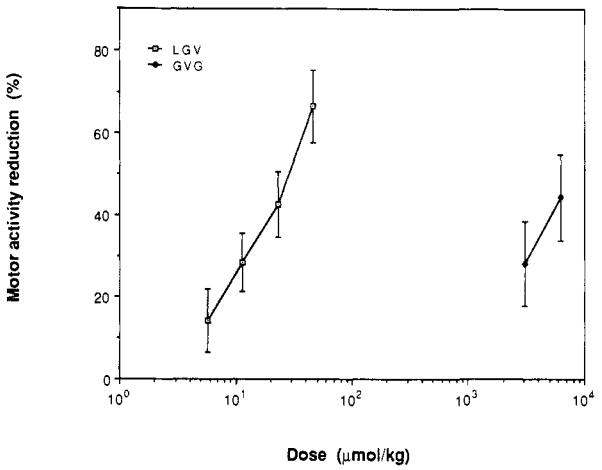

Figure 5. Effect of LGV and GVG on spontaneous locomotor activity of mice. Each point represents the mean (±SEM) for nine mice compared to nine vehicle-injected controls. All injections were intraperitoneal with a volume of 0.3 mL. Note the about 300-fold increase of LGV activity as compared to GVG.

Experimental Section

Chemistry. IR spectra were recorded on a Perkin-Elmer Infracord spectrophotometer and are reported in reciprocal centimeters. NMR spectra were recorded on a CFT 20 spectrometer. The NMR and IR spectra were characteristic for the compounds. Elemental analyses were performed by the Midwest Microlab Ltd., Indianapolis, IN. Thin-layer chromatography (TLC) was performed on 100-μM-thick precoated silica gel chromatogram sheets by Eastman. For column chromatography, silica gel (230–400 mesh) was used as the adsorbant.

1-*O*-Linolenoyl-3-*O*-[4-vinyl-4-(Boc-amino)butyryl]-*rac*-glycerol (2). A solution of monolinolenoylglycerol[3,6] (31 mg, 0.088 mmol), γ-vinyl-*N*-Boc-GABA (18 mg, 0.079 mmol), dicyclohexylcarbodiimide (18 mg), and 4-(dimethylamino)pyridine (3 mg) in 5 mL of methylene chloride was stirred overnight at room temperature under a nitrogen atmosphere. The reaction mixture was filtered and the filtrate was washed with 0.1 N HCl (ice-cold), 5% sodium bicarbonate solution, and brine. The solution was dried (Na_2SO_4) and concentrated to give 47 mg (0.087 mmol, 99% yield) of crude product, which was purified by column chromatography on silica gel, eluting with a mixture of ethyl acetate and petroleum ether. IR (neat): 3450, 1730, 1720, 1690, 1650, 1170 cm^{-1}.

1-*O*-Linolenoyl-2-*O*-[4-(Boc-amino)butyryl]-3-[4-vinyl-4-(Boc-amino)butyryl]-*rac*-glycerol (3). A solution of glycerol diester **2**, (89 mg, 0.166 mmol), *N*-Boc-GABA anhydride[3] (90 mg, 0.25 mmol), and 4-(dimethylamino)pyridine (22 mg, 0.18 mmol) in 20 mL of benzene was stirred overnight at room temperature. The mixture was extracted with 0.1 N HCl (ice-cold), 5% sodium bicarbonate, and brine. The solution was evaporated to dryness. The product was dissolved in ethyl acetate and fractionated by column chromatography on silica gel, eluting with a mixture of ethyl acetate and petroleum ether to yield 115 mg (0.159 mmol, 96% yield) of the compound. IR (neat): 3500, 2350, 1725, 1680, 1650 cm^{-1}. NMR ($CDCl_3$) δ: 5.0–5.7 (m, 10 H), 3.85–4.4 (m, 6 H), 3.15 (m, 1 H), 2.8 (m, 4 H, 2.35 (m, 6 H), 1.5–2.0 (m, 10 H), 1.55 (s, 7 H), 1.45 (s, 18 H), 1.32 (s, 9 H), 1.0 (t, J = 16 Hz, 3 H).

1-*O*-Linolenoyl-2-*O*-(4-aminobutyryl)-3-*O*-(4-vinyl-4-aminobutyryl)-*rac*-glycerol Dihydrochloride (LGV, 4). (a) A solution of di-Boc-amino derivative **3** (53 mg, 0.071 mmol) in 10 mL of methylene chloride was cooled to 0 °C under a nitrogen

atmosphere, and 0.5 mL of trifluoroacetic acid was added. The mixture was stirred for 5 h and was concentrated under vacuum. The residue was taken up in 10 mL of chloroform and was washed with 5% $NaHCO_3$ solution containing brine (2 × 5 mL). The washings were extracted with chloroform. The combined chloroform extracts were washed once with brine, dried over anhydrous Na_2SO_4, and concentrated to give 35 mg (0.063 mmol, 89% yield) of LGV. TLC: R_f 0.38 [chloroform/methanol/acetic acid (9:3:0.5)]. Compound 4 was also synthesized as a radioactive derivative by using the above methods on a microscale. The product had all of its label as ^{14}C in the GABA moiety of the molecule as U-^{14}C. It had the same TLC properties as the unlabeled product and a specific activity of 55 μCi/mmol.

(b) To a solution of the di-Boc-amino derivative (28 mg) in 4 mL of methylene chloride was added 0.3 mL of 4 N HCl in dioxane, and the mixture was stirred at room temperature for 3 h. The mixture was concentrated in vacuum and the residue was purified on a silica gel column eluting first with chloroform and then with 10% methanol in chloroform. The product was isolated from the $CHCl_3$/MeOH fractions, yielding 9 mg of the dihydrochloride. TLC: R_f 0.38 [$CHCl_3/CH_3OH/CH_3COOH$ (9:3:0.5)]. Anal. Calcd for $C_{31}H_{54}N_2O_6Cl_2 \cdot 3H_2O$: C, 55.86; H, 8.86; N, 4.20. Found: C, 55.45; H, 8.51; N, 4.66.

Measurements of BPI. The ^{14}C-labeled compounds LGV and LGV·2HCl were dissolved in 25% propylene glycol in water and injected subcutaneously (sc) into Balb-c mice (20 ± 2 g). The amount of radioactivity injected was 0.041–0.066 μCi. After 5 min the animals were sacrificed and the brain and liver were removed, weighed, and homogenized in 8 and 10 mL, respectively, of brain protein solvent[12] (BPS, phosphate buffer containing urea, sodium dodecyl sulfate, and EDTA at pH 7.6), and the radioactivity per gram wet weight of tissue was determined. The BPI was calculated as the ratio of the quantity present in brain to the amount in liver per gram of tissue.[3] A compound for which there is no blood–brain barrier would have a BPI of 100%. This procedure is particularly applicable to sparingly soluble lipid esters that tend to remain largely at the site of injection and slowly diffuse into the circulation.

Studies of the in Vivo Breakdown of Compound 4. ^{14}C-labeled LGV·2HCl (23 mg/kg sc; specific activity 0.088 mCi/mg) was administered to a mouse. After 1 h the animal was sacrificed and brain and liver were homogenized in 3 mL of pyridine. The homogenates were centrifuged, and the clear pyridine supernatant fractions were concentrated under a flow of nitrogen. The residues were taken in 1 mL each of ethanol, and the ethanol solution was centrifuged to produce clear supernatants which were analyzed in triplicate by TLC on silica gel on plastic sheets in chloroform/methanol/acetic acid (9:3:0.5). The plates were cut into segments and analyzed for ^{14}C. In the brain, 2% of the radioactivity was found to cochromatograph with the lipid LGV, and about 1% comigrated with GABA. In the liver, 6% of the label corresponded to the lipid LGV and 16% corresponded to GABA. Most of the remaining radioactivity (brain 78%, liver 46%) was present mostly in the high-R_f, non-ninhydrin-positive region of the chromatogram. Similar high-R_f labeled products were observed when ^{14}C-GABA was injected into mice.

