6,8-Dimethyl-1*H*-imidazo[4,5-*f*]quinazoline-7,9-(6*H*,8*H*)-dione (3f, *prox*-Benzotheophylline). A mixture of 1 g (4 mmol) of **12a** in 20 mL of 97% formic acid, to which 100 mg of 10% Pd-C was added under N_2, was shaken under H_2 (initial pressure 52 psi) for 3 h. The catalyst was removed by filtration and the filtrate was then refluxed under N_2 for 2 h. This solution was evaporated to dryness on a rotary evaporator and a mixture of 25 mL of formic acid and 25 mL of toluene was added to the residue and the reflux was resumed for an additional 5 h under N_2. This solution was also evaporated to dryness to result in **3f** (Table IV).

1,3-Dimethyl-5-(methylamino)-6-nitro-2,4(1*H*,3*H*)-quinazolinedione (20). A mixture of 1 g (3.7 mmol) of **19** and 10 mL of absolute 1-BuOH (which had been saturated with anhydrous $MeNH_2$ at room temperature) was heated at 140 °C for 24 h in a sealed, stainless steel reaction vessel. Following this period, the vessel was cooled to -20 °C for 12 h and the precipitated solid was isolated by filtration and washed with Et_2O to give **20** as described in Table II.

1,6,8-Trimethyl-1*H*-imidazo[4,5-*f*]quinazoline-7,9-(6*H*,8*H*)-dione (3g, *prox*-Benzocaffeine). A mixture of 0.5 g (1.89 mmol) of **20** and 50 mL of 97% formic acid, to which 100 mg of 10% Pd-C was added under N_2, was shaken under H_2 (initial pressure 50 psi) for 3 h. The catalyst was removed by filtration and the filtrate was refluxed for 3 h under N_2. The formic acid was then removed in vacuo and 50 mL of toluene was added to the residue. The toluene was, in turn, removed to dryness and the residue was purified to give **3g** as described in Table IV.

Adenosine-Receptor Assay. A_1 affinities were determined in [^{3}H]-N^6-cyclohexyladenosine binding in rat brain membranes,[3f] and A_2 affinities were determined in [^{3}H]NECA binding in rat striatal membranes in the presence of 50 nM N^6-cyclopentyladenosine.[3f] A_1 and A_2 assays were carried out at 25 °C for 1 h with 1 nM [^{3}H]-N^6-cyclohexyladenosine (25 Ci/mmol) or 4 nM [^{3}H]NECA (30 Ci/mmol), respectively. All values are means of two or more independent determinations.

Acknowledgment. We appreciate the excellent assistance of Nancy Raber in preparing this manuscript and Gina Lu and Jim Fergus for performing the binding assays.

Registry No. **2a**, 121496-93-3; **2b**, 121496-94-4; **2c**, 121496-95-5; **2d**, 121496-96-6; **2e**, 101031-51-0; **2f**, 76822-71-4; **2g**, 76832-42-3; **3a**, 121496-97-7; **3b**, 121496-98-8; **3c**, 121496-99-9; **3d**, 121497-00-5; **3e**, 101031-57-6; **3f**, 78754-88-8; **3g**, 78754-89-9; **4**, 76822-66-7; **5b**, 107731-67-9; **5c**, 121496-85-3; **5d**, 121496-86-4; **6a**, 76822-72-5; **6b**, 107710-65-6; **6c**, 121496-87-5; **6d**, 121496-88-6; **7b**, 93355-82-9; **9a**, 101031-64-5; **9b**, 101031-66-7; **10**, 121497-01-6; **11a**, 78754-82-2; **11b**, 78754-83-3; **11c**, 78754-81-1; **12a**, 78754-86-6; **12b**, 121496-89-7; **12c**, 121496-90-0; **12d**, 121496-91-1; **13a**, 41632-04-6; **13b**, 19407-42-2; **13c**, 70625-65-9; **14a**, 118470-98-7; **14b**, 121496-92-2; **15a**, 101031-68-9; **15b**, 101031-70-3; **16**, 54166-95-9; **18**, 76822-74-7; **19**, 78754-85-5; **20**, 78754-87-7; 3-chloro-2-methylaniline, 87-60-5.

