3′-Amino-2′,3′-dideoxy-5-fluorouridine (6, 3′-NH_2-FUdR). 5-Fluorouracil (7.07 g, 54 mmol), 1 (4.1 g, 17 mmol), and KH_2PO_4 (3.5 mmol) were suspended in 393 mL of water. The pH of the suspension was adjusted to 6.5. Thymidine phosphorylase (30 000 units) was then added, and the suspension was maintained at 37 °C for 3 days. More enzyme (40 000 units) and water (200 mL) were then added. After 2 more days at 37 °C, the reaction mixture was filtered, and the volume of the filtrate was decreased to 150 mL in vacuo. The resulting suspension was filtered, and the solvent was removed from the filtrate. The residue was extracted with 35 mL of chloroform–methanol–2.5 N NH_4OH (4:5:1). After centrifugation of the suspension, the supernatant fluid was applied to a column of silica gel (5 × 15 cm), and the solvent mixture described above was used as the eluent. Solvent was removed from those fractions containing product. This provided 1.4 g (29% yield on the basis of 1 used): mp 119–121 °C; UV λ_{max} ($\epsilon \times 10^{-3}$) at pH 1, 268 (8.06) nm; at pH 13, 269 (6.01) nm; $[\alpha]^{20}_D$ 58.65° (*c* 1.0, DMF); ^{1}H NMR δ 8.32 (d, 1 H, *J* = 7.4 Hz, H_6), 6.03 (m, 1 H, $H_{1'}$), 3.62 (m, $H_{3'}$, $H_{4'}$, $H_{5'}$), 2.1 (m, 2 H, $H_{2'}$); ^{13}C NMR δ 157.2 (d, J_{CF} = 25.9 Hz, C_4), 149.0 (s, C_2), 139.8 (d, J_{CF} = 229.5 Hz, C_5), 124.8 (d, J_{CF} = 34.7 Hz, C_6), 87.6 (s, $C_{4'}$), 84.3 (s, $C_{1'}$), 60.1 (s, $C_{5'}$), 50.0 (s, $C_{3'}$),[24] 40.7 (s, $C_{2'}$). Anal. ($C_9H_{12}FN_3O_4 \cdot 2H_2O$) C, H, N.

3′-Azido-2′,3′-dideoxycytidine. 3′-Azido-2′,3′-dideoxyuridine (2.2 g, 8.7 mmol), formamide (0.693 mL, 17.4 mmol), hexamethyldisilazane (27.5 mL, 130.5 mmol), and ammonium sulfate (0.13 g, 1 mmol) were combined in a steel bomb and stirred in a 120 °C oil bath for 71 h. After cooling, the contents of the bomb were dissolved in methanol and refluxed for 6 h. After removal of the solvents in vacuo, the resulting foam was applied to a column of silica gel (2.5 × 23 cm). The column was washed with a mixture of ethyl acetate and methanol (85:15). Solvent was removed from the fractions containing the product and then applied to a second column of silica gel (5 × 18 cm). The gel was washed with a mixture of chloroform and methanol (85:15). The product was then eluted with a mixture having a higher proportion of methanol (3:2). After the solvent was removed, the residue was dissolved in methanol and 1 equiv of HCl in dioxane was added. Ethyl ether precipitated the product (1.29 g, 51% yield) as the HCl salt from the solution: mp 174.5–176.5 °C; UV λ_{max} ($\epsilon \times 10^{-3}$) at pH 1, 279 (13.4) nm; at pH 13, 271 (8.6) nm; NMR δ 8.22 (d, 1 H, $J_{5,6}$ = 7.9 Hz, H_6), 6.16 (d, 1 H, H_5), 6.0 (t, 1 H, $H_{1'}$), 4.32 (m, 1 H, $H_{3'}$), 3.92 (m, 1 H, $H_{4'}$), 3.64 (m, 2 H, $H_{5'}$). Anal. ($C_9H_{12}N_6O_3 \cdot HCl$) C, H, Cl, N.

3′-Amino-2′,3′-dideoxycytidine (7, 3′-NH_2-dCyd). 3′-Azido-2′,3′-dideoxycytidine hydrochloride (0.42 g, 1.45 mmol) was converted to the product by catalytic hydrogenation as described above for **2**. Purification was achieved by chromatography on a column of silica gel (1.25 × 15 cm) with chloroform–methanol–water (5:4:1) as the eluent. The yield was 0.28 g (65%) of a hygroscopic powder: mp 180 °C dec; UV λ_{max} at pH 1, 278 nm; at pH 13, 272 nm; NMR δ 7.82 (d, 1 H, $J_{5,6}$ = 7.5 Hz, H_6), 5.72 (d, 1 H, H_5), 7.1 (br s, 2 H, 4-NH_2), 6.15 (t, 1 H, $H_{1'}$), 3.5 (m, $H_{3'}$, $H_{4'}$, $H_{5'}$), 2.15 (m, 2 H, $H_{2'}$). Anal. ($C_9H_{15}N_4O_3 \cdot HCl \cdot 2H_2O$) C, H, Cl, N.

Acknowledgment. The authors acknowledge the contributions of J. Peck, R. Crouch, H. LeBlanc, E. H. Dark, L. A. Bridges, V. C. Knick, P. Collins, S. M. Tisdale, J. L. Rideout and G. B. Elion to this study.

Registry No. 1, 52450-18-7; **2**, 84472-86-6; **3**, 85236-89-1; **4**, 85236-93-7; **5**, 85236-94-8; **6**, 85236-95-9; **7**·HCl, 85236-97-1; 1-(3-chloro-2,3-dideoxy-β-D-*threo*-pentofuranosyl)-2,4(1*H*,3*H*)pyrimidinedione, 85236-87-9; 2′-deoxyuridine, 951-78-0; 2′-deoxy-5′-*O*-(triphenylmethyl)uridine, 14270-73-6; 1-[3-chloro-2,3-dideoxy-5-*O*-(triphenylmethyl)-β-D-*threo*-pentofuranosyl]-2,4-(1*H*,3*H*)pyrimidinedione, 85236-88-0; 3′-azido-2′,3′-dideoxyuridine, 84472-85-5; 2′-deoxy-5-iodouridine, 54-42-2; 2′-deoxy-5-iodo-5′-*O*-(triphenymethyl)uridine, 15414-61-6; 1-[3-chloro-2,3-dideoxy-5-*O*-(triphenymethyl)-β-D-*threo*-pentofuranosyl]-5-iodo-2,4-(1*H*,3*H*)-pyrimidinedione, 85236-90-4; 1-(3-chloro-2,3-dideoxy-β-D-*threo*-pentofuranosyl)-5-iodo-2,4(1*H*,3*H*)-pyrimidinedione, 85236-91-5; 3′-azido-2′,3′-dideoxy-5-iodouridine, 85236-92-6; 5-bromouracil, 51-20-7; thymidine phosphorylase, 9030-23-3; 5-chlorouracil, 1820-81-1; 5-fluorouracil, 51-21-8; 3′-azido-2′,3′-dideoxycytidine hydrochloride, 85236-96-0.