Neurophysiological Studies. The neurophysiological effects of LGV were assessed with slices of rat hippocampus maintained in vitro.[9,10] Transverse slices of hippocampus (350–375 μm thick) from male Sprague–Dawley rats (ca. 250 g) were prepared and suspended on nylon nets over a 1.5-mL chamber filled with medium up to but not covering the upper surface of the slices. Medium flowed through the chamber at a rate of about 0.2 mL/min. The chamber temperature was maintained at 32–33 °C, and the atmosphere consisted of 95% O_2 with 5% CO_2. The incubation medium consisted of (in mM) NaCl, 125; KCl, 3.0; $CaCl_2$, 2.0; $MgSO_4$, 1.3; NaH_2PO_4, 1.0; $NaHCO_3$, 24; and glucose, 10.

Extracellular field potentials were recorded with NaCl-filled micropipettes, and stimulus pulses were applied through bipolar platinum wire electrodes. The inhibitory effectiveness of the compounds was investigated by measuring their ability to suppress the extracellular population spike[13] recorded from the hippocampal CA1 pyramidal layer and elicited by stimulation of stratum radiatum axons.[9,10] The compounds to be tested were dissolved in water, neutralized, diluted in fresh medium, and applied to the surface of the slice as microdroplets pressure ejected from a micropipette near the recording electrode. The volume of a microdroplet of a drug solution was about 200 pL.

General Motor Activity. The effect of LGV and GVG on spontaneous motor activity of mice was assessed with a Stoelting electronic activity monitor apparatus. Male albino mice (weight about 30 g) were acclimated to the apparatus by at least four 60-min sessions (1 per day) in the apparatus. Then general motor activity was monitored for 60 min following intraperitoneal injections of one of the compounds dissolved in saline with 25% propylene glycol. Control animals were injected with vehicle only. Nine mice were injected with each compound (or vehicle) at each dose level. The injection volume was 0.3 mL at all doses.

GABA-T Assay. A brain tissue homogenate was prepared for GABA-T assays from the brains of male mice. An animal was sacrificed by decapitation and the brain was quickly removed, rinsed and homogenized in approximately 8 volumes of a chilled buffer consisting of Triton X-100, 0.5% (v/v); dithiothreitol, 5 mM; pyridoxal phosphate, 1 mM; and sodium phosphate buffer, 10 mM, pH 7.0. This homogenate was frozen, thawed, and then centrifuged at 2000*g* for 20 min at 0 °C. The supernatant was removed and used for GABA-T determinations.

A second brain homogenate preparation was used to assay the effect of hydrolysis of LGV on its inhibition of GABA-T. Mouse brains were homogenized in 8 volumes of cold HEPES-buffered saline (0.005 M, pH 7.4) with 1 mM calcium acetate. This homogenate was centrifuged at 2000*g* for 20 min at 0 °C. The supernatant was removed and stored at −20 °C until used in LGV hydrolysis assays. This homogenate preparation had no measurable GABA-T activity.

GABA-T was assayed spectrophotometrically.[14,15] The assay buffer consisted of GABA, 20 mM; α-ketoglutarate, 10 mM; and NAD, 0.5 mM; in sodium phosphate buffer (0.05 M, pH 8.0). The GABA, α-ketoglutarate, and NAD solutions were neutralized before use. Aliquots of brain homogenate (200 μL) were mixed with assay buffer (800 μL) and incubated for 30 min at 21 °C, and then the formation of NADH was followed by measuring the optical density of the sample at 340 nm. In the absence of inhibitors the reaction mixture was stable for at least 90 min.

The effect of the GABA-T inhibitors GVG and LGV was evaluated by adding the inhibitor to the tissue homogenate in the appropriate concentration and incubating at 21 °C for 10 min and then assaying for GABA-T activity as described above. To determine the effect of enzymatic hydrolysis on the GABA-T inhibitory activity of LGV, the compound was added to the hydrolysis homogenate to a concentration of 1 mM. This mixture was incubated at 21 °C, at various times aliquots were removed, and the GABA-T inhibitory effect of the aliquot was determined.

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Registry No. 1, 75685-85-7; 2, 124099-16-7; 3, 124099-17-8; 4, 124099-18-9; 4·2HCl, 124099-20-3; BOC-GVG, 124099-19-0; [BOC-NH-$(CH_2)_3CO]_2O$, 89231-63-0.

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