GABA Agonists. Resolution, Absolute Stereochemistry, and Enantioselectivity of (*S*)-(+)- and (*R*)-(−)-Dihydromuscimol

Povl Krogsgaard-Larsen,*† Lone Nielsen,† Erik Falch,† and David R. Curtis‡

Department of Chemistry BC, The Royal Danish School of Pharmacy, DK-2100 Copenhagen, Denmark, and Department of Pharmacology, The Australian National University, Canberra, ACT 2601, Australia. *Received March 15, 1985*

(*RS*)-5-(Aminomethyl)-2-isoxazolin-3-ol (dihydromuscimol, DHM) is a potent 4-aminobutyric acid (GABA) agonist, the inhibitory effects of which on neurons are sensitive to the antagonist bicuculline methochloride (BMC), and it also interacts with the GABA uptake system in vitro. (*S*)-(+)-DHM (**4**) and (*R*)-(−)-DHM (**5**) were obtained in optically pure forms via resolution of *tert*-butyloxycarbonyl-protected DHM (**1**) using cinchonidine as the only resolving agent. The optical purity and absolute stereochemistry of **4** and **5** were established by chemical correlation to the (*S*)-(+) enantiomer of 3-hydroxy-4-aminobutyric acid (GABOB). While **4** was a specific and potent BMC-sensitive GABA agonist in vivo and in vitro, possibly the most potent GABA agonist so far described, the inhibition of GABA uptake by DHM proved to reside exclusively in the (*R*)-(−) enantiomer (**5**). The affinity of **5** for BMC-sensitive GABA receptor sites in vitro was some 50 times lower than that of **4**. Compounds **4** and **5** can be considered semirigid isosteres of the conformationally flexible GABA analogues (*S*)-(+)- and (*R*)-(−)-GABOB, respectively, which show a very low degree of enantioselectivity with respect to GABA synaptic mechanisms. This correlation between the degree of enantioselectivity and conformational mobility of chiral GABA analogues might be of importance for the design of new drugs with specific actions at synapses at which GABA is the transmitter.

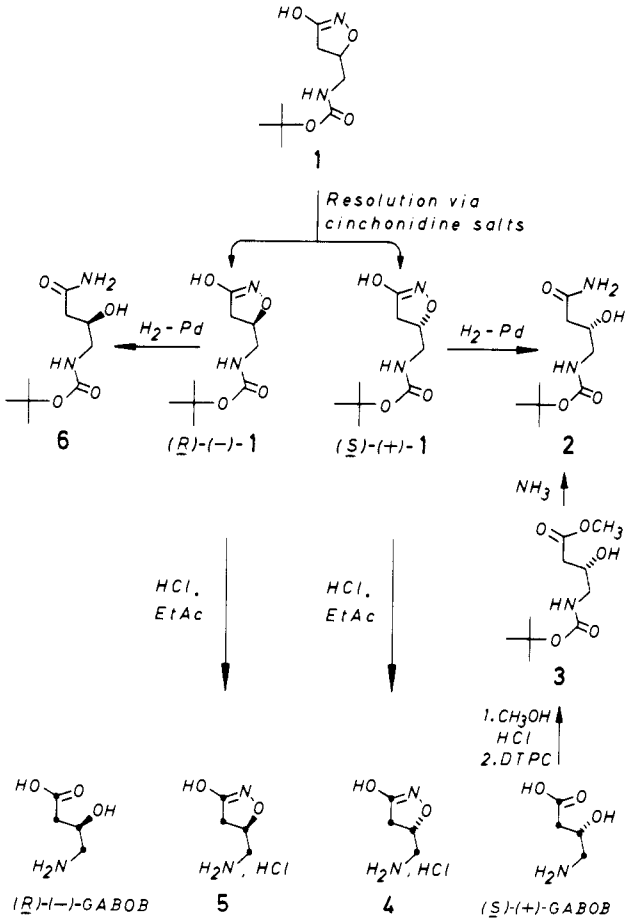
4-Aminobutyric acid (GABA) is an inhibitory transmitter concerned with the control of neuronal activity in virtually all regions of the mammalian central nervous system.[1-3] GABA is involved in the regulation of a variety of physiological mechanisms,[4-7] and impaired transmission at central synapses using GABA may be important in a number of neurological disorders.[8-10] As a consequence, there is an increasing interest in the postsynaptic receptors and the uptake mechanisms at GABA-mediated synapses as sites for pharmacological and therapeutic attack.

Extensive structure–activity studies on conformationally restricted analogues of GABA have shown that different structural constraints are imposed on agonists acting at GABA receptors and inhibitors of GABA uptake.[11,12] While the relatively flexible GABA analogue *trans*-4-aminocrotonic acid (*trans*-ACA) interacts with both mechanisms,[13] isoguvacine is a potent and specific GABA agonist sensitive to the antagonist bicuculline methochloride (BMC),[14,15] and guvacine is an effective inhibitor/substrate for GABA uptake systems but shows no detectable affinity for GABA postsynaptic receptors[11,16,17] (Figure 1).

In continuation of these studies we have been examining the correlation between conformational mobility, absolute stereochemistry, and biological activity of chiral GABA analogues.[18-21] (*S*)-(−)- and (*R*)-(+)-4-aminovaleric acid (4-Me-GABA) are almost equipotent in vitro as inhibitors of the receptor binding of GABA and a number of GABA agonists, as stimulators of the binding of diazepam, and as inhibitors of neuronal and glial GABA uptake.[18,22] However, reduction of the molecular flexibility of these compounds by incorporation of double bonds into the carbon backbones has pronounced effects on the pharmacological profiles. Thus, the (*S*)-(−) form of *trans*-4-aminopent-2-enoic acid (4-Me-*trans*-ACA) interacts specifically with the BMC-sensitive GABA receptors, whereas (*R*)-(+)-4-Me-*trans*-ACA is an inhibitor of GABA uptake showing no receptor affinity.[11,18,19]

(*RS*)-5-(Aminomethyl)-2-isoxazolin-3-ol (dihydromuscimol, DHM),[23] which is a conformationally restricted bioisoster of 3-hydroxy-4-aminobutyric acid (GABOB) (Figure 1), is a very potent BMC-sensitive GABA agonist and an inhibitor of GABA uptake.[15] In this paper we describe the resolution of the DHM derivative **1**, the determination of the absolute stereochemistry of (*S*)-(+)-

Chart I



† The 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., Ed. "The GABA Receptors"; The Humana Press: New Jersey, 1983.
(3) Krogsgaard-Larsen, P., Scheel-Krüger, J., Kofod, H., Eds. "GABA-Neurotransmitters. Pharmacochemical, Biochemical and Pharmacological Aspects"; Munksgaard: Copenhagen, 1979.
(4) Enna, S. J. *Biochem. Pharmacol.* **1981**, *30*, 907.

0022-2623/85/1828-1612$01.50/0

Table I. Biological and in Vitro Effects of GABA and Some GABA Analogues[a]

compd	GABA agonism in vivo (rel potency)	in vitro effects: IC_{50}, μm			
		GABA binding	THIP binding	P4S binding	GABA uptake
GABA	- - -	0.033	0.015	0.025	3
muscimol	- - - -[b]	0.006	0.005	0.006	320
(*RS*)-DHM	- - - -[b]	0.008	0.005	0.018	130
4	- - - -	0.004	0.003	0.008	>500
5	- - -	0.25	0.14	0.54	70
(*S*)-(+)-GABOB	- - (-)	0.40	0.20	0.90	29
(*R*)-(-)-GABOB	- - (-)	1.0	0.30	1.9	67

[a] IC_{50} values for GABA receptor binding were determined by incubation of rat brain synaptic membranes for 15 min at 4 °C in 0.05 Tris–citrate buffer (pH 7.1) containing 0.005 μM [^{3}H]GABA, [^{3}H]THIP, or [^{3}H]P4S 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.[29] The value at each concentration of analogue was the average of three experiments, and SEM's 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. IC_{50} values for GABA uptake were determined by using a crude preparation of rat brain synaptosomes in a phosphate medium containing 0.05 μM [^{3}H]GABA and the inhibitor. [b] Reference 15.

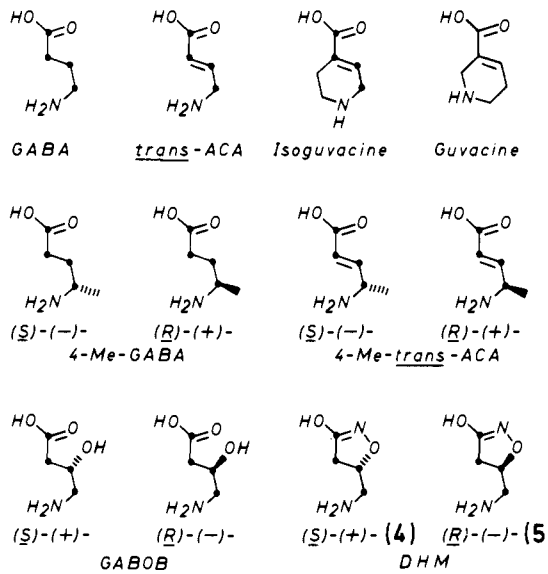


Figure 1. Structure of GABA and some GABA analogues.

DHM (4) and (*R*)-(-)-DHM (5), prepared from (*S*)-(+)-1 and (*R*)-(-)-1, respectively, and the results of comparative biological and in vitro studies on 4, 5, and (*S*)-(+)- and (*R*)-(-)-GABOB.

Chemistry. The resolution of 1, using cinchonidine, is based on the weakly acidic character (pK_a 6.5) of the 2-isoxazolin-3-ol unit. The crystalline cinchonidine salt of 1 was recrystallized twice to apparent optical purity. The (+) isomer of 1 (Chart I), recovered from this salt, had a melting point at 142.0–142.5 °C, which is substantially higher than that of racemic 1 (89.0–90.0 °C).[24] This pronounced difference in melting points opened up the possibility of developing a procedure for the isolation of both of the enantiomers of 1 using cinchonidine as the only resolving agent. Consequently, from the crystalline, but not recrystallized, cinchonidine salt of 1 partially resolved (+)-1 was recovered and recrystallized to optical purity. The resolution process was monitored by IR spectroscopy as well as by determination of melting points and optical rotations. Similarly, the crystalline evaporated mother liquor from the first precipitation of the cinchonidine salt of 1 was worked up to give optically pure (-)-1.

The absolute configuration of 4 was established by chemical correlation to (*S*)-(+)-GABOB, which has been synthesized from D-arabinose through chemical reactions not affecting bonds to the pertinent chiral center.[25] The absolute configuration of the enantiomers of GABOB has recently been confirmed.[26] Reductive ring opening of (*S*)-(+)-1 gave 2, which was also synthesized in two steps from (*S*)-(+)-GABOB (Chart I). The two samples of 2 synthesized by these two independent routes were identical, thus confirming the optical purity of (*S*)-(+)-1. Deprotection of (*S*)-(+)-1 and (*R*)-(-)-1 gave 4 and 5, respectively, under conditions that are unlikely to affect the bonds to the chiral centers of the compounds. Within experimental error the numerical values of the optical rotations of (*S*)-(+)-1 and (*R*)-(-)-1, of 2 and 6, and of 4 and 5 were identical.

(5) DeFeudis, F. V. *Neurochem. Int.* **1981**, *3*, 113.
(6) Grognet, A.; Hertz, F.; DeFeudis, F. V. *Gen. Pharmacol.* **1983**, *14*, 585.
(7) Hoehn-Saric, R. *Psychopharmacology* **1983**, *80*, 338.
(8) Meldrum, B. S. *Clin. Neuropharmacol.* **1982**, *5*, 293.
(9) Morselli, P. L., Lloyd, K. G., Löscher, W., Meldrum, B., Reynolds, E. H., Eds. "Neurotransmitters, Seizures and Epilepsy"; Raven Press: New York, 1981.
(10) Chase, T. N., Wexler, N. S., Barbeau, A., Eds. "Huntington's Disease"; Raven Press: New York, 1979.
(11) Krogsgaard-Larsen, P. *J. Med. Chem.* **1981**, *24*, 1377.
(12) Krogsgaard-Larsen, P.; Brehm, L.; Schaumburg, K. *Acta Chem. Scand., Ser. B* **1981**, *B35*, 311.
(13) Johnston, G. A. R.; Allan, R. D.; Kennedy, S. M. E.; Twitchin, B. In "GABA-Neurotransmitters. Pharmacochemical, Biochemical and Pharmacological Aspects"; Krogsgaard-Larsen, P., Scheel-Krüger, J., Kofod, H., Eds.; Munksgaard: Copenhagen, 1979; p 149.
(14) Krogsgaard-Larsen, P.; Johnston, G. A. R.; Lodge, D.; Curtis, D. R. *Nature (London)* **1977**, *268*, 53.
(15) Krogsgaard-Larsen, P.; Hjeds, H.; Curtis, D. R.; Lodge, D.; Johnston, G. A. R. *J. Neurochem.* **1979**, *32*, 1717.
(16) Johnston, G. A. R.; Krogsgaard-Larsen, P.; Stephanson, A. L. *Nature (London)* **1975**, *258*, 627.
(17) Lodge, D.; Johnston, G. A. R.; Curtis, D. R.; Brand, J. *Brain Res.* **1977**, *136*, 513.
(18) Schousboe, A.; Thorbek, P.; Hertz, L.; Krogsgaard-Larsen, P. *J. Neurochem.* **1979**, *33*, 181.
(19) Krogsgaard-Larsen, P.; Falch, E.; Peet, M. J.; Leah, J. D.; Curtis, D. R. In "CNS Receptors—From Molecular Pharmacology to Behavior"; Mandel, P., DeFeudis, F. V., Eds.; Raven Press: New York, 1983; p 1.
(20) Falch, E.; Krogsgaard-Larsen, P. *J. Neurochem.* **1982**, *38*, 1123.
(21) Krogsgaard-Larsen, P.; Falch, E.; Jacobsen, P. In "Actions and Interactions of GABA and Benzodiazepines"; Bowery, N. G., Ed.; Raven Press: New York, 1984; p 109.
(22) Krogsgaard-Larsen, P. *Mol. Cell. Biochem.* **1980**, *31*, 105.
(23) Krogsgaard-Larsen, P.; Larsen, A. L. N.; Thyssen, K. *Acta Chem. Scand., Ser. B* **1978**, *B32*, 469.
(24) Brehm, L.; Jacobsen, P.; Johansen, J. S.; Krogsgaard-Larsen, P. *J. Chem. Soc., Perkin Trans. 1* **1983**, 1459.
(25) Bock, K.; Lundt, I.; Pedersen, C. *Acta Chem. Scand., Ser. B* **1983**, *B37*, 341.
(26) Rossiter, B. E.; Sharpless, K. B. *J. Org. Chem.* **1984**, *49*, 3707.

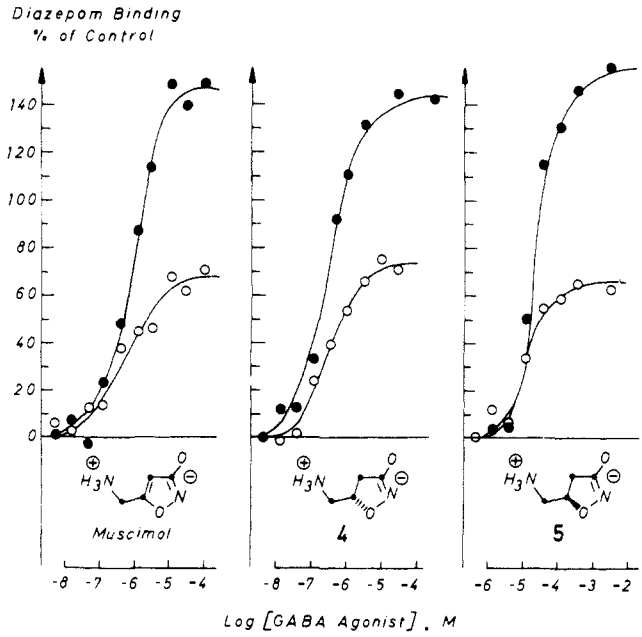


Figure 2. Effects on [^{3}H]diazepam binding at 30 °C of muscimol, 4, and 5 in the absence (○) or in the presence (●) of 0.15 M NaCl.

***I*/*U* Ratios.** The *I*/*U* ratios for the enantiomers of 1 were calculated following the procedure described earlier.[27] Using the pK_a values for (*RS*)-DHM (5.8, 9.3[23]) and 1 (6.5), the approximate *I*/*U* ratio for (*RS*)-DHM, and thus for 4 and 5, was calculated to be 630 ($10^{-6.5}/10^{-9.3}$).

Effects on the Binding of GABA and GABA Agonists. The ability of 4, 5, and (*S*)-(+)- and (*R*)-(−)-GABOB to displace radioactive GABA and the radioactive GABA agonists THIP[14] and piperidine-4-sulfonic acid (P4S)[28] from GABA binding sites on rat brain synaptic membranes was measured by using published procedures.[20] These data were compared with the effects of GABA and muscimol. In agreement with the results of earlier studies using these radioactive ligands,[11,20,21] the relative potencies of the chiral compounds under study as inhibitors of the binding of GABA, THIP, and P4S were comparable, whereas differences in their absolute potencies were observed (Table I). Thus, the IC_{50} values for (*RS*)-DHM, 4, 5, and (*S*)-(+)- and (*R*)-(−)-GABOB as inhibitors of the binding of radioactive P4S were typically 2–4 times higher than those measured with labeled GABA or THIP as ligands. Similar differences were not observed for GABA or muscimol (Table I). While (*S*)-(+)-GABOB was approximately twice as potent as the (*R*)-(−) isomer in these test systems, 4 was some 50 times more active than its (*R*)-(−) isomer (5). Like muscimol, 4 binds substantially more tightly to the GABA binding sites than GABA (Table I).

Effects on the Binding of Diazepam. The relative potencies of (*S*)-(+)- and (*R*)-(−)-GABOB, GABA, and muscimol in enhancing the binding of radioactive diazepam to rat brain synaptic membranes in vitro have been measured earlier.[21] In order to describe in detail the agonist profile of 4 and 5 with respect to the BMC-sensitive GABA receptor complex, the effects of these compounds on diazepam binding were studied and compared with that of muscimol under different conditions (Figure 2). In agreement with earlier findings for other BMC-sensitive

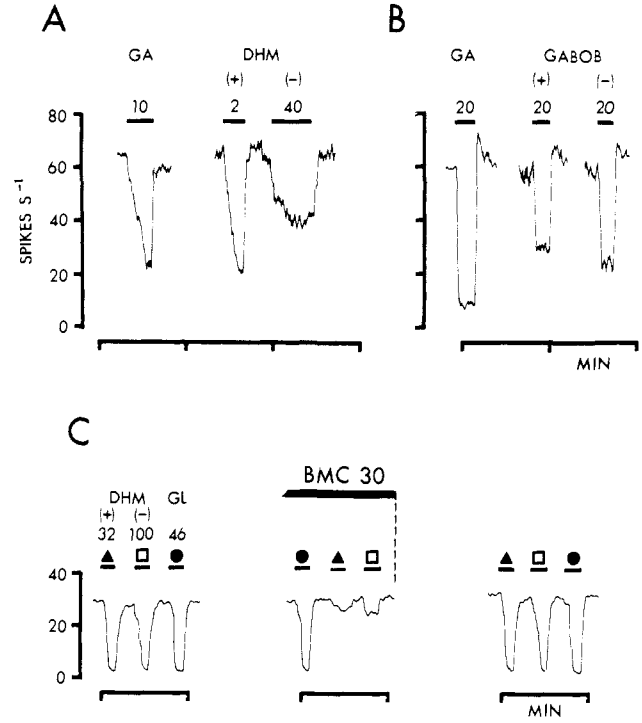


Figure 3. Comparison of the inhibitory effects of GABA (GA), glycine (GL), 4 [(+)-DHM], 5 [(−)-DHM], (*S*)-(+)-GABOB, and (*R*)-(−)-GABOB on the firing of two spinal interneurons (A and C) and a Renshaw cell (B), maintained with continuously ejected (*RS*)-homocysteic acid: A, 19 nA; B, 8 nA;C, 13 nA. The amino acids were ejected for the times and with the currents (nA) indicated by the horizontal bars and figures from aqueous solutions (for details, see the Experimental Section). C: before, during and 2 min after the microelectrophoretic ejection of BMC for a total time of 2.5 min, ceasing at the vertical broken line. Ordinates: firing rate, spikes/s. Abscissae: time, min.

GABA agonists,[11,21,29-32] the relative potencies of 4, 5, and muscimol as stimulators of diazepam binding, in the presence or absence of chloride ions, were comparable to those measured for the compounds as inhibitors of GABA agonist binding, but the compounds are typically 2 orders of magnitude weaker in the former test system. The very similar maximum levels of stimulation of diazepam binding by these GABA agonists is in accord with the similar structure and conformational mobility of these compounds.[21,29]

Effects on Synaptosomal GABA Uptake. The affinities of the GABA analogues listed in Table I for the GABA uptake mechanisms in a crude preparation of synaptosomes isolated from rat brains were examined following a published procedure.[29] (*S*)-(+)-GABOB, which is slightly more potent than the (*R*)-(−) isomer as an inhibitor of the binding of GABA agonists, also inhibits GABA uptake slightly more potently, whereas the GABA uptake affinity of (*RS*)-DHM resides exclusively in the (*R*)-(−) isomer (5) (Table I).

Single-Cell Pharmacology. Microelectrophoretic techniques[33] were used to compare the effects on single neurons (Renshaw cells and interneurons) in the cat spinal

(27) Krogsgaard-Larsen, P.; Mikkelsen, H.; Jacobsen, P.; Falch, E.; Curtis, D. R.; Peet, M. J.; Leah, J. D. *J. Med. Chem.* **1983**, *26*, 895.
(28) Krogsgaard-Larsen, P.; Falch, E.; Schousboe, A.; Curtis, D. R.; Lodge, D. *J. Neurochem.* **1980**, *34*, 756.
(29) Falch, E.; Jacobsen, P.; Krogsgaard-Larsen, P.; Curtis, D. R. *J. Neurochem.* **1985**, *44*, 68.
(30) Braestrup, C.; Nielsen, M.; Krogsgaard-Larsen, P.; Falch, E. *Nature (London)* **1979**, *280*, 331.
(31) Karobath, M.; Placheta, P.; Lippitsch, M.; Krogsgaard-Larsen, P. *Nature (London)* **1979**, *278*, 748.
(32) Supavilai, P.; Karobath, M. *Neurosci. Lett.* **1980**, *19*, 337.
(33) Curtis, D. R.; Duggan, A. W.; Felix, D.; Johnston, G. A. R. *Brain Res.* **1971**, *32*, 69.

cord of GABA, glycine, (S)-(+)- and (R)-(−)-GABOB, 4, and 5. In agreement with the results of the in vitro binding studies, 4 was considerably more potent (10–40 times) than GABA as a BMC-sensitive depressant of neuronal firing, whereas 5 was approximately equipotent with or, on some neurons, 2–4 times more potent than GABA (Table I; Figure 3). (S)-(+)- and (R)-(−)-GABOB were approximately equipotent as neuronal depressants. They were slightly less potent than GABA, and the inhibitory effects were also sensitive to BMC (not illustrated). Glycine does not interact with GABA receptors[1,33] and, accordingly, the inhibitory effect of glycine was not significantly reduced by simultaneous administration of BMC (Figure 3).