4,5,6,7-Tetrahydroisothiazolo[5,4-*c*]pyridin-3-ol and Related Analogues of THIP. Synthesis and Biological Activity

Povl Krogsgaard-Larsen,*[†] Hans Mikkelsen,[†] Poul Jacobsen,[†] Erik Falch,[†] David R. Curtis,[‡] Martin J. Peet,[‡] and John D. Leah[‡]

Department of Chemistry BC, Royal Danish School of Pharmacy, DK-2100 Copenhagen Ø, Denmark, and Department of Pharmacology, The Australian National University, Canberra City, A.C.T. 2601, Australia. *Received November 2, 1982*

The thio analogues of the GABA (γ-aminobutyric acid) agonist THIP (4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol), the GABA uptake inhibitor THPO (4,5,6,7-tetrahydroisoxazolo[4,5-*c*]pyridin-3-ol), and the glycine antagonist THAZ (5,6,7,8-tetrahydro-4*H*-isoxazolo[4,5-*d*]azepin-3-ol) have been synthesized and tested biologically on single neurons in the cat spinal cord and in vitro by using synaptic membrane preparations obtained from rat brains. In contrast to THIP, thio-THIP (4,5,6,7-tetrahydroisothiazolo[5,4-*c*]pyridin-3-ol, **5**) was only a weak GABA agonist. Thio-THPO (4,5,6,7-tetrahydroisothiazolo[4,5-*c*]pyridin-3-ol, **10**) was slighlty weaker than THPO as an inhibitor of GABA uptake in vitro, and these two compounds were approximately equipotent in enhancing the inhibition of the firing of cat spinal neurons by GABA. Like THAZ and structurally related bicyclic isoxazole zwitterions, thio-THAZ (5,6,7,8-tetrahydro-4*H*-isothiazolo[4,5-*d*]azepin-3-ol, **15**) was an antagonist at glycine receptors on cat spinal neurons. The *I*/*U* ratios, which reflect the ability of neutral amino acids to penetrate the blood–brain barrier (BBB), were calculated for **5** (*I*/*U* = 16), **10** (63), and **15** (200). These low *I*/*U* ratios, compared with the findings that THIP (*I*/*U* = 500 or 1500) and THPO (*I*/*U* = 2500) enter the brain after systemic administration, suggest that the thio analogues may penetrate the BBB very easily.

γ-Aminobutyric acid (GABA) is an inhibitory transmitter concerned with the control of neuronal activity in virtually all regions of the mammalian central nervous system,[1-3] and impaired transmission at such GABA-ergic synapses may be important in a number of neurological and psychiatric disorders.[4-6] As a consequence, com-

[†] Royal Danish School of Pharmacy.
[‡] The Australian National University.

(1) Curtis, D. R.; Johnston, G. A. R. *Ergeb. Physiol. Biol. Exp. Pharmakol.* **1974**, *69*, 97.
(2) Enna, S. J. *Biochem. Pharmacol.* **1981**, *30*, 907.
(3) DeFeudis, F. V. *Neurochem. Int.* **1981**, *3*, 113.

0022-2623/83/1826-0895$01.50/0

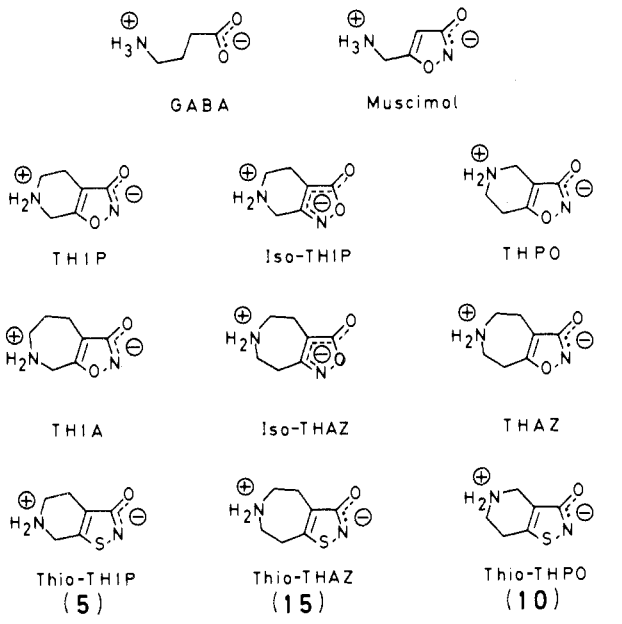


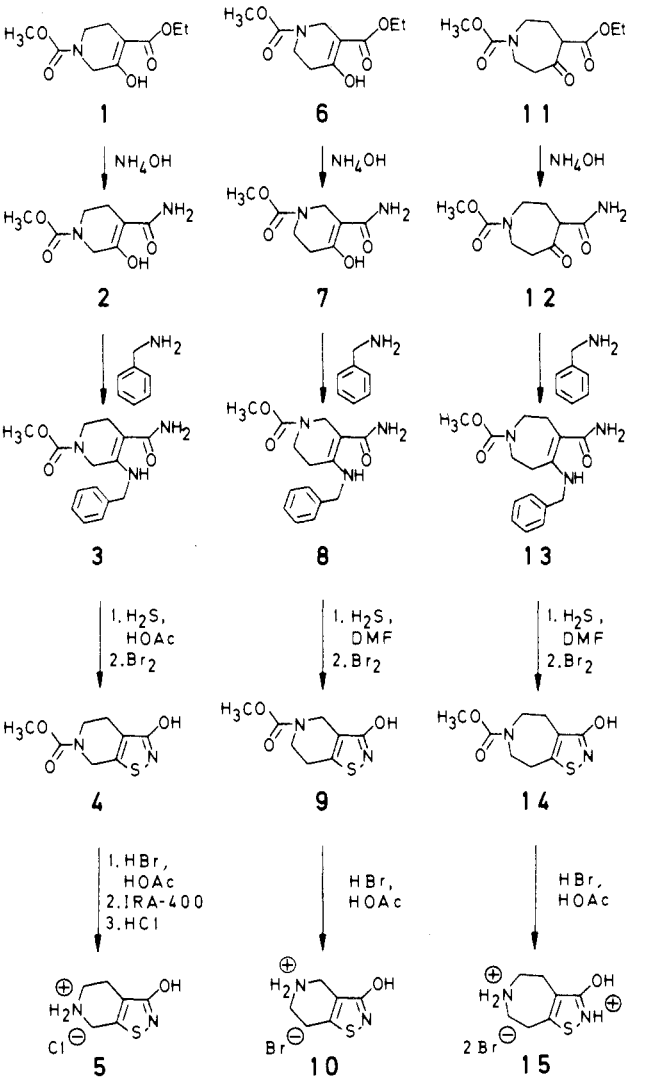
Figure 1. The structures of GABA and some heterocyclic GABA agonists, antagonists, and uptake inhibitors and glycine antagonists.

pounds that are GABA agonists, or inhibitors of the cellular uptake of this amino acid, are potentially important as therapeutic agents.[1,7]

Postsynaptic receptors for GABA,[8] and transport sites associated with GABA uptake,[9] have high degrees of agonist/antagonist and substrate specificities, as exemplified in Figure 1. Only zwitterionic compounds are "accepted" by these receptors and transport sites, and while THIP (4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol) is an agonist at GABA receptors,[10] the isomeric bicyclic muscimol analogue THPO (4,5,6,7-tetrahydroisoxazolo-[4,5-*c*]pyridin-3-ol) appears not to activate such receptors but to inhibit GABA uptake, possibly with a preferential effect on the uptake system in glia cells.[7,9] Whereas the actions of iso-THIP (4,5,6,7-tetrahydroisoxazolo[3,4-*c*]-pyridin-3-ol) and iso-THAZ (5,6,7,8-tetrahydro-4*H*-isoxazolo[3,4-*d*]azepin-3-ol) when directly injected into the brain have been interpreted in terms of antagonism of GABA,[11] iso-THAZ, THAZ (5,6,7,8-tetrahydro-4*H*-isoxazolo[4,5-*d*]azepin-3-ol), and THIA (5,6,7,8-tetrahydro-4*H*-isoxazolo[5,4-*c*]azepin-3-ol) appeared not to influence GABA receptors in the spinal cord but to antagonize the inhibitory effect of glycine.[12]

While THIP[13] and, to some extent, THPO[14] appear to enter the brain after systemic administration, iso-THAZ and THIA, which represent a new class of glycine antagonists,[12] apparently penetrate blood–brain barriers (BBB) poorly.[11] As an attempt to convert these heterocyclic zwitterions with effects on GABA or glycine receptors into analogues with improved pharmacokinetic properties, the thio analogues of THIP, THPO, and THAZ have been synthesized.

Scheme I



The ability of neutral amino acids to penetrate the BBB is largely determined by the ratio between the concentrations of the ionized and un-ionized forms of the compounds (I/U ratio), which is a function of the difference between the pK_a^I and pK_a^{II} values.[13,15] A small difference between the pK_a values is concomitant with a low I/U ratio[16] and with an increased ability to penetrate the BBB,[13,15] and the synthesis of the thio analogues of THIP, THPO, and THAZ has served the dual purpose of developing more lipophilic analogues with lower I/U ratios.

This paper describes the syntheses of thio-THIP (4,5,6,7-tetrahydroisothiazolo[5,4-*c*]pyridin-3-ol), thio-THPO (4,5,6,7-tetrahydroisothiazolo[4,5-*c*]pyridin-3-ol), and thio-THAZ (5,6,7,8-tetrahydro-4*H*-isothiazolo[4,5-

(4) Chase, T. N.; Wexler, N. S.; Barbeau, A., Eds. "Huntington's Disease"; Raven Press: New York, 1979.
(5) Morselli, P. L.; Lloyd, K. G.; Löscher, W.; Meldrum, B; Reynolds, E. H., Eds. "Neurotransmitters, Seizures, and Epilepsy"; Raven Press: New York, 1981.
(6) Van Kammen, D. P. *Am. J. Psychiatry* **1977**, *134*, 138.
(7) Krogsgaard-Larsen, P. *J. Med. Chem.* **1981**, *24*, 1377.
(8) Krogsgaard-Larsen, P.; Falch, E. *Mol. Cell. Biochem.* **1981**, *38*, 129.
(9) Krogsgaard-Larsen, P. *Mol. Cell. Biochem.* **1980**, *31*, 105.
(10) Krogsgaard-Larsen, P.; Johnston, G. A. R.; Lodge, D.; Curtis, D. R. *Nature (London)* **1977**, *268*, 53.
(11) Arnt, J.; Krogsgaard-Larsen, P. *Brain Res.* **1979**, *177*, 395.
(12) Krogsgaard-Larsen, P.; Hjeds, H.; Curtis, D. R.; Leah, J. D.; Peet, M. J. *J. Neurochem.* **1982**, *39*, 1319.
(13) Krogsgaard-Larsen, P.; Schultz, B.; Mikkelsen, H.; Aaes-Jørgensen, T.; Bøgesø, K. P. In "Amino Acid Neurotransmitters"; DeFeudis, F. V.; Mandel, P., Eds.; Raven Press: New York, 1981; p 69.
(14) Wood, J. D.; Schousboe, A.; Krogsgaard-Larsen, P. *Neuropharmacology* **1980**, *19*, 1149.
(15) Krogsgaard-Larsen, P.; Falch, E.; Mikkelsen, H.; Jacobsen, P. In "Optimization of Drug Delivery"; Bundgaard, H.; Hansen, A. B.; Kofod, H., Eds.; Munksgaard: Copenhagen, 1982; p 225.
(16) Edsall, J. T.; Wyman, J. "Biophysical Chemistry"; Academic Press: New York, 1958; Vol. 1, p 485.

Table I. pK_a Values, I/U Ratios, and in Vivo and in Vitro Effects of Some GABA Analogues[a]

compd	pK_a values	I/U ratios	IC_{50}, μM: effect in vitro on GABA binding	IC_{50}, μM: effect in vitro on GABA uptake	effects on cat spinal neurons: GABA agonism	GABA enhancement	glycine antagonism
GABA	4.0, 10.7	800 000	0.033[b]	2	---		
THIP	4.4, 8.5	500, 1500	0.13[b]	>500	---		
THPO	4.3, 9.1	2500	72	160		+	
THAZ	4.8, 9.2	4000	15[c]	>500	0		Δ
5	6.1, 8.5	16	42	220	-		
10	6.1, 9.2	63	>100	230		+	
15	2.6, 7.3, 9.9	200	31	400	0		Δ

[a] IC_{50} values for GABA receptor binding were determined by incubation of rat brain synaptic membranes for 5 min at 4 °C in 0.05 M Tris–citrate buffer (pH 7.1) containing 5 nM [^{3}H]GABA and the analogues at different concentrations (maximum 100 μM). The displacement of GABA from GABA binding sites was calculated and used to determine the IC_{50} values as earlier described.[23] The value at each concentration of analogue was the average of three experiments, and standard errors of the mean were less than 10%. The BMC-sensitive inhibitory effects of some of the compounds on cat spinal neurons are expressed relative to that of GABA (---), the number of symbols indicating less, equal, or greater activity. Zero indicates no significant activity. Glycine antagonists are indicated by the symbols Δ. [b] Reference 23. [c] Reference 11.

d]azepin-3-ol), isolated as the respective salts **5**, **10**, and **15**, and the calculation of their I/U ratios. The effects of these compounds were also studied on single cells in the cat spinal cord by using microelectrophoretic techniques. The affinities of **5**, **10**, and **15** for the GABA receptor sites and the GABA uptake system(s) have been determined in vitro by appropriate test systems.