Excitatory Amino Acid Agonists. Enzymic Resolution, X-ray Structure, and Enantioselective Activities of (*R*)- and (*S*)-Bromohomoibotenic Acid

Jan J. Hansen,*[†] Birgitte Nielsen,[†] Povl Krogsgaard-Larsen,[†] Lotte Brehm,[†] Elsebet Ø. Nielsen,[†] and David R. Curtis[‡]

PharmaBiotec, Department of Chemistry BC, The Royal Danish School of Pharmacy, DK-2100 Copenhagen Ø, Denmark, and Department of Pharmacology, The Australian National University, Canberra, A.C.T 2601, Australia.
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The enantiomers of α-amino-4-bromo-3-hydroxy-5-isoxazolepropionic acid (4-bromohomoibotenic acid, Br-HIBO, 1), a selective and potent agonist at one class of the central (*S*)-glutamic acid receptors, were prepared with an enantiomeric excess higher than 98.8% via stereoselective enzymic hydrolysis of (*RS*)-α-(acetylamino)-4-bromo-3-methoxy-5-isoxazolepropionic acid (**4**) using immobilized aminoacylase. The absolute configuration of the enantiomers of Br-HIBO was established by X-ray crystallographic analysis, which confirmed the expected preference of the enzyme for the *S* form of the substrate **4**. (*S*)- and (*RS*)-Br-HIBO were potent neuroexcitants on cat spinal neurones in vivo, while (*R*)-Br-HIBO was a very weak excitant. Correspondingly, the *S* enantiomer of Br-HIBO (IC_{50} = 0.34 μM) was considerably more potent than the *R* form (IC_{50} = 32 μM) as an inhibitor of [^{3}H]-(*RS*)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid ([^{3}H]AMPA) binding to rat brain synaptic membranes in vitro. In contrast, (*S*)- and (*R*)-Br-HIBO were approximately equipotent (IC_{50} values of 0.22 and 0.15 μM, respectively) as inhibitors of [^{3}H]-(*S*)-glutamic acid binding in the presence of $CaCl_2$. The enantiomers of Br-HIBO showed no significant affinity for those binding sites on rat brain membranes which are labeled by [^{3}H]kainic acid or [^{3}H]-(*R*)-aspartic acid.

(*S*)-Glutamic acid [(*S*)-Glu] and (*S*)-aspartic acid [(*S*)-Asp] are now widely recognized as excitatory neurotransmitters in the mammalian central nervous system (CNS).[1,2] In analogy to other neurotransmitters, multiple receptors seem to exist in the CNS for excitatory amino acids (EAA's), and on the basis of electrophysiological in vivo experiments and in vitro binding studies at least three receptor classes[3,4] for EAA's have been characterized by their relative sensitivity to a number of agonists and antagonists: NMDA receptors, where *N*-methyl-(*R*)-aspartic acid (NMDA) is a potent and selective agonist, and a number of compounds, notably (*R*)-2-amino-5-phosphonopentanoic acid [(*R*)-AP5] and (*RS*)-[3-(2-carboxypiperazin-4-yl)propyl]phosphonic acid (CPP), are potent and selective antagonists;[3] QUIS/AMPA receptors, where (*S*)-quisqualic acid (QUIS) is a potent but nonselective agonist,[4] (*RS*)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) is a potent and selective agonist,[5] and (*S*)-Glu diethyl ester [(*S*)-GDEE] is a weak but selective antagonist;[6] and KAIN receptors, where

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

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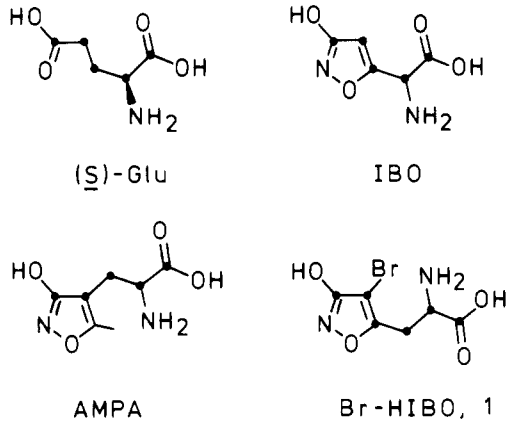
Chart I