Discussion. Dihydromuscimol (DHM) was resolved to give (S)-(+)-DHM (4) and (R)-(−)-DHM (5) in optically pure forms, with cinchonidine as the resolving agent (Chart I). From the crystalline salt formed from 1 and cinchonidine and from the corresponding mother liquor partially resolved, (+)-1 and (−)-1, respectively, were recovered, and both of these products were recrystallized to optical purity. This very convenient resolution procedure was based on the pronounced difference between the melting points of 1 (89.0–90.0 °C) and (+)- and (−)-1 (142.0–142.5 °C), suggesting that 1 is a racemic mixture ("conglomerate") rather than a racemic compound or racemic solution.[34] The absolute configurations of 4 and 5 were established via chemical correlation with (S)-(+)-GABOB (Chart I).

(RS)-DHM[23] is a very potent BMC-sensitive GABA agonist, which also interacts with the GABA uptake system.[15,18] This lack of specificity reduces its importance as a neurochemical and pharmacological tool. These dual effects of (RS)-DHM were quite effectively "separated" via resolution. While the inhibitory effect on GABA uptake of (RS)-DHM was shown to reside exclusively in the (R)-(−) isomer (5), the (S)-(+) isomer (4) proved to be almost exclusively responsible for the interaction of (RS)-DHM with GABA postsynaptic receptors (Table I; Figure 3). Accordingly, 4 is much more potent than 5 in displacing GABA and the specific GABA agonists THIP and P4S from GABA binding sites in vitro, and 4 is more effective than 5 as an enhancer of the binding of diazepam (Figure 2). This latter effect of BMC-sensitive GABA agonists, which is sensitive to chloride ions,[29,32] suggests that the "benzodiazepine receptor" is a structural unit of the postsynaptic GABA receptor complex, the function of which is to regulate a membrane chloride channel.[2,11,35,36]

As shown in Table I the relative potencies of the chiral GABA analogues as inhibitors of the binding of radioactive GABA, THIP, and P4S are similar, whereas their absolute potencies are different, the compounds being significantly weaker as inhibitors of P4S binding (Table I). Such a difference was not observed for GABA and muscimol, but a similar effect has been observed earlier on other chiral GABA analogues.[20,21] These observations strongly suggest that the inhibitors are competing with GABA, THIP, or P4S for binding to the same chiral receptor macromolecule, which apparently binds GABA and THIP in a manner different from that in which P4S is bound. While the nature of this difference in GABA agonist–receptor interaction is still unknown, a number of observations support the view that the receptor interaction of the specific BMC-sensitive GABA agonist P4S[28,29] is somehow unique. Thus, P4S shows a low "efficacy" in enhancing the binding of diazepam in vitro,[29,30,32] and the binding of P4S is unusually sensitive to stimulation by barbiturates,[35] which appear to interact with a distinct site at the postsynaptic GABA receptor complex.[36]

(S)-(−)- and (R)-(+)-4-Me-*trans*-ACA, but not the conformationally more mobile analogues (S)-(−)- and (R)-(+)-4-Me-GABA (Figure 1), show a high degree of stereoselectivity with respect to interaction with GABA synaptic mechanisms.[11,18,19] Accordingly, 4 and 5, but not the enantiomers of GABOB, show very different effects on GABA receptors and uptake mechanisms (Table I). This enantioselecitivty of conformationally restricted GABA analogues supports the view, derived from structure–activity studies on relatively rigid GABA analogues such as isoguvacine and guvacine (Figure 1), that the "active conformations" of GABA with respect to the GABA receptors and GABA uptake systems are different.[14,21,22,37,38] It is reasonable to assume that the flexible GABA analogues such as 4-Me-GABA and GABOB relatively easily can relieve the strain caused by unfavorable steric arrangements of groups during the interactions with receptors and binding sites.

Compound 4 is an extremely potent BMC-sensitive GABA agonist, which binds more tightly to the receptor sites than any other GABA analogue so far described. This demonstrates the ability of this compound to adopt conformations, which almost perfectly mimic the "receptor-active conformation(s)" of GABA. In light of its high degree of selectivity (Table I), 4 is an important model compound for GABA receptor studies. The low I/U ratio for 4 and 5 (630) is comparable with that calculated for THIP (1500 or 500),[27] which is capable of penetrating the blood–brain barrier very easily.[11] Thus, 4 and 5 may be pharmacologically active after systemic administration. Animal behavioral studies must, however, await the production of 4 and 5 on a larger scale.

Experimental Section

General Chemistry. Melting points are corrected and were determined in capillary tubes. Elemental analyses were performed by P. Hansen, Chemical Laboratory II, University of Copenhagen. A Perkin-Elmer grating infrared spectrophotometer, a Varian 360L spectrometer, and a Perkin-Elmer polarimeter 141 were used. Me_4Si was used as an internal standard in the ^1H NMR spectra except for compounds dissolved in D_2O, where sodium 3-(trimethylsilyl)propanesulfonate was used. Thin-layer chromatography (TLC) and gravity column chromatography (CC) were performed by using silica gel F_{254} plates (Merck) and silica gel (Woelm, 0.063–0.200 mm), respectively. Compounds containing the 2-isoxazolin-3-ol unit were visualized on TLC plates with use of a $FeCl_3$ spraying reagent (purple color). Compounds containing amino or (*tert*-butyloxycarbonyl)amino groups were visualized with use of a ninhydrin spray reagent, and all compounds under study were detected on TLC plates with a $KMnO_4$ spray reagent. All evaporations were performed at ca. 15 mmHg on a rotatory evaporator.

(S)-(+)-5-[[(*tert*-Butyloxycarbonyl)amino]methyl]-2-isoxazolin-3-ol [(S)-(+)-1]. Method A. To a solution of 1[23,24] (562 mg, 2.6 mmol) in chloroform (3 mL) was added a solution of cinchonidine (765 mg, 2.6 mmol) in chloroform (8 mL), and the solution was left at 5 °C for 24 h. The precipitated cinchonidine salt [445 mg; $[\alpha]^{25}_D$ −45.6° (MeOH, *c* 1.0)] was recrystallized to give 385 mg of product [$[\alpha]^{25}_D$ −42.7° (MeOH, *c* 1.0)]. Repeated recrystallization gave 306 mg of product (enantiomeric yield 46%): mp 150–160 °C; $[\alpha]^{25}_D$ −42.6° (MeOH, *c* 1.0). Anal. ($C_{28}H_{38}N_4O_5$) C, H, N.