Chemistry. The halides of thio-THIP (**5**), thio-THPO (**10**), and thio-THAZ (**15**) were synthesized as outlined in Scheme I. The appropriate β-oxo esters, of which **1**[17] and **6**,[18] in contrast to **11**,[19] are completely enolized, were converted into the corresponding β-oxo amides **2**, **7**, and **12** by using saturated aqueous solutions of ammonia. Attempts to improve the moderate yields of **7** and **12** (ca. 35%) by using various nonaqueous solvents were unsuccessful. ^{1}H NMR, IR, and UV data suggest that **2** and **7** are almost completely enolized. Stable β-enamino derivatives of **2**, **7**, and **12** could only be prepared by using benzylamine, probably due to stabilization of **3**, **8**, and **13** by *intra*molecular hydrogen bonds, in analogy with the findings for a series of β-*N*-benzylenamino esters.[20,21] The two-step conversions of **3**, **8**, and **13** into the 3-isothiazolols **4**, **9**, and **14** caused problems. In no case could the structures of the products from the reactions between **3**, **8**, and **13** and hydrogen sulfide be unequivocally determined. After column chromatographic treatments of the complex reaction mixtures, the major constituents were isolated as chromatographically homogeneous amorphous products having extremely complex ^{1}H NMR spectra. However, oxidation of these purified intermediates or the crude reaction mixtures with bromine gave **4**, **9**, and **14** in rather poor to moderate yields. While the use of glacial acetic acid as a solvent for the reaction between **3** and hydrogen sulfide was decisive for the preparation of **4**, detectable amounts of **9** and **14** were only formed with dimethylformamide as a solvent (Scheme I). Thio-THIP and thio-THPO were isolated as the monohalides **5** and **10**, the former compound via an ion-exchange purification step, whereas thio-THAZ formed the dihydrobromide **15**.

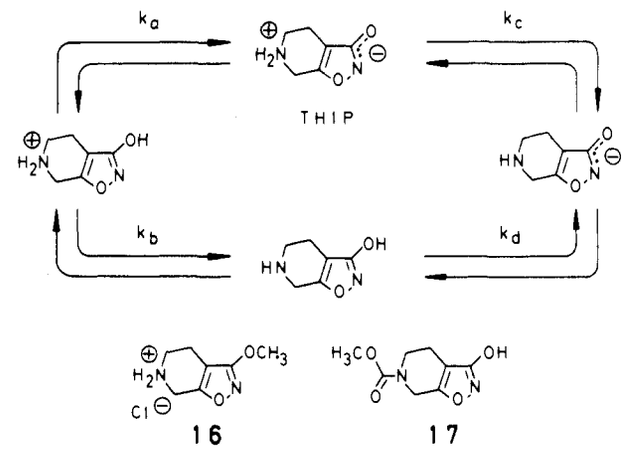


Figure 2. The ionization scheme for THIP and the structures of two derivatives (**16** and **17**) used for the estimation of the ionization constants (k_b and k_d, respectively) for THIP.

For all of the new compounds **2–5**, **7–10**, and **12–15**, the ^{1}H NMR, IR, and UV data were consistent with the proposed structures (Scheme I).

***I/U* Ratios.** The I/U ratio for neutral amino acids can be calculated in two ways, namely, as the ratios between the intrinsic constants k_a and k_b or between k_d and k_c[16] as exemplified for THIP in Figure 2. While approximate values of k_a and k_c can be obtained by titration of the amino acid, k_b and k_d have to be estimated indirectly via titration of derivatives, in which either the acid or the basic functions, respectively, have been blocked.[16] In the case of THIP (pK_a = 4.4, 8.5[17]), titrations of 3-methoxy-4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridinium chloride[22] (**16**) (pK_a = 7.1) and methyl 3-hydroxy-4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridine-6-carboxylate[17] (**17**) (pK_a = 5.3) gave approximate values for k_b and k_d, respectively. From these data, I/U ratios of 500 ($10^{-4.4}/10^{-7.1}$) and 1500 ($10^{-5.3}/10^{-8.5}$) were calculated for THIP. Titrations of THPO (pK_a = 4.3, 9.1[18]) and 3-methoxy-4,5,6,7-tetrahydroisoxazolo[4,5-*c*]pyridinium chloride[22] (pK_a = 7.7) gave an I/U ratio of 2500 for THPO. Titrations of THAZ (pK_a = 4.8, 9.2[18]) and of ethyl 3-hydroxy-5,6,7,8-tetrahydro-4*H*-isoxazolo[4,5-*d*]azepine-6-carboxylate[18] (pK_a = 5.6) gave an I/U ratio of 4000 for THAZ. In the cases of **5**, **10**, and **15**, the I/U ratios were calculated from the pK_a values of the compounds and of the respective N-protected derivatives **4**

(17) Krogsgaard-Larsen, P. *Acta Chem. Scand., Ser. B* **1977**, *B31*, 584.
(18) Krogsgaard-Larsen, P.; Hjeds, H. *Acta Chem. Scand., Ser. B* **1974**, *B28*, 533.
(19) Krogsgaard-Larsen, P.; Christiansen, T. R. *Eur. J. Med. Chem.* **1979**, *14*, 157.
(20) Krogsgaard-Larsen, P.; Christensen, S. B.; Hjeds, H. *Acta Chem. Scand.* **1973**, *27*, 2802.
(21) Krogsgaard-Larsen, P.; Hjeds, H.; Christensen, S. B.; Brehm, L. *Acta Chem. Scand.* **1973**, *27*, 3251.
(22) Krogsgaard-Larsen, P.; Johansen, J. S.; Falch, E. *J. Labelled Compd.* **1982**, *19*, 689.

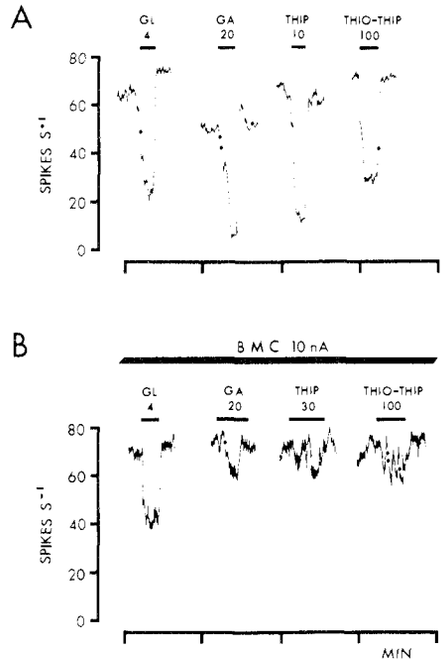


Figure 3. Comparison of the inhibitory effects of glycine (GL), GABA (GA), THIP, and thio-THIP (**5**) on the firing of a cat dorsal horn interneuron, maintained with continuously ejected DLH before (A: DLH, 15 nA) and during (B: DLH, 3 nA) the ejection of BMC (10 nA). The amino acids were ejected electrophoretically with the indicated currents (nanoamperes) from aqueous solutions (for details, see Experimental Section).

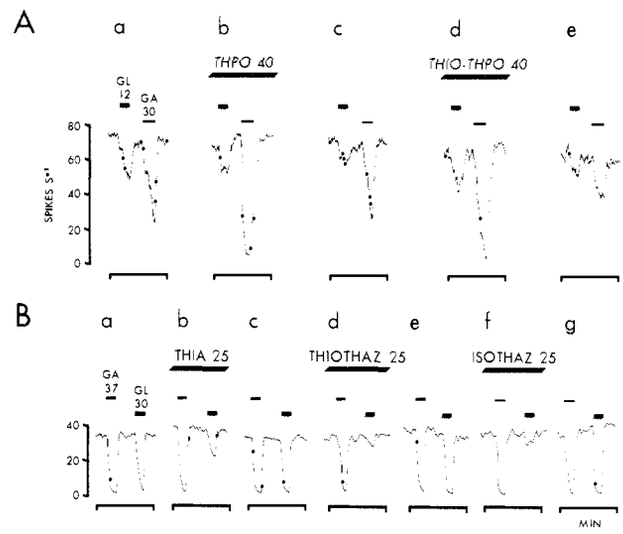


Figure 4. (A) The effects of THPO and thio-THPO (**10**) on the inhibition of the firing of a cat spinal Renshaw cell, maintained with continuously ejected DLH (6–8 nA), by glycine (GL) and GABA (GA), ejected for the periods and with the currents indicated by the horizontal lines: a, before; b, 2.5 min during the ejection of THPO; c, 2 min after THPO; d, 2.5 min during the ejection of **10**; e, 2 min after **10**. (B) The effects of THIA, thio-THAZ (**15**), and iso-THAZ on the inhibition of the spontaneous firing of a cat dorsal horn interneuron by glycine (GL) and GABA (GA): a, before; b, 2 min during THIA; c, 1 min after THIA; d, 1 min during **15**; e, 3 min after **15**; f, 1 min during iso-THAZ; g, 6 min after iso-THAZ (for details, see Experimental Section).

(pK_a = 7.3), **9** (pK_a = 7.4), and **14** (pK_a = 7.6) (Table I). Based on the pK_a values for GABA (4.0, 10.7) and for GABA methyl ester hydrochloride (9.9), an I/U ratio of 800 000 was calculated for GABA.

Effects on GABA Binding and GABA Uptake. The affinities were studied of **5**, **10**, and **15** for the GABA binding sites on purified synaptic membranes and for the GABA uptake mechanisms in a crude preparation of synaptosomes, both prepared from rat brains (Table I). Replacement of the heterocyclic oxygen atom of THIP by sulfur has pronounced consequences for the affinity for the GABA receptors sites, **5** being almost three orders of magnitude weaker than THIP. Futhermore, in contrast to THIP,[10,23] **5** proved to be an inhibitor of GABA uptake approximately equipotent with THPO and **10** (Table I). Like THPO,[24] **10** did not significantly affect the binding of GABA to the receptor sites. Both THAZ[11] and **15** were more than two orders of magnitude weaker than THIP as inhibitors of GABA binding. However, in contrast to THAZ,[24] **15** did affect GABA uptake in vitro, although weaker than THPO, **5**, or **10**.

Single-Cell Pharmacology. Microelectrophoretic techniques[25] were used to compare the effects on single neurons (Renshaw cells and interneurons) in the cat spinal cord of **5**, **10**, and **15** and structurally related bicyclic isoxazole zwitterions.

In agreement with the results of the in vitro receptor binding studies, **5** was much weaker than THIP as a depressant of neuronal firing (Figure 3). Like the effect of THIP,[10] the weak neuronal depression induced by **5** was reduced by the GABA antagonist bicuculline methochloride (BMC),[1,26] suggesting that both effects were mediated by GABA receptors. Glycine, which does not interact with the GABA receptors,[1,25] was used as a control substance. As shown in Figure 3, the inhibitory effect of glycine was much less sensitive to BMC than were the effects of GABA, THIP, and **5**.

In agreement with earlier findings for THPO,[27] simultaneous microelectrophoretic administration of GABA and **10** on cat spinal neurons enhanced the depressant effect of GABA, whereas glycine-induced depression of neuronal firing was not significantly modified (Figure 4A). Neither THPO nor **10** significantly reduced the firing of interneurons, and the GABA-enhancing effects of both, which were reversible on all cells studied, were probably the consequence of inhibition of the uptake of the microelectrophoretically administered GABA from the cellular environments. As expected from the similar potencies of THPO and **10** as GABA uptake inhibitors in vitro, these compounds has comparable GABA-enhancing effects in vivo. The direct GABA agonist activity of **5** prevented examination of a possible GABA-enhancing effect in vivo of this compound (Table I).

The effect of **15** on cat spinal neurons was compared with those of THIA, iso-THAZ (Figure 4B), and THAZ. All of these latter compounds are more than two orders of magnitude weaker than THIP as inhibitors of GABA binding,[11,24] and none showed detectable GABA agonist activity in vivo.[12,27] However, all three isoxazole zwitterions antagonized the depressant effect of glycine on cat spinal neurons,[12] and a similar effect was demonstrated for the structurally related 3-isothiazolol zwitterion **15** (Figure 4B). This reversible glycine antagonist activity of **15** was dem-

(23) Krogsgaard-Larsen, P.; Hjeds, H.; Curtis, D. R.; Lodge, D.; Johnston, G. A. R. *J. Neurochem.* **1979**, *32*, 1717.
(24) Krogsgaard-Larsen, P.; Johnston, G. A. R. *J. Neurochem.* **1978**, *30*, 1377.
(25) Curtis, D. R.; Duggan, A. W.; Felix, D.; Johnston, G. A. R. *Brain Res.* **1971**, *32*, 69.
(26) Johnston, G. A. R.; Beart, P. M.; Curtis, D. R.; Game, C. J. A.; McCulloch, R. M.; Maclachlan, R. M. *Nature (London), New Biol.* **1972**, *240*, 219.
(27) Krogsgaard-Larsen, P.; Johnston, G. A. R.; Curtis, D. R.; Game, C. J. A.; McCulloch, R. M. *J. Neurochem.* **1975**, *25*, 803.

onstrated on all (seven) neurons tested and shown to be comparable with that of iso-THAZ, the most potent compound within this class of glycine antagonists.[12] In general, the duration of action of iso-THAZ exceeded that of **15**.

Discussion

Replacement of the ring oxygen atom of THIP by sulfur has substantial effects on biological activity. While THIP is a potent and specific GABA agonist,[10,23] **5** has very low affinity for, and intrinsic activity at, the GABA receptors; in contrast to THIP, it also interacts with GABA uptake mechanisms in vitro (Table I). It has been demonstrated that the degree of delocalization of the negative charge of GABA agonists is a factor of critical importance for the interaction with the GABA receptors.[7,11,23] Although the degree of charge delocalization of the 3-isothiazolol anion of **5** (Figure 1) is not yet known, it is assumed to be somewhat different from that of the 3-isoxazolol anion of THIP. If so, this factor may explain the very weak effect of **5** on the GABA receptors.

The biological effects of **10**, on the other hand, are very similar to those of THPO.[27,28] Both compounds are virtually devoid of affinity for the GABA receptor sites, and the thio analogue is only slightly weaker than THPO, both as an inhibitor of GABA uptake in vitro and in enhancing the depressant effect of GABA on cat spinal neurons. The comparable effects of **5**, **10**, and **15** on GABA uptake in vitro is surprising in light of the very strict substrate/inhibitor specificity of the GABA transport carriers;[9] in the case of the corresponding 3-isoxazolols THIP, THPO, and THAZ, only THPO interferes significantly with GABA uptake (Table I).[24]

In agreement with the structural similarity between **15** and the glycine antagonists THAZ, THIA, and iso-THAZ,[12] **15** was a glycine antagonist in the cat spinal cord equipotent with iso-THAZ. The effects of all of these compounds on glycine receptors were reversible.

A major objective of synthesizing **5**, **10**, and **15** was the development of compounds active at GABA and glycine synapses and capable of penetrating the BBB very easily. The I/U ratio of neutral amino acids appears to correlate with their ability to penetrate the BBB, compounds with low I/U ratios having the highest ability to enter the brain after systemic administration.[7,13,15] The I/U ratios of these thio analogues are actually very low compared with those of aliphatic amino carboxylic acids (typically higher than 100 000)[7,16] and lower than those of the corresponding 3-isoxazolol zwitterions (Table I). In light of the very weak and nonspecific effects of **5**, behavioral studies of this compound in animals are not under consideration. In the case of the other two agents, such studies must await the preparation of these compounds on a larger scale.

Experimental Section

Chemistry. Melting points are corrected and were determined in capillary tubes. Elemental analyses were performed by P. Hansen, Chemical Laboratory II, University of Copenhagen. IR spectra, obtained on a Perkin-Elmer Grating infrared spectrophotometer, Model 247, were taken in KBr pellets. ^{1}H NMR spectra were recorded on a Varian T60 spectrometer or a JEOL FX 90 Q spectrometer with Me_4Si as an internal standard, except for the compounds dissolved in D_2O, where sodium 3-(trimethylsilyl)propanesulfonate was used as an internal standard. UV spectra were recorded with methanol as a solvent on a Perkin-Elmer ultraviolet–visible spectrophotometer, Model 402. Thin-layer chromatography (TLC) and gravity column chromatography (CC) were performed with silica gel F_{254} plates (Merck) and silica gel (Woelm, 0.063–0.200 mm), respectively. Compounds containing 3-isothiazolol were visualized on TLC plates with UV light and a $FeCl_3$ spraying reagent (purple color), and compounds containing amino groups were visualized with a ninhydrin spraying reagent (yellow color). pH values were measured at 25 °C on a Radiometer pH meter 26, and pK_a values were determined according to the method described by Albert and Serjeant.[29]

1-(Methoxycarbonyl)-3-hydroxy-1,2,5,6-tetrahydropyridine-4-carboxamide (2). A mixture of 1[17] (9.2 g, 40 mmol) and aqueous ammonia (115 mL, ρ 0.87) was stirred for 2 h at 0 °C and the solution was left at 25 °C for 3 days. The solution was evaporated in vacuo to give an oil. CC [silica gel, 400 g; eluent: ethyl acetate–methanol (9:1)] afforded TLC-pure **2** (5.0 g, 62%) [R_f 0.40 (ethyl acetate–methanol, 9:1)]. A sample was recrystallized (ethyl acetate) to give analytically pure **2**, mp 132.5–133.5 °C. Anal. ($C_8H_{12}N_2O_4$) C, H, N.

1-(Methoxycarbonyl)-3-(benzylamino)-1,2,5,6-tetrahydropyridine-4-carboxamide (3). A mixture of **2** (1.50 g, 7.5 mmol), benzylamine (900 mg, 8.4 mmol), toluene (90 mL), and molecular sieve (Union Carbide 3A, 3 g) was refluxed for 100 h with a Dean-Stark water separator. The rection mixture was diluted with dichloromethane and filtered, and the filtrate was evaporated in vacuo to give crude **3** (2.20 g). Recrystallization (ethyl acetate) gave **3** (1.50 g, 69%), mp 147.5–149.5 °C. Anal. ($C_{15}H_{19}N_3O_3$) C, H, N.

Methyl 3-Hydroxy-4,5,6,7-tetrahydroisothiazolo[5,4-*c*]pyridine-6-carboxylate (4). To a solution of **3** (21.5 g, 74 mmol) in glacial acetic acid (140 mL) was added, with stirring, excess dry hydrogen sulfide for 4 h at 80 °C. The reaction was followed by TLC. After 4 h, a spot with R_f 0.45 (**3**) has disappeared, and a spot with R_f 0.29 had maximal intensity [eluent: ethyl acetate–methanol (9:1); visualizer: iodine vapor, UV light]. The reaction mixture was evaporated in vacuo to give a treacly mass. CC [silica gel, 600 g; eluents: ethyl acetate to which methanol (10–18%) was added] afforded 10.9 g of a TLC-pure, amorphous substance (R_f 0.29). To a solution of 7.00 g of this product with unknown structure in ethyl acetate (40 mL) was added dropwise, at 25 °C and with stirring, a solution of bromine (8.30 mL, 155 mmol) in ethyl acetate (30 mL). The solution was left at 25 °C for 48 h and then evaporated in vacuo to give a treacly mass. CC [silica gel, 500 g; eluent: ethyl acetate–methanol–formic acid (90:10:1)] gave TLC-pure **4** (1.25 g, 12%, based on the starting compound **3**]. A sample was recrystallized (ethyl acetate) to give analytically pure **4**: mp 177.5–179.0 °C; IR 3700–3300 (m), 3100–2500 (several bands, w–m), 1695 (s), 1610 (m), 1480 (s), 1430 (s), 1400 (s) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 4.58 (2 H, s), 3.66 (3 H, s), 3.62 (2 H, t, J = 6 Hz), 2.49 (2 H, t, J = 6 Hz); UV 264 nm (log ϵ 3.82), 216 (3.61). Anal. ($C_8H_{10}N_2O_3S$) C, H, S; N: calcd, 13.07; found, 12.48.

4,5,6,7-Tetrahydroisothiazolo[5,4-*c*]pyridin-3-ol Hydrochloride (5). A solution of **4** (1.80 g, 8.4 mmol) in a solution of hydrogen bromide in glacial acetic acid (10 mL, 43%) was left at 25 °C for 20 h. Evaporation in vacuo gave 1.40 g of a crystalline product, which was dissolved in water (10 mL) and passed through a column containing an ion-exchange resin [Amberlite IRA-400 (OH), 130 mL] with acetic acid (1 M) as an eluent. The fractions containing material, which on TLC plates gave spots sensitive to $FeCl_3$ and ninhydrin spraying reagents, were acidified with hydrochloric acid (4 M), treated with a mixture of activated charcoal and diatomaceous earth, and filtered, and the filtrate was evaporated in vacuo to give TLC-pure **5** (817 mg, 50%). Recrystallization (water) of a small sample gave **5**: mp 230 °C (dec); IR 3700–3300 (m), 3000–2300 (several bands, s–m), 1620 (s), 1495 (s), (1430 (m) cm^{-1}; ^{1}H NMR (D_2O) δ 4.49 (2 H, q, J = 1.0 Hz), 3.57 (2 H, t, J = 6 Hz), 3.00–2.60 (2 H, m); UV 263 nm (log ϵ 3.68). Anal. ($C_6H_9N_2OSCl$) C, H, N; S: calcd, 16.64; found, 17.50; Cl: calcd, 18.40; found, 17.65.

1-(Methoxycarbonyl)-4-hydroxy-1,2,5,6-tetrahydropyridine-3-carboxamide (7) was synthesized as described above for **2** by using **6**[18] (10.0 g, 44 mmol) and aqueous ammonia (200 mL, ρ 0.87). Reaction for 5 days and CC of the crude product [silica gel, 300 g; eluent: ethyl acetate–methanol (9:1)] afforded

(28) Krogsgaard-Larsen, P.; Johnston, G. A. R. *J. Neurochem.* **1975**, *25*, 797.

(29) Albert, A.; Serjeant, E. P. "The Determination of Ionization Constants"; Chapman and Hall: London, 1979; p 9.

TLC-pure **7** (2.85 g, 33%) [R_f 0.43, ethyl acetate–methanol (9:1)]. Recrystallization (ethyl acetate) of a small sample gave analytically pure **7**, mp 169.5–170.5 °C. Anal. ($C_6H_{12}N_2O_4$) C, H, N.

1-(Methoxycarbonyl)-4-(benzylamino)-1,2,5,6-tetrahydropyridine-3-carboxamide (**8**) was synthesized as described above for **3** by using **7** (4.0 g, 20 mmol), benzylamine (2.35 g, 22 mmol), toluene (200 mL), and molecular sieve (Union Carbide, 3A, 3 g). Recrystallization (ethyl acetate) of crude **8** gave **8** (2.80 g, 49%), mp 157.0–160.0 °C. Anal. ($C_{15}H_{19}N_3O_3$) C, H, N.

Methyl 3-Hydroxy-4,5,6,7-tetrahydroisothiazolo[4,5-*c*]pyridine-6-carboxylate (**9**). A solution of **8** (1.5 g, 5.2 mmol) in dimethylformamide (DMF, 15 mL) was treated with excess dry hydrogen sulfide for 2 h at 80 °C as described above for **4**. Evaporation in vacuo gave a treacly mass (2.5 g), in which a component with R_f 0.27 was the main constituent [eluent: ethyl acetate–methanol (9:1); visualizer: iodine vapor, UV light]. To a solution of this crude product in ethyl acetate (5 mL) was added dropwise, at 25 °C and with stirring, a solution of bromine (1.32 mL, 24 mmol) in ethyl acetate (5 mL). The solution was left at 25 °C for 24 h and then evaporated in vacuo to give a treacly mass. CC [silica gel, 200 g; eluent: ethyl acetate–methanol–formic acid (95:5:1)] gave crude crystalline **9** (1.34 g). Recrystallization (ethyl acetate) afforded **9** (278 mg, 25%, based on the starting compound **8**): mp 190.0–192.0 °C; IR 3700–3300 (w), 3100–2500 (several bands, m–w), 1695 (s), 1600 (m), 1485 (s), 1445 (s), 1400 (s) cm^{-1}; ^1H NMR (Me_2SO-d_6) δ 5.54 (1 H, s), 4.30 (2 H, s), 3.71 (3 H, s), 3.67 (2 H, t, J = 5 Hz), 2.86 (2 H, t, J = 5 Hz); UV 263 nm (log ϵ 3.79), 214 (3.57). Anal. ($C_8H_{10}N_2O_3S$) C, H, N, S.

4,5,6,7-Tetrahydroisothiazolo[4,5-*c*]pyridin-3-ol Hydrobromide (**10**). A solution of **9** (1.30 g) in a solution of hydrogen bromide in glacial acetic acid (40 mL, 43%) was left at 25 °C for 20 h. The solution was evaporated in vacuo, and curde **10** recrystallized (methanol) to give **10** (351 mg, 24%): mp 240 °C (dec); IR 3600–3300 (m), 3100–2300 (several bands, s–m) 1630 (s), 1590 (m) cm^{-1}; ^1H NMR (Me_2SO-d_6) δ 7.49 (3 H, s), 4.10 (2 H, s), 3.60–3.40 (2 H, m), 3.30–3.15 (2 H, m); UV 263 nm (log ϵ 3.68). Anal. ($C_6H_9N_2OSBr$) C, H, N, S, Br.

1-(Methoxycarbonyl)-4-oxoperhydroazepine-5-carboxamide (**12**) was synthesized as described above for **2** with **11**[19] (9.5 g, 39 mmol) and aqueous ammonia (100 mL, ρ 0.87). After reaction for 3 days the solution was evaporated in vacuo to give crude **12** (7.4 g). CC [silica gel, 300 g; eluent: ethyl acetate–methanol (9:1)] afforded TLC-pure **12** (3.0 g, 36%), [R_f 0.28; eluent: ethyl acetate–methanol (9:1)]. Recrystallization (ethyl acetate) of a small sample gave analytically pure **12**, mp 130.5–132.0 °C. Anal. ($C_9H_{14}N_2O_4$) C, H, N.

1-(Methoxycarbonyl)-4-(benzylamino)-2,3,6,7-tetrahydro-1*H*-azepine-5-carboxamide (**13**) was synthesized as described above for **3** by using **12** (3.0 g, 14 mmol), benzylamine (1.88 g, 17.5 mmol), toluene (200 mL), and molecular sieve (Union Carbide, 3A, 3 g). Recrystallization (ethyl acetate) of crude **13** (4.8 g) gave **13** (2.12 g, 50%), mp 141.0–143.0 °C. Anal. ($C_{16}H_{21}N_3O_3$) C, H, N.

Methyl 3-Hydroxy-4,5,7,8-tetrahydro-6*H*-isothiazolo[4,5-*d*]azepine-6-carboxylate (**14**). A solution of **13** (2.58 g) in DMF (20 mL) was treated with excess dry hydrogen sulfide for 2 h at 80 °C as described above for **9**. The reaction mixture was evaporated in vacuo to give 3.25 g of treacly mass, in which a component with R_f 0.26 was the main constituent [eluent: ethyl acetate–methanol (9:1); visualizer: iodine vapor, UV light]. To a solution of this product in ethyl acetate (10 mL) was added dropwise a solution of bromine (1.78 mL, 32 mmol) in ethyl acetate (5 mL). The solution was left at 25 °C for 18 h and then evaporated in vacuo to give a treacly mass. CC [silica gel, 150 g; eluent: ethyl acetate–methanol–formic acid (95:5:1)] afforded crude **14** (2.02 g). Recrystallization (carbon tetrachloride) gave **14** (540 mg, 28%, based on the starting compound **13**): mp 119.0–121.0 °C; IR 3600–3350 (m), 3100–2500 (several bands, m), 1695 (s), 1620 (m), 1590 (m), 1480 (s), 1440 (s), 1410 (s) cm^{-1}; ^1H NMR (CCl_4) δ 12.04 (1 H, s), 3.74 (s) and 3.70–3.55 (m) (a total of 7 H), 3.00–2.65 (m, a total of 4 H); UV 266 nm (log ϵ 3.71). Anal. ($C_9H_{12}N_2O_3S$) C, H, N, S.

5,6,7,8-Tetrahydro-4*H*-isothiazolo[4,5-*d*]azepin-3-ol Dihydrobromide (**15**). A solution of **14** (590 mg) in a solution of hydrogen bromide in glacial acetic acid (15 mL, 43%) was left at 25 °C for 22 h. Evaporation in vacuo and recrystallization (methanol) of crude **15** gave **15** (310 mg, 36%): mp 215–225 °C (dec); IR 3600–3300 (m), 3100–2300 (several bands, s–m), 1580 (s), 1540 (m), 1455 (m) cm^{-1}; ^1H NMR (D_2O) δ 3.50–3.27 (4 H, m), 3.22–3.10 (2 H, m), 2.84–2.76 (2 H, m); UV 264 nm (log ϵ 3.52). Anal. ($C_7H_{12}N_2OSBr_2$) H, N, S; C: calcd, 25.32; found, 24.78; Br: calcd, 48.13; found, 46.48.

Microelectrophoretic Studies. Experiments were performed on lumbar dorsal horn interneurons and Renshaw cells of six cats anesthetized with pentobarbitone sodium (35 mg/kg intraperitoneally initially, supplemented intravenously when required). Extracellular action potentials were recorded by means of the center barrel of seven-barrel micropipets, which contained 3.6 M NaCl. The compounds were administered electrophoretically from the outer barrels of the micropipets,[25] which contained aqueous solutions: glycine, 0.5 M, pH 3; GABA, 0.2 M, pH 3; THIP, 0.2 M, pH 3.5; **5**, 0.2 M, pH 3.2; THPO, 0.2 M, pH 3; **10**, 0.2 M, pH 3.3; THIA, 0.2 M, pH 3; iso-THAZ, 0.2 M, pH 3.4; **15**, 0.15 M, pH 3; BMC, 0.01 M in 0.15 M NaCl. As in previous studies of this type,[10,12,23,25,27] relative potencies of agonists and antagonists were estimated from the ratios of equieffective electrophoretic currents. Cell firing rates were maintained (30–60 Hz) by continuous ejection of DL-homocysteic acid (DLH, 0.2 M, pH 7.5), and the inhibitory amino acids were administered for fixed periods of regular intervals to produce submaximal inhibition of firing. Antagonism was apparent from a slower onset and reduced degree of inhibition.

GABA Receptor Binding. An earlier method for the preparation of synaptic membranes from the cerebral cortices of adult rats[30] was modified as described previously.[23] The membrane preparation I was frozen rapidly at −70 °C and kept at −20 °C for at least 18 h before use in the receptor-binding assay. For the [^3H]GABA-binding assay procedures, aliquots of synaptic membranes (0.8–1.2 mg of protein) were incubated in triplicate at 4 °C for 5 min in 2 mL of 0.05 M Tris–citrate buffer (pH 7.1) containing 0.005 μM [^3H]GABA, and the IC_{50} values of the agents tested were determined as described elsewhere.[23]

GABA Uptake. The rat brain synaptosomes were prepared as described in detail elsewhere.[31] In the GABA uptake procedure, 500 μL of the synaptosome suspension was preincubated for 10 min at 25 °C with 1.9 mL of phosphate medium containing the inhibitor. Then [^3H]GABA was added to give a final GABA concentration of 0.05 μM, and the incubation was continued for a further 10 min. The synaptosomes were isolated by rapid filtration through Whatman GF/C glass-fiber filters, and the filters were washed with phosphate medium (10 mL). The filters were transferred to scintillation vials, and the radioactivity was measured by liquid scintillation counting after addition of Liposolve/Lipolume/water (1:10:0.2, 3 mL) (Lumac, Basel). The IC_{50} values for inhibition of high-affinity neuronal (synaptosomal) GABA uptake at 0.05 μM GABA with preincubation of the tissue for 10 min in the presence of inhibitor were determined as described elsewhere in detail.[32]

Acknowledgment. This work was supported by grants from the Danish Medical Research Council and The Australian National University. The technical assistance of Mrs. P. Searle and the secretarial assistance of Mrs. B. Hare are gratefully acknowledged.

Registry No. 1, 85250-57-3; **2**, 85250-58-4; **3**, 85250-59-5; **4**, 85250-60-8; **5**, 85250-61-9; **6**, 68710-81-6; **7**, 85250-62-0; **8**, 85250-63-1; **9**, 85250-64-2; **10**, 85250-65-3; 11, 71233-14-2; **12**, 85250-66-4; **13**, 85250-67-5; **14**, 85250-68-6; **15**, 85250-69-7; GABA, 56-12-2; glycine, 56-40-6; benzylamine, 100-46-9.

(30) Enna, S. J.; Snyder, S. H. *Brain Res.* **1975**, *100*, 81.
(31) Fjalland, B. *Acta Pharmacol. Toxicol.* **1978**, *42*, 73.
(32) Beart, P. M.; Johnston, G. A. R.; Uhr, M. L. *J. Neurochem.* **1972**, *19*, 1855.