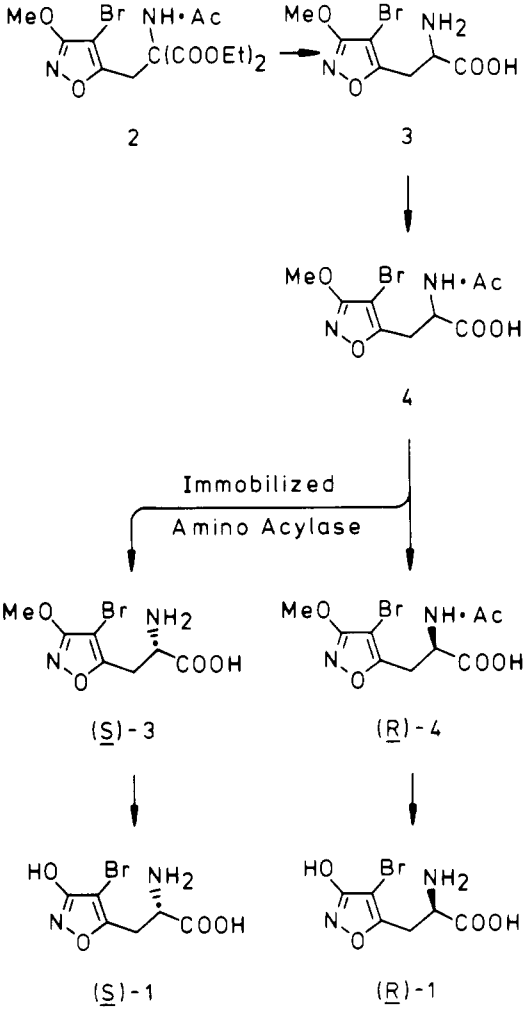


(2*S*,3*S*,4*S*)-α-kainic acid (kainic acid, KAIN) is a relatively selective agonist not affected by any of the above antagonists.[4] In addition, a fourth receptor class has been proposed[7] and named for (*S*)-2-amino-4-phosphonobutanoic acid [(*S*)-AP4], which at these receptors acts as an antagonist of synaptic transmission[8] but does not affect excitations induced by NMDA, QUIS, AMPA, or KAIN. Further subclassification of the NMDA and the QUIS/AMPA receptors has also been suggested.[9,10]

Ibotenic acid (IBO) is a constituent of the fly agaric mushroom, *Amanita muscaria*, and may be regarded as a conformationally restricted analogue of (*S*)-Glu, the acidic 3-hydroxyisoxazole moiety being a bioisostere of the γ-carboxyl group (Chart I). IBO shows potent neuroexcitatory properties and preferentially interacts with NMDA receptors[6,11] but, in addition, shows some affinity to the QUIS/AMPA and KAIN receptors.[5,11] By structural manipulations of IBO, we have designed and synthesized a number of isoxazole amino acids with potent and selective actions at either QUIS/AMPA or NMDA receptors.[12] As stated above, the (*S*)-Glu analogue AMPA is a highly selective agonist for QUIS/AMPA receptors,[5] and radiolabeled AMPA has been used extensively for the characterization of this class of receptors. AMPA was previously resolved by using an enzymic procedure.[13] The neuroexcitatory properties were mainly associated with the *S* enantiomer,[14] and (*S*)-AMPA, accordingly, was considerably more potent than the *R* form as an inhibitor of [^{3}H]AMPA binding.[13] Both enantiomers were essentially inactive as inhibitors[15] of [^{3}H]-(*S*)-Glu binding in the presence of $CaCl_2$.

(*RS*)-α-Amino-4-bromo-3-hydroxy-5-isoxazolepropionic acid (4-bromohomoibotenic acid, Br-HIBO, 1) is a structural homologue of (*S*)-Glu[16] and, accordingly, an analogue of the NMDA receptor antagonist α-aminoadipic acid (αAA). Yet, Br-HIBO was, like AMPA, a selective agonist at QUIS/AMPA receptors in vivo and in vitro with no affinity for NMDA receptors in vivo or KAIN receptors in vitro.[5,17] However, in contrast to AMPA, Br-HIBO was a relatively potent inhibitor of $CaCl_2$-dependent (*S*)-Glu binding.[17] We therefore found it worthwhile to investigate the in vivo and in vitro activities of the enantiomers of this compound after an enzymic resolution procedure analogous to the one previously performed on AMPA.[13] A preliminary account of the results has been presented.[18]

Scheme I



Results

Chemistry. The resolution of Br-HIBO was performed as outlined in Scheme I. The previously described intermediate, diethyl acetamido[(4-bromo-3-methoxyisoxazol-5-yl)methyl]malonate[16] (**2**), was hydrolyzed with concomitant monodecarboxylation into (*RS*)-α-amino-4-bromo-3-methoxy-5-isoxazolepropionic acid (*O*-Me-Br-HIBO) (**3**) and subsequently acetylated to yield (*RS*)-α-(acetylamino)-4-bromo-3-methoxy-5-isoxazolepropionