Pergamon

Bioorganic & Medicinal Chemistry, Vol. 3, No. 5, pp. 553–558, 1995

0968-0896/95 $9.50 + .00

0968-0896(95)00044-5

Excitatory Amino Acid Receptor Ligands: Asymmetric Synthesis, Absolute Stereochemistry and Pharmacology of (*R*)- and (*S*)-Homoibotenic Acid

F. Bischoff, T. N. Johansen, B. Ebert, P. Krogsgaard-Larsen and U. Madsen*

PharmaBiotec Research Center, The Royal Danish School of Pharmacy, Department of Medicinal Chemistry, 2 Universitetsparken, DK-2100 Copenhagen, Denmark

Abstract—The (*R*)- and (*S*)-forms of 2-amino-3-(3-hydroxyisoxazol-5-yl)propionic acid (homoibotenic acid, HIBO) were synthesized, using (*S*)-BOC-phenylalanine as a chiral auxiliary and their absolute stereochemistry correlated with that of (*R*)-Br-HIBO. The enantiomeric excesses for (*R*)-HIBO (1) (> 99.5%) and (*S*)-HIBO (2) (99.5%) were determined using chiral HPLC. Whereas compounds 1 and 2 were equipotent inhibitors of the binding of [^{3}H]glutamic acid in the presence of calcium chloride, 2 showed AMPA agonist activity and 1 very weak NMDA agonist activity.

Introduction

(*S*)-Glutamic acid (Glu) is the major excitatory amino acid (EAA) in the mammalian central nervous system (CNS) and operates through multiple ionotropic and G protein-coupled (metabotropic) receptors.[1,2] The former class of EAA receptors comprises three different heterogeneous families of receptors, named *N*-methyl-D-aspartic acid (NMDA), 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionic acid (AMPA), and kainic acid receptors.

The NMDA receptor complex is activated by the naturally occurring amino acid, ibotenic acid (Fig. 1) in a non-selective manner,[3] whereas NMDA and (*RS*)-2-amino-2-(3-hydroxy-5-methylisoxazol-4-yl)acetic acid (AMAA)[4] (Fig. 1) are selective agonists. NMDA receptors are competitively blocked by [(*RS*)-3-(2-carboxypiperazin-4-yl)propyl]phosphonic acid (CPP).[5] AMPA receptors are stereospecifically activated by (*S*)-AMPA[3,6] and (*S*)-2-amino-3-(4-bromo-3-hydroxyisoxazol-5-yl)propionic acid [(*S*)-Br-HIBO][3,7] (Fig. 1) and competitively blocked by 6-nitro-7-sulfamoylbenzo(*f*)quinoxaline-2,3-dione (NBQX).[8] Kainic acid receptors are selectively activated by kainic acid, whereas NBQX is a relatively effective antagonist.[8]

EAA receptors have also been identified in a number of invertebrates.[1] The pharmacology of such receptors has, so far, not been elucidated in great detail, but the isoxazole amino acids ibotenic acid, AMPA, Br-HIBO, and (*RS*)-2-amino-3-(3-hydroxyisoxazol-5-yl)propionic acid (HIBO) (Fig. 1) have been used to characterize EAA receptors in central neurones of snail (*Helix aspersa*), leech (*Hirudo medicinalis*), and horse-shoe crab (*Limulus polyphemus*).[9] In agreement with the findings for AMPA receptors in the mammalian CNS,[3,10] the AMPA agonists, AMPA and Br-HIBO are very potent agonists at leech EAA receptors, whereas ibotenic acid and, in particular, HIBO are somewhat weaker. However, whereas AMPA and Br-HIBO are totally inactive at snail and horse-shoe crab EAA receptors, HIBO is a moderately potent agonist at inhibitory EAA receptors in the horse-shoe crab, and slightly weaker at excitatory EAA receptors in this animal and at inhibitory EAA receptors in the snail.[9]

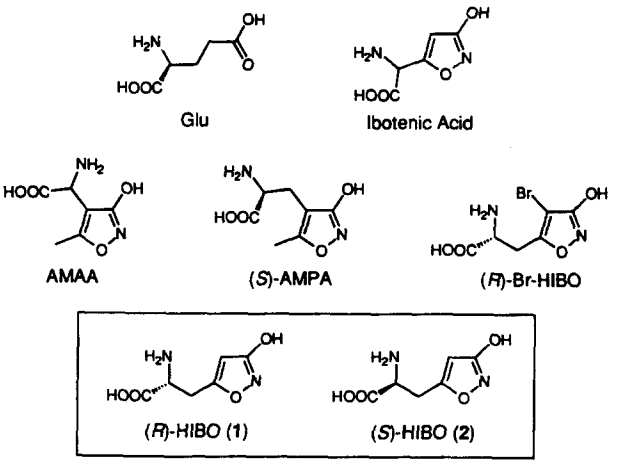


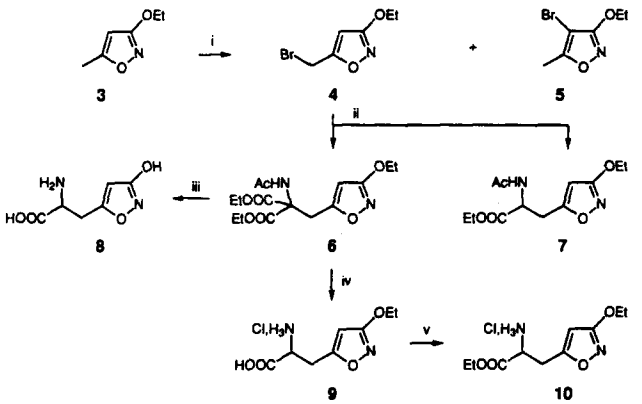
Figure 1. Structures of a number of excitatory amino acids including the enantiomers of HIBO.