(34) Eliel, E. L. "Stereochemistry of Carbon Compounds"; McGraw-Hill: New York, 1962; p 44.
(35) Olsen, R. W.; Snowman, A. M. *J. Neurosci.* **1982**, *12*, 1812.
(36) Olsen, R. W. *J. Neurochem.* **1981**, *37*, 1.
(37) Krause, D. N.; Ikeda, K.; Roberts, E. *Brain Res.* **1981**, 225, 319.
(38) Johnston, G. A. R.; Allan, R. D.; Andrews, P. R.; Kennedy, S. M. E.; Twitchin, B. In "Advances in Pharmacology and Therapeutics"; Simon, P., Ed.; Pergamon Press: Oxford and New York, 1978; Vol. 2, p 11.

The cinchonidine salt (306 mg) was dissolved in a solution of glacial acetic acid (0.2 mL) in water (7 mL). The pH of this solution was adjusted to 9.5 by addition of aqueous sodium carbonate (5 mL, 1 M), and after the mixture was stirred for 1 h, the precipitate was isolated and washed with water (8 mL). The combined aqueous phases (ca. 15 mL) were extracted with ethyl acetate (4 × 15 mL), and the pH of the aqueous phase was then adjusted to 4.5 by addition of glacial acetic acid (3 mL) followed by extraction with ethyl acetate (4 × 15 mL). The latter organic phases were dried ($MgSO_4$) and evaporated and the residue was recrystallized (ethyl acetate–petroleum ether) to give (*S*)-(+)-1: 117 mg (90%); mp 142.0–142.5 °C; $[\alpha]^{25}_D$ +36.6° (MeOH, *c* 1.0). Anal. ($C_9H_{16}N_2O_4$) C, H, N. The IR spectrum (KBr) of (*S*)-(+)-1 was similar to that of 1,[23,24] notable differences being an absorption band at 1410 (m) cm^{-1} in the spectrum of (*S*)-(+)-1 but not in that of 1 and an absorption band at 895 (m) cm^{-1} in the spectrum of 1 but not in that of (*S*)-(+)-1. This latter absorption band was used to monitor the optical purity of (*S*)-(+)-1 and (*R*)-(−)-1 during the subsequent resolution procedures.

Method B. To a solution of 1[23,24] (562 mg, 2.6 mmol) in chloroform (3 mL) was added a solution of cinchonidine (765 mg, 2.6 mmol) in chloroform (8 mL). The solution was left at 5 °C for 24 h. The precipitated cinchonidine salt [475 mg; $[\alpha]^{25}_D$ −45.2° (MeOH, *c* 1.0)] was, without recrystallization, decomposed following a procedure analogous to that described above (method A) to give 170 mg of (*S*)-(+)-1 of unknown optical purity. This product was recrystallized four times (ethyl acetate–petroleum ether) to give (*S*)-(+)-1 (100 mg; enantiomeric yield 36%): mp 142.0–142.5 °C; $[\alpha]^{25}_D$ +37.1° (MeOH, *c* 1.0). The IR spectrum (KBr) was identical with that of (*S*)-(+)-1 prepared according to method A.

(*R*)-(−)-5-[[(*tert*-Butyloxycarbonyl)amino]methyl-2-isoxazolin-3-ol [(R)-(−)-1]. The mother liquor from the 475 mg of cinchonidine salt mentioned above (method B) was evaporated to dryness, and the crystalline residue [849 mg; $[\alpha]^{25}_D$ −68.1° (MeOH, *c* 1.0)] was decomposed following a procedure analogous to that described above (method A) to give 296 mg of (*R*)-(−)-1 of unknown optical purity. This product was recrystallized four times (ethyl acetate–petroleum ether) to give (*R*)-(−)-1 (149 mg; enantiomeric yield 53%): mp 142.0–142.5 °C; $[\alpha]^{25}_D$ −36.5° (MeOH, *c* 1.0). Anal. ($C_9H_{16}N_2O_4$) C, H, N. The IR spectrum (KBr) was identical with that of (*S*)-(+)-1.

(*S*)-(−)-3-Hydroxy-4-[(*tert*-butyloxycarbonyl)amino]-butyramide (2). Method A. To a solution of (*S*)-(+)-1 (100 mg, 0.46 mmol) in methanol (30 mL) was added a Pd/C catalyst (100 mg, 10%), and the suspension was hydrogenated in a Parr apparatus at 35 psi for 10 h. The catalyst was filtered off, the solvent evaporated, and the residue recrystallized (ethanol–toluene) to give 2 (26 mg, 26%): mp 114.0–115.0 °C; $[\alpha]^{25}_D$ −6.1° (MeOH, *c* 1.0). Anal. ($C_9H_{18}N_2O_4$) C, H, N. IR (KBr): 3390 (s), 3360 (s), 3205 (s), 2985–2915 (m–w, several bands), 1765 (s), 1680 (s), 1640 (s), 1530 (s) cm^{-1}. ^1H NMR [$CDCl_3$–Me_2SO-d_6 (4:1)]: δ 7.4–7.1 (1 H, m), 6.7–6.3 (1 H, m), 6.3–5.8 (1 H, m), 5.0–4.7 (1 H, m), 4.1–3.8 (1 H, m), 3.3–2.9 (2 H, m), 2.28 (2 H, d, J = 6 Hz), 1.45 (9 H, s).

Method B. To a solution of hydrogen chloride in methanol prepared from methanol (20 mL) and acetyl chloride (4 mL) was added (*S*)-(+)-GABOB[25] (618 mg, 5.2 mmol). The solution was refluxed for 16 h. The evaporated reaction product was dissolved in water (5 mL) and the solution cooled to 5 °C. Upon addition of triethylamine (2.5 mL, 18 mmol), a solution of di-*tert*-butyl pyrocarbonate (DTPC; 1.5 mL, 6.4 mmol) in tetrahydrofuran (3 mL) was added with stirring. The mixture was stirred at 5 °C for 3 h. About 5 mL of solvent was evaporated and the residue extracted with ethyl acetate (3 × 15 mL). The combined and dried (Na_2SO_4) organic phases were evaporated, and the oily residue was subjected to CC [silica gel; 50 g; eluents toluene containing ethyl acetate (30–50%)] to give TLC-pure 3 (300 mg, 25%) [R_f 0.18; eluent toluene–ethyl acetate (3:2)] as an oil. ^1H NMR (CCl_4): δ 5.2–4.9 (1 H, m), 4.2–3.9 (1 H, m), 3.65 (3 H, s), 3.5–3.4 (1 H, m), 3.3–3.0 (2 H, m), 2.45 (2 H, d, J = 6 Hz), 1.45 (9 H, s). Without further characterization, 3 (296 mg, 1.3 mmol) was dissolved in aqueous ammonia (8 mL, ρ 0.88), and the solution was left at 25 °C for 16 h. The solution was continuously extracted with ether–dichloromethane (4:1) for 4 h. The dried (Na_2SO_4) and evaporated organic phase was recrystallized (ethanol–toluene) to give 2 (66 mg (24%) based on 3): mp 115.0–116.0 °C; $[\alpha]^{25}_D$ −6.0° (MeOH, *c* 1.0). The IR spectrum (KBr) was identical with that of 2 prepared according to method A.

(*R*)-(+)-3-Hydroxy-4-[(*tert*-butyloxycarbonyl)amino]-butyramide (6). Compound 6 was prepared from (*R*)-(−)-1 (30 mg, 0.14 mmol) as described above for 2 (method A). Obtained was 6 (9 mg, 30%): mp 113.5–114.5 °C, $[\alpha]^{25}_D$ +6.0° (MeOH, *c* 1.0). Anal. ($C_9H_{18}N_2O_4$) C, H, N. The IR spectrum (KBr) was identical with that of 2.

(*S*)-(+)-5-(Aminomethyl)-2-isoxazolin-3-ol Hydrochloride (4). To a solution of (*S*)-(+)-1 (60 mg, 0.28 mmol) in ethyl acetate (2 mL) was added dropwise a solution of hydrogen chloride in ethyl acetate (5 mL, 3.4 M). The solution was left at 25 °C for 1 h and evaporated, and the residue was recrystallized from *N*,*N*-dimethylformamide–acetonitrile to give 4 (38 mg, 89%): mp 166–172 °C (sublimation); $[\alpha]^{25}_D$ +102° (H_2O, *c* 1.0). Anal. ($C_4H_9N_2O_2Cl$) C, H, N, Cl. IR (KBr): 3600–3250 (m), 3250–2500 (s), 1675 (s), 1610 (m) cm^{-1}. The IR spectrum was similar to that of (*RS*)-DHM.HCl.[23] ^1H NMR (D_2O): δ 5.3–4.8 (1 H, m), 3.8–3.3 (2 H, m), 3.3–2.5 (2 H, m).

(*R*)-(−)-5-(Aminomethyl)-2-isoxazolin-3-ol Hydrochloride (5). Compound 5 was prepared from (*R*)-(−)-1 (60 mg, 0.28 mmol) as described for 4. Obtained was 5 (36 mg, 85%): mp 163–168 °C (sublimation); $[\alpha]^{25}_D$ −103° (H_2O, *c* 1.0). Anal. ($C_4H_9N_2O_2Cl$) C, H, N, Cl. The IR spectrum of 5 was identical with that of 4.

Microelectrophoretic Studies. Experiments were performed on lumbar dorsal horn interneurons and Renshaw cells of cats anaesthetized 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 that contained 3.6 M NaCl. The compounds were administered electrophoretically from the outer barrels of the micropipets[33] that contained aqueous solutions: GABA (0.2 M, pH 3.0); 4 (0.05 M in 0.15 M NaCl, pH 3.2); 5 (0.05 M in 0.15 M NaCl, pH 3.2); (*S*)-(+)-GABOB (0.1 M, pH 3.4); (*R*)-(−)-GABOB (0.1 M, pH 3.4); glycine (0.5 M, pH 3.0); BMC (0.01 M in 0.15 M NaCl). As in previous studies of this type,[14,15,27-29] relative potencies of agonists were estimated from the ratios of equieffective electrophoretic currents, allowance being made for the dilution of 4 and 5 in NaCl solution. Cell firing rates were maintained (30–60 Hz) by continuous ejection of (*RS*)-homocysteic acid (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.

Inhibition of GABA Agonist Binding. An earlier method for the preparation of synaptic membranes from the cerebral cortices of adult rats[39] was modified as described previously.[20] For the binding assay procedures, aliquots of synaptic membranes (0.8–1.2 mg of protein) were incubated in triplicate at 4 °C for 15 min in 2 mL of 0.05 M Tris–citrate buffer (pH 7.1) containing 0.005 μM [^3H]GABA, [^3H]THIP, or [^3H]P4S. The IC_{50} values of the GABA analogues tested were determined as described elsewhere.[20]

Stimulation of Diazepam Binding. The method used for study of the binding of [^3H]diazepam to rat brain membranes[29] was a modification of a previously published procedure.[30] The final suspension of membranes was diluted to 160 volumes (mL/g original tissue) in 0.1 M Tris–citrate (pH 7.1), and, optionally, a solution of sodium chloride resulting in a final concentration of chloride of 0.15 M was added. The GABA analogue was added to 2.5-mL aliquots of the membrane suspension, which were then incubated in triplicate with 0.0008 μM [^3H]diazepam for 20 min at 30 °C in a total of 2.7 mL. After incubation, the samples were diluted to 10 mL with ice-cold buffer and filtered immediately through Whatman GF/C glass fiber filters. The filters were washed with an additional 10 mL of buffer, and the radioactivity was measured by conventional scintillation counting methods. All binding values were calculated as specific binding, which is total binding minus binding in the presence of 3 μM diazepam.