In light of the results of these comparative studies, we consider HIBO a useful tool for pharmacological characterization of mammalian as well as invertebrate EAA receptors. Such studies may have interest in the attempts to design EAA receptor-active drugs and new types of pesticides, respectively. We here describe the synthesis, absolute stereochemistry and pharmacology at rat brain EAA receptors of (*R*)-HIBO (1) and (*S*)-HIBO (2) (Fig. 1).

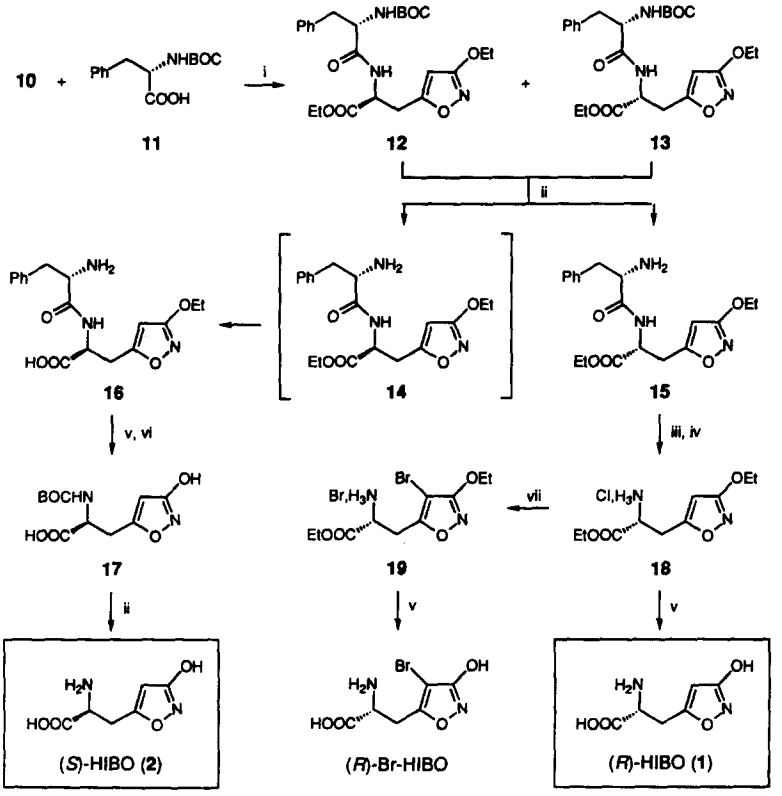
Synthesis

The synthesis of ethyl (*RS*)-2-amino-3-(3-ethoxy-isoxazol-5-yl)propionate (**10**), which is the key intermediate in the asymmetric syntheses of (*R*)-HIBO and (*S*)-HIBO, is outlined in Scheme 1, which also shows an improved synthesis of (*RS*)-HIBO (**8**). This latter synthesis, using 3-hydroxy-5-methylisoxazole as starting material, afforded **8** on a larger scale (69 mg) than obtained by a previously described synthesis.[11] Bromination of **3** using *N*-bromosuccinimide (NBS) under free radical conditions invariably led to mixtures of compounds **4** and **5**. The formation of **5** was minimized under the reaction conditions described here, but attempts to eliminate the formation of this undesired product were unsuccessful. A Sorensen reaction converted **4** into a separable mixture of **6** and **7**, and whereas **8** was synthesized by deprotection of purified **6**, a mixture of **6** and **7** was converted into **9**, which was esterified to give **10**.

In analogy with a published procedure,[12] (*S*)-*N*-(*tert*-butoxycarbonyl)phenylalanine [(*S*)-BOC-phenylalanine, **11**] was used as a chiral auxiliary for the conversion of **10** into (*R*)-HIBO and (*S*)-HIBO (Scheme 2). Compound **11** was coupled to **10** to give an inseparable mixture of the diastereomeric compounds **12** and **13**. This mixture showed no separation on TLC plates using 28 different eluents. Treatment of the mixture of **12** and **13** with trifluoroacetic acid did, however, furnish a mixture of **14** and **15** which could be separated by analytical as well as preparative TLC. The ester group of **14** turned out to be extremely susceptible to hydrolysis. Thus, during the extraction of **14** from silica gel, after preparative TLC purification, using a mixture of ethyl acetate and methanol and the subsequent evaporation of the solvent *in vacuo*, **14** was largely converted into the corresponding amino carboxylic acid **16**. Similarly, the ester group of **15** underwent spontaneous hydrolysis, though at a much slower rate making it possible to isolate **15** after preparative TLC purification. Attempt to obtain separable diastereomeric derivatives of **10** by using *N*-phthaloyl-, *N*-methoxy-carbonyl-, or *N*-benzyloxycarbonyl-(*S*)-phenyl-alanine as alternative chiral auxiliary groups, were unsuccessful. In no case could the mixtures of diastereomeric analogues be separated by TLC using a large variety of solvent systems.



Scheme 1. (i) NBS, benzoyl peroxide; (ii) AcNH–CH(COOEt)$_2$, EtOH, Na; (iii) 48% HBr/H_2O; (iv) 1 N HCl; (v) EtOH/CH_3COCl.



Scheme 2. (i) Na_2CO_3, 1,1'-carbonyldiimidazole; (ii) CF_3COOH; (iii) PhNCS; (iv) EtOH/CH_3COCl; (v) 48% HBr/H_2O; (vi) BOCOBOC, TEA; (vii) Br_2.

(*R*)-HIBO was obtained by Edman degradation of **15** followed by reflux of the intermediate **18** in 48% aqueous hydrobromic acid. A similar deprotection of **16** could not be accomplished due to insolubility of this amino acid in organic solvents. Instead, full deprotection was achieved by reflux in 48% aqueous hydrobromic acid followed by BOC-protection of the resulting mixture of (*S*)-phenylalanine and (*S*)-HIBO. These two BOC-protected amino acids were separated by column chromatography and the latter compound (**17**) deprotected by treatment with trifluoroacetic acid to give (*S*)-HIBO.

Absolute Configuration and Enantiomeric Purity

The absolute configurations of (*R*)-HIBO and (*S*)-HIBO were established by conversion of ethyl (*R*)-2-amino-3-(3-ethoxyisoxazol-5-yl)propionate hydrochloride (**18**) into the corresponding 4-bromo analogue (**19**) by treatment with neat bromine (Scheme 2). Compound **19** was deprotected by reflux in 48% aqueous hydrobromic acid to give (*R*)-Br-HIBO, the absolute configuration of which has previously been established by an X-ray crystallographic analysis.[7] The synthesized sample of (*R*)-Br-HIBO was subjected to ligand-exchange HPLC using a chiral stationary phase containing (*S*)-proline and shown to co-elute with an authentic sample of (*R*)-Br-HIBO[7] (Fig. 2).

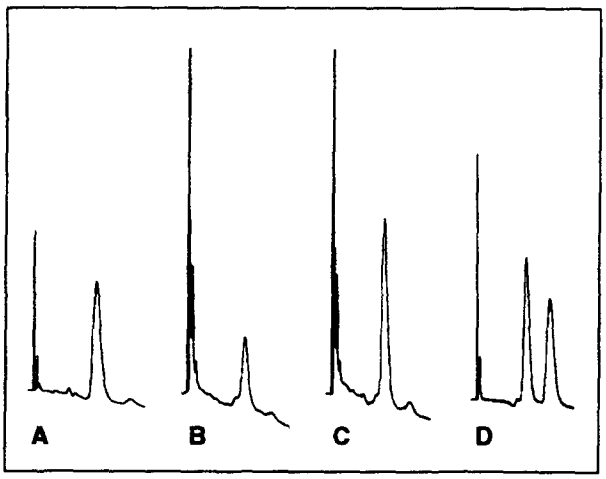


Figure 2. Chiral HPLC analysis of the Br-HIBO enantiomer obtained from compound **18** (Scheme 2). (A) Injection of an authentic sample of (*R*)-Br-HIBO; (B) injection of the prepared Br-HIBO enantiomer; (C) co-injection of the two former samples; (D) injection of (*RS*)-HIBO.

The enantiomeric purities of (*R*)-HIBO and (*S*)-HIBO were likewise determined by chiral HPLC analysis using a stationary phase containing (*S*)-proline. The enantiomeric excess was found to be 99.5% for (*S*)-HIBO and > 99.5% (detection limit) for (*R*)-HIBO.

In Vitro Pharmacology

The *in vitro* pharmacological effects of (*R*)-HIBO and (*S*)-HIBO were examined using receptor binding techniques and the rat cortical slice preparation[13] for electropharmacological investigations (Table 1). NMDA, AMPA and kainic acid receptor affinities were determined using [^{3}H]CPP,[14] [^{3}H]AMPA[15] and [^{3}H]kainic acid,[16] respectively as radioligands. The [^{3}H]AMPA binding site showed a marked stereoselectivity towards the enantiomers of HIBO, the (*S*)-form (**2**) having approximately 60 times higher affinity for this receptor site than the (*R*)-form (**1**) (Table 1). Accordingly, (*S*)-HIBO was, in the rat cortical slice preparation, shown to be a moderately potent AMPA receptor agonist (EC_{50} = 329 μM), whereas (*R*)-HIBO was virtually inactive (EC_{50} > 1 mM), though with detectable excitatory activity at 1 mM. The excitatory effect of (*S*)-HIBO was fully antagonized by the AMPA antagonist, NBQX[8] (Fig. 3), whereas this effect of (*S*)-HIBO was not detectably reduced by the NMDA antagonist, CPP.[5] Conversely, the very weak excitatory effect elicited by (*R*)-HIBO at a concentration of 1 mM was blocked by CPP but not by NBQX (Fig. 3). This very weak NMDA receptor interaction of (*R*)-HIBO escaped detection in [^{3}H]CPP binding experiments, and neither (*R*)- nor (*S*)-HIBO showed significant receptor affinities (IC_{50} > 100 μM) in [^{3}H]CPP and [^{3}H]kainic acid binding assays (Table 1).

(*R*)-HIBO as well as (*S*)-HIBO were shown to be effective inhibitors of the binding of [^{3}H]Glu in the presence of calcium chloride, the (*R*)-form being slightly more potent than the (*S*)-enantiomer (Table 1). The physiological relevance of this binding affinity is unclear but is believed to represent a transport mechanism[17] located within nerve terminals at Glu-operated synapses.[18]

Discussion

(*S*)-HIBO was found to be the most potent HIBO enantiomer, eliciting moderate AMPA receptor agonist

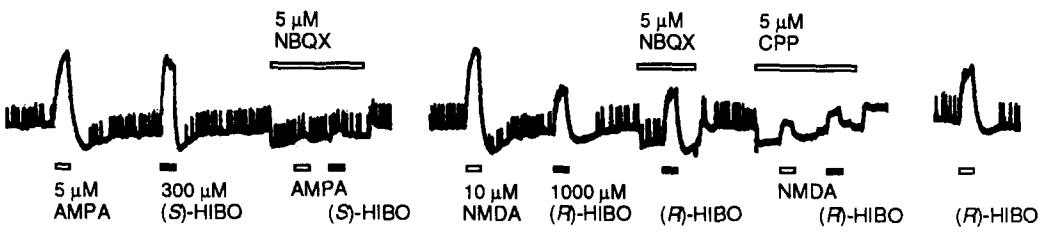


Figure 3. Electrophysiological recordings from the rat cortical slice preparation showing antagonism of NBQX towards AMPA and (*S*)-HIBO induced responses, whereas the weak activity of (*R*)-HIBO could be antagonized by CPP.

Table 1. *In vitro* pharmacology of compounds **1**, **2** and **8** (values ± S.E.M., $n = 3$–6)

Compound	Receptor binding [^{3}H]AMPA	[^{3}H]Glu	[^{3}H]CPP	[^{3}H]Kainic acid	Electro-pharmacology
	IC_{50} (μM)				EC_{50} (μM)
(*R*)-HIBO	49 ± 34	0.3 ± 0.1	> 100	> 100	>1000
(*S*)-HIBO	0.8 ± 0.4	0.5 ± 0.1	> 100	> 100	329 ± 45
(*RS*)-HIBO	1.5 ± 0.8	0.3 ± 0.1	> 100	> 100	374 ± 10

activity. This is in agreement with the findings for the AMPA agonistic effects shown to reside in the (*S*)-forms of AMPA,[6] Br-HIBO[7] and 2-amino-3-(3-hydroxy-5-phenylisoxazol-4-yl)propionic acid (APPA).[19] However, whereas (*R*)-HIBO is a very weak NMDA agonist, (*R*)-AMPA[6] and (*R*)-Br-HIBO[7] are inactive, and (*R*)-APPA is a moderately potent AMPA antagonist.[19] The more potent enantiomers are normally twice as potent as the racemate, but in this case (*S*)-HIBO and (*RS*)-HIBO were shown to be approximately equipotent in electrophysiological experiments (Table 1). This probably reflects a potentiating effect of (*R*)-HIBO on the activity of (*S*)-HIBO in analogy with what has previously been shown for (*S*)- and (*R*)-Br-HIBO.[18] The potentiating effect observed for (*R*)-Br-HIBO is not observed for (*R*)-AMPA and it is believed to be correlated to the affinity for calcium chloride dependent [^{3}H]Glu binding.[18]

AMPA does not affect the binding of [^{3}H]Glu in the presence of calcium chloride,[7] but (*R*)- and (*S*)-HIBO are effective inhibitors of this binding (Table 1), both enantiomers with slightly lower affinity than the respective enantiomers of Br-HIBO. Similarly, (*S*)-Br-HIBO (EC_{50} = 40 μM)[18] is more potent than (*S*)-HIBO as an AMPA receptor agonist. The molecular mechanism(s) underlying these positive effects of the presence of a bromine atom, and also of a methyl group,[3,10] in the 4-position of the rings of these compounds are unknown, but steric effects of these groups may force the amino acid side chains into favourable conformations.[3]

It is interesting to note that HIBO shows agonistic effects at certain invertebrate neuronal EAA receptors, which do not recognize AMPA or Br-HIBO[9] (see Introduction). It thus seems unlikely that the AMPA agonistic effect of (*S*)-HIBO or the effects of (*S*)- or (*R*)-HIBO on [^{3}H]Glu binding (Table 1) are responsible for these effects of HIBO. (*R*)-HIBO shows very weak NMDA agonist effects in the rat cortical wedge preparation, and the effects of HIBO on invertebrate neurones may be mediated by NMDA-like receptors showing higher affinity for (*R*)-HIBO than mammalian NMDA receptors.

In any case, it seems likely that the effects of HIBO on certain types of invertebrate neurones are mediated by unique isoforms of NMDA, AMPA, or kainic acid receptors or by receptors distinctly different from the EAA receptors so far identified in the mammalian CNS. (*R*)-HIBO and (*S*)-HIBO may prove to be useful tools for studies of these aspects.

Experimental

Chemistry

Melting points were determined in capillary tubes and are uncorrected. Compounds were visualized on TLC plates (Merck silica gel 60 F_{254}) using UV light or $KMnO_4$ spraying reagent. Compounds containing amino groups were visualized using a ninhydrin spraying reagent. 200 MHz ^{1}H NMR spectra were recorded on a Bruker AC 200 spectrometer. IR spectra were recorded on a Perkin–Elmer 781 grating infra red spectrophotometer in KBr discs. Elemental analyses were performed by Mr P. Hansen, Department of General and Organic Chemistry, University of Copenhagen and were within ± 0.4% of calculated values, unless otherwise indicated. Optical rotations were determined on a Perkin–Elmer polarimeter 141 in buffer solution pH 9.5 (0.025 M sodium tetraborate, 0.1 M sodium hydroxide).

Analyses of enantiomers on chiral HPLC were performed on a Waters HPLC Model 510 with an analytical column containing a stationary phase of silica gel to which (*S*)-proline was chemically bound via a 3-glycidoxypropyl spacer. Chromatography was performed with the column at 50 °C, an aqueous buffer (50 mM KH_2PO_4) as the mobile phase and for detection a Water Lambda-Max LC spectrophotometer Model 480 and a Hitachi D-200 Chromato-Integrator were used.

5-Bromomethyl-3-ethoxyisoxazole (4) and 4-bromo-3-ethoxy-5-methylisoxazole (5). To 3-ethoxy-5-methylisoxazole[11] (**3**) (16.0 g, 125.8 mmol) dissolved in tetrachloromethane (400 mL) were added in quarter portions at 90 min intervals benzoyl peroxide (400 mg) and NBS (24.6 g, 138.4 mmol) freshly recrystallized from water. The mixture was refluxed for 24 h and then further benzoyl peroxide (200 mg) and NBS (12.3 g, 69.1 mmol) were added in quarter portions at 90 min intervals. The reaction mixture was refluxed for a further 24 h and then cooled, filtered and evaporated. The product was column chromatographed (petroleum ether/dichloromethane) to yield crude **5** (2.2 g, 8%). ^{1}H NMR ($CDCl_3$) δ: 1.44 (*t*, 3H, *J* = 7 Hz), 2.34 (*s*, 3H); 4.35 (*q*, 2H, *J* = 7 Hz). Further elution gave crude **4** (7.5 g, 29%). ^{1}H NMR ($CDCl_3$) δ: 1.41 (*t*, 3H, *J* = 7 Hz), 4.29 (*q*, 2H, *J* = 7 Hz), 4.34 (*s*, 2H), 5.95 (*s*, 1H). Crude **4** was used in the next step without further purification.

Ethyl 2-acetamido-2-ethoxycarbonyl-3-(3-ethoxyisoxazol-5-yl)propionate (6) and ethyl (RS)-2-acetamido-3-(3-ethoxyisoxazol-5-yl)propionate (7). Diethyl acetamido-

malonate (7.7 g, 35.4 mmol) was added to a solution of sodium ethoxide (from 0.9 g of sodium, 39.1 mmol) in ethanol (50 mL) and stirred for 10 min at room temperature. Crude 5-bromomethyl-3-ethoxyisoxazole (**4**) (7.3 g, *ca* 35.4 mmol) in ethanol (20 mL) was added and refluxed for 4 h. The resulting suspension was evaporated, diluted with water, and extracted with dichloromethane. The organic phase was dried ($MgSO_4$), evaporated and column chromatographed (toluene/ethyl acetate containing 1% of glacial acetic acid) to give **6** which was recrystallized from water (4.56 g, 38%); mp 120–121 °C. ^1H NMR ($CDCl_3$) δ: 1.28 (*t*, 6H, *J* = 7 Hz), 1.39 (*t*, 3H, *J* = 7 Hz), 2.03 (*s*, 3H), 3.77 (*s*, 2H), 4.1–4.5 (*m*, 6H), 5.61 (*s*, 1H), 6.75 (*br s*, 1H). Analysis ($C_{15}H_{22}N_2O_7$) C, H, N. Further elution gave compound **7** after recrystallization from ethyl acetate/petroleum ether (2.2 g, 23%); mp 104–105 °C. ^1H NMR ($CDCl_3$) δ: 1.30 (*t*, 3H, *J* = 7 Hz), 1.40 (*t*, 3H, *J* = 7 Hz), 2.03 (*s*, 3H), 3.20 (*dd*, 1H, *J* = 5 Hz and 15 Hz), 3.30 (*dd*, 1H, *J* = 5 Hz and 15 Hz), 4.24 (*q*, 2H, *J* = 7 Hz), 4.26 (*q*, 2H, *J* = 7 Hz), 4.85 (*dt*, 1H, *J* = 5 Hz and 7 Hz), 5.67 (*s*, 1H), 6.22 (*br d*, 1H, *J* = 7 Hz). Analysis ($C_{12}H_{18}N_2O_5$) C, H, N.

(RS)-2-Amino-3-(3-hydroxyisoxazol-5-yl)propionic acid (HIBO, 8). Compound **6** (0.25 g, 0.86 mmol) was dissolved in 48% hydrobromic acid (15 mL) and refluxed at 140 °C for 30 min. The solution was rapidly cooled and evaporated, then twice dissolved in water and re-evaporated. After drying *in vacuo* (over KOH) the crude hydrobromide salt was dissolved in water (100 μL) and ethanol (1 mL) and neutralized with a solution of triethylamine in ethanol to pH 3–4. The precipitate was recrystallized from water to give zwitterionic HIBO (**8**) (69 mg, 47%). The prepared HIBO was identical with an authentic sample,[11] as determined by IR spectroscopy.

(RS)-2-Amino-3-(3-ethoxyisoxazol-5-yl)propionic acid hydrochloride (9). A mixture of **7** (4.26 g, 12.4 mmol) and **6** (2.2 g, 8.14 mmol) suspended in 1 M hydrochloric acid (200 mL) was refluxed for 8 h. The resulting solution was evaporated and dried to give **9** (4.2 g, 87%) after recrystallization from ethanol/ether; mp 182 °C (decomp.). ^1H NMR (D_2O) δ: 1.30 (*t*, 3H, *J* = 7 Hz), 3.33 (*d*, 2H, *J* = 6 Hz), 4.18 (*q*, 2H, *J* = 7 Hz), 4.28 (*t*, 1H, *J* = 6 Hz), 6.02 (*s*, 1H). Analysis ($C_8H_{13}N_2O_4Cl$) C, H, N, Cl: calcd 14.97, found 14.31.

Ethyl (RS)-2-amino-3-(3-ethoxyisoxazol-5-yl)propionate hydrochloride (10). Compound **9** (3.78 g, 15.9 mmol) was suspended in a solution of ethanol (50 mL) containing 5% hydrogen chloride prepared by adding acetyl chloride to ethanol at 0 °C. The mixture was refluxed for 5 h. Evaporation and recrystallization from ethanol/ether afforded the ester hydrochloride **10** (3.11 g, 74%); mp 143–145 °C. ^1H NMR (D_2O) δ: 1.28 (*t*, 3H, *J* = 7 Hz), 1.39 (*t*, 3H, *J* = 7 Hz), 3.47 (*d*, 2H, *J* = 6 Hz), 4.2–4.4 (*m*, 4H), 4.55 (*t*, 1H, *J* = 6 Hz), 6.12 (*s*, 1H). Analysis ($C_{10}H_{17}N_2O_4Cl$, 1/4 H_2O) C, H, N, Cl.

Ethyl (SS)- and (2R,2'S)-2-[2-(tert-butoxycarbonyl)amino-1-oxo-3-phenylpropyl]amino-3-(3-ethoxyisoxazol-5-yl)propionate (12) and (13). 1,1'-Carbonyldiimidazole (0.50 g, 3.09 mmol) was added at 0 °C to a stirred solution of (*S*)-BOC-phenylalanine (**11**) (0.78 g, 2.94 mmol) in THF (8 mL). After stirring for 30 min at 0 °C, a solution of compound **10** [0.74 g prepared by partitioning the hydrochloride salt (0.92 g, 3.47 mmol) between ethyl acetate and Na_2CO_3 (1.1 eq. solution)] in THF (5 mL) was added. The solution was stirred overnight (0 °C → 25 °C) and THF removed under reduced pressure. The crude product was dissolved in dichloromethane and the organic layer washed with water, dried ($MgSO_4$) and evaporated. Column chromatography (dichloromethane/ethyl acetate) afforded a mixture of **12** and **13** (1.13 g, 81%) as a white powder; mp 113–115 °C. ^1H NMR ($CDCl_3$) δ: 1.26 (*t*, 6H, *J* = 7 Hz), 1.3–1.4 (*m*, 24H), 3.0–3.3 (*m*, 8H), 4.1–4.3 (*m*, 10H), 4.7–5.0 (*m*, 2H), 5.54 (*s*, 1H), 5.59 (*s*, 1H), 6.5–6.7 (*m*, 2H), 7.1–7.4 (*m*, 10H). Analysis ($C_{24}H_{33}N_3O_7$) C, H, N.

Ethyl (2R,2'S)-2-[(2-amino-1-oxo-3-phenyl)propyl]amino-3-(3-ethoxyisoxazol-5-yl)propionate (15) and (SS)-2-[(2-amino-1-oxo-3-phenyl)propyl]amino-3-(3-ethoxyisoxazol-5-yl)propionic acid (16). A mixture of compounds **12** and **13** (0.475 g, 1.0 mmol) was dissolved in dichloromethane (4 mL) and trifluoroacetic acid (TFA) (0.77 mL, 10.0 mmol) was added. The solution was stirred at room temperature for 2 h. Dichloromethane and TFA were removed under reduced pressure, and the residual product dissolved in water, treated with K_2CO_3 (until pH 11) and extracted with dichloromethane. The organic phase was washed with brine, dried ($MgSO_4$) and evaporated. The two diastereomers were separated on preparative TLC using ethyl acetate containing 2% MeOH as eluent. Compound **15** (R_f = 0.36): ^1H NMR ($CDCl_3$) δ: 1.28 (*t*, 3H, *J* = 7 Hz), 1.38 (*t*, 3H, *J* = 7 Hz), 2.67 (*dd*, 1H, *J* = 9.9 Hz, *J* = 13.7 Hz), 3.2–3.4 (*m*, 3H), 3.65 (*dd*, 1H, *J* = 4.2 Hz, *J* = 9.9 Hz), 4.24 (*t*, 2H, *J* = 7 Hz), 4.26 (*t*, 2H, *J* = 7 Hz), 4.85 (*dt*, 1H, *J* = 8.2 Hz, *J* = 6.0 Hz), 5.65 (*s*, 1H), 7.1–7.4 (*m*, 5H), 7.98 (*d*, 1H, *J* = 8.2 Hz). Compound **14** (R_f = 0.31) was unstable and decomposed to the carboxylic acid derivative **16**: ^1H NMR (D_2O) δ: 1.30 (*t*, 3H, *J* = 7 Hz), 1.88 (*dd*, 1H, *J* = 15 Hz, *J* = 7 Hz), 2.36 (*dd*, 1H, *J* = 15 Hz, *J* = 5 Hz), 2.83 (*dd*, 1H, *J* = 13 Hz, *J* = 5 Hz), 3.04 (*dd*, 1H, *J* = 13 Hz, *J* = 5 Hz), 3.9–4.1 (*m*, 1H), 4.13 (*t*, 2H, *J* = 7 Hz), 4.1–4.3 (*m*, 1H), 5.54 (*s*, 1H), 7.1–7.4 (*m*, 5H), 8.12 (*br s*, 1H).

Ethyl (R)-2-amino-3-(3-ethoxyisoxazol-5-yl)propionate hydrochloride (18). Phenylisocyanate (0.22 g, 1.6 mmol) was added at 0 °C to a stirred solution of **15** (0.40 g, 1.06 mmol) in THF (8 mL). The solution was allowed to warm to room temperature and evaporated after 1 h. The crude thiourea product obtained was dissolved in ethanol (3 mL) and added to a solution of ethanol (7 mL) containing acetyl chloride (0.38 mL, 5.3 mmol). The solution was stirred at room temperature for 30 min, evaporated and the remaining precipitate washed with ether and filtered. Recrystallization from ethanol/ether afforded compound **18** (0.14 g, 50%). ^1H NMR (D_2O) δ: 1.28 (*t*, 3H, *J* = 7.0 Hz), 1.38 (*t*, 3H, *J* = 7 Hz), 3.47 (*d*, 2H, *J* = 6.3 Hz), 4.2–4.4 (*m*, 4H), 4.54

(*t*, 1H, *J* = 6.3 Hz), 6.11 (*s*, 1H). Analysis ($C_{10}H_{17}N_2O_4Cl$) C, H, N, Cl.

(R)-2-Amino-3-(3-hydroxyisoxazol-5-yl)propionic acid [(R)-HIBO, 1]. Compound **18** (0.13 g, 0.49 mmol) was dissolved in 48% hydrobromic acid (2 mL) and refluxed at 140 °C for 30 min. The solution was rapidly cooled and evaporated, then twice dissolved in water and re-evaporated. After drying *in vacuo* (over KOH) the crude hydrobromide was dissolved in water (50 µL) and ethanol (100 µL) and neutralized with a solution of triethylamine in ethanol to pH 3–4 to afford zwitterionic (*R*)-HIBO after recrystallization from water (27 mg, 37%); mp 225 °C (decomp.). ^{1}H NMR (D_2O) δ (hydrobromide): 3.33 (*d*, 2H, *J* = 6.1 Hz), 4.37 (*t*, 1H, *J* = 6.1 Hz), 5.95 (*s*, 1H). IR: 3010 (br s) 1640 (s), 1620 (s), 1590 (s), 1510 (s), 1420 (s) cm^{-1}. Analysis ($C_6H_8N_2O_4$, 1/6 H_2O) C, H, N. $[\alpha]_{365}^{25}$ = +86.7° (*c* 0.3, pH 9.5).

(S)-2-Amino-3-(3-hydroxyisoxazol-5-yl)propionic acid [(S)-HIBO, 2]. Compound **16** (0.2 g, 0.58 mmol) was dissolved in 48% hydrobromic acid (3 mL) and refluxed at 140 °C for 30 min. The solution was rapidly cooled and evaporated, then twice dissolved in water and re-evaporated. To a solution of this crude mixture in water (1 mL) was added triethylamine (0.212 g, 2.1 mmol) and a solution of di-*tert*-butyl dicarbonate (0.172 g, 0.75 mmol) in THF (1 mL). After stirring for 16 h at 20 °C, THF was evaporated and the aqueous mixture was acidified with 4 M hydrochloric acid to pH 2 and extracted with ethyl acetate (3 × 8 mL). The combined organic phases were dried ($MgSO_4$), evaporated and the residue was submitted to column chromatography (eluent: ethyl acetate containing 10% acetone and 1% AcOH) to give **17** (104 mg, 66%). ^{1}H NMR ($CDCl_3$) δ: 1.39 (*t*, 3H, *J* = 7.1 Hz), 1.43 (*s*, 9H), 3.2–3.3 (*m*, 2H), 4.25 (*q*, 2H, *J* = 7.1 Hz), 4.5–4.7 (*m*, 1H), 5.3–5.4 (*m*, 1H), 5.75 (*s*, 1H). Compound **17** (100 mg, 0.37 mmol) was dissolved in dichloromethane (2 mL) and trifluoroacetic acid (0.28 mL, 3.6 mmol) was added. The solution was stirred at room temperature for 2 h and then evaporated. The crude product was dissolved in water (50 µL) and ethanol (100 µL) and neutralized with a solution of triethylamine in ethanol to pH 3–4 to afford zwitterionic (*S*)-HIBO after recrystallization from water (20 mg, 32%); mp 224 °C (decomp.). ^{1}H NMR similar to that of (*R*)-HIBO. IR: 2990 (br s) 1650 (s), 1630 (s), 1540 (s), 1510 (s), 1430 (s). $[\alpha]_{365}^{25}$ = –86.0° (*c* 0.3, pH 9.5).

In vitro *pharmacology*

Binding experiments using the ligands [^{3}H]CCP, [^{3}H]AMPA, [^{3}H]kainic acid and [^{3}H]glutamic acid were performed as described in Refs 14, 15, 16 and 20, respectively. A rat cortical slice preparation for determination of excitatory amino acid-evoked depolarizations described by Harrison and Simmonds[13] was used in a slightly modified version.[18]

Acknowledgements

This work was supported by grants from the EEC (BIO2-CT93-0243), the Danish State Biotechnology Programme (1991–1995), and the Lundbeck Foundation. The secretarial assistance of Mrs Anne Nordly and Dorthe Persson is gratefully acknowledged.

References

1. Hollmann, M.; Heinemann, S. *Ann. Rev. Neurosci.* **1994**, *17*, 31.

2. Nakanishi, S. *Science* **1992**, *258*, 597.

3. Krogsgaard-Larsen, P.; Honoré, T.; Hansen, J. J.; Curtis, D. R.; Lodge, D. *Nature* **1980**, *284*, 64.

4. Madsen, U.; Ferkany, J. W.; Jones, B. E.; Ebert, B.; Johansen, T. N.; Holm, T.; Krogsgaard-Larsen, P. *Eur. J. Pharmacol. Mol. Pharmacol. Sect.* **1990**, *189*, 381.

5. Watkins, J. C.; Olverman, H. J. *Trends Neurosci.* **1987**, *10*, 265.

6. Hansen, J. J.; Lauridsen, J.; Nielsen, E.; Krogsgaard-Larsen, P. *J. Med. Chem.* **1983**, *26*, 901.

7. Hansen, J. J.; Nielsen, B.; Krogsgaard-Larsen, P.; Brehm, L.; Nielsen, E. Ø.; Curtis, D. R. *J. Med. Chem.* **1989**, *32*, 2254.

8. Sheardown, M. J.; Nielsen, E. Ø.; Hansen, A. J.; Jacobsen, P.; Honoré, T. *Science* **1990**, *247*, 571.

9. Roberts, C. J.; Nielsen, E.; Krogsgaard-Larsen, P.; Walker, R. J. *Comp. Biochem. Physiol.* **1982**, *73C*, 439.

10. Hansen, J. J.; Krogsgaard-Larsen, P. *Med. Res. Rev.* **1990**, *10*, 55.

11. Hansen, J. J.; Krogsgaard-Larsen, P. *J. Chem. Soc. Perkin Trans 1* **1980**, 1826.

12. Moon, M. W.; Hsi, R. S. P. *J. Labelled Compd Radiopharm.* **1992**, *31*, 933.

13. Harrison, N. L.; Simmonds, M. A. *Br. J. Pharmacol.* **1985**, *84*, 381.

14. Murphy, D. E.; Schneider, J.; Boehm, C.; Lehmann, J.; Williams, K. *J. Pharmacol. Exp. Ther.* **1987**, *240*, 778.

15. Honoré, T.; Nielsen, M. *Neurosci. Lett.* **1985**, *54*, 27.

16. Braitman, D. J.; Coyle, J. T. *Neuropharmacology* **1987**, *26*, 1247.

17. Pin, J.-P.; Bockaert, J.; Recasens, M. *FEBS Lett.* **1984**, *175*, 31.

18. Madsen, U.; Ebert, B.; Hansen, J. J.; Krogsgaard-Larsen, P. *Eur. J. Pharmacol.* **1993**, *230*, 383.

19. Ebert, B.; Lenz, S.; Brehm, L.; Bregnedal, P.; Hansen, J. J.; Frederiksen, K.; Bøgesø, K. P.; Krogsgaard-Larsen, P. *J. Med. Chem.* **1994**, *37*, 878.

20. Honoré, T.; Drejer, J.; Nielsen, M.; Braestrup, C. *J. Neural Trans.* **1986**, *65*, 93.

(*Received in U.S.A.* 20 *November* 1994; *accepted* 14 *February* 1995)