Inhibition of GABA Uptake. The rat brain synaptosomes were prepared as described in detail elsewhere.[40] In the GABA

(39) Enna, S. J.; Snyder, S. H. *Brain Res.* **1975**, *100*, 81.
(40) Fjalland, B. *Acta Pharmacol. Toxicol.* **1978**, *42*, 73.

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 [^{3}H]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.[41]

Acknowledgment. This work was supported by a grant from NOVO Research Institute, Bagsvaerd, Copenhagen. R. Malik assisted with the microelectrophoretic investigations, and the technical assistance of P. Searle and the secretarial and technical assistance of B. Hare and S. Stilling are gratefully acknowledged.

(41) Beart, P. M.; Johnston, G. A. R.; Uhr, M. L. *J. Neurochem.* **1972**, *19*, 1855.

Synthesis and Antidiarrheal Activity of *N*-(Aminoiminomethyl)-1*H*-pyrrole-1-acetamides Related to Guanfacine

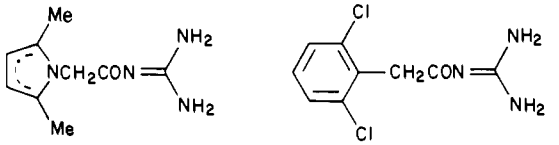
Doreen E. Beattie, Gillian M. Dover, and Terence J. Ward*

Wyeth Research (UK), Taplow, Maidenhead, Berkshire SL6 OPH, England. *Received January 22, 1985*

A series of *N*-(aminoiminomethyl)-1*H*-pyrrole-1-acetamides, related to guanfacine, were prepared and tested for antidiarrheal activity in castor oil dosed rats. *trans*-*N*-(Aminoiminomethyl)-2,5-dihydro-2,5-dimethyl-1*H*-pyrrole-1-acetamide (**2**), in which the dichlorophenyl ring of guanfacine is replaced by 2,5-dimethyl-2,5-dihydropyrrole, showed potent antidiarrheal activity but possessed only minimal cardiovascular activity in rats.

Most currently available antidiarrheal agents exert their activity by an action on cholinergic or opiate receptors.[1] The usefulness of anticholinergic agents is limited by their short duration of action and other anticholinergic side effects such as mydriasis, dry mouth, and blurred vision. Drugs acting at opiate receptors have the potential to produce undesirable CNS effects including addiction. In the search for more specific antidiarrheal agents our attention was directed toward the gastrointestinal actions of clonidine and related α_2-agonists.[2,3] In addition to its well-known cardiovascular actions, clonidine increases electrolyte absorption in the intestine[4] and inhibits castor oil[5] or naloxone[6] induced diarrhea in the rat. Antidiarrheal activity has also been reported for the clonidine-related compounds lofexidine[5] and lidamidine.[7] The latter compound has been shown to be an effective antidiarrheal agent in man,[8] recently launched as liderral in Mexico. In order to exploit the antidiarrheal activity of clonidine-like α-agonists, it is necessary to achieve a separation of their desired gastrointestinal effect from their cardiovascular activity. During the course of studies on a series of heterocyclic analogues of the clonidine-like antihypertensive agent guanfacine,[9] we synthesized an analogue that retained marked antidiarrheal activity but was free of significant cardiovascular effects.

Chemistry. The primary structural types desired were acylguanidines related to I, in which the dichlorophenyl ring of guanfacine (II) was replaced by a pyrrole or dihydropyrrole ring (Table I).



I II

All of the acylguanidines listed in Table I, except compound **3**, were prepared by reaction of an appropriate methyl or ethyl ester with a guanidine (Scheme I). a number of novel dihydropyrrole esters (Table II) were required as part of this work. These were prepared by alkylation of 2,5-dihydro-2,5-dimethylpyrrole[10] with halo acid esters and generally consisted of mixtures of cis and trans isomers in around 1:4 ratio. These mixed isomers were used for the preparation of the *trans*-acylguanidines (**2** and **4**–**8**), since the residual cis isomer was readily removed by crystallization at the final stage of the synthesis. The trans configuration of these acylguanidines was confirmed by examination of their NMR spectra in trifluoroacetic acid. Under these conditions protonation of the ring nitrogen inhibits its rate of inversion and results in nonequivalence of the *trans*-dimethyl groups that then

(1) Niemegeers, C. J. E. *Clin. Res. Rev.* **1981**, *1* (Suppl. 1), 125.
(2) Taylor, J. A.; Mir, G. N. *Drug Dev. Res.* **1982**, *2*, 105.
(3) Doherty, N. S.; Hanock, A. A. *J. Pharmacol. Exp. Ther.* **1983**, *225*, 269.
(4) Dharmsathaphorn, K.; Yamashiro, D. J.; Lindeborg, D.; Mandel, K. G.; McRoberts, J.; Ruffolo, R. R. *Gastroenterology* **1984**, *86*, 120.
(5) Lal, H.; Shearman, G. T.; Ursillo, R. C. *J. Clin. Pharmacol.* **1981**, *21*, 16.
(6) Nakaki, T.; Chang, P. C. J.; Tokunaga, Y.; Kato, R. *J. Pharm. Pharmacol.* **1981**, *33*, 397.
(7) Mir, G. N.; Alioto, R. I.; Aperow, J. W.; Eash, J. R.; Krebs, J. R.; Yelnosky, J. *Arzneim. Forsch. Drug Res.* **1978**, *28*, 1448.
(8) Leibach, J. R.; Sninsky, C. A.; Justus, P. G.; Riley, R. L.; Mathias, J. R. *Gastroenterology*, **1981**, *80*, 1207.
(9) Scholtysik, G.; Jerie, P.; Picard, C. W. In "Pharmacology of Antihypertensive Drugs"; Scriabine, A., Ed.; Raven Press: New York, 1980; pp 79.
(10) Evans, G. G. *J. Am. Chem. Soc.* **1951**, *73*, 5230.

0022-2623/85/1828-1617$01.50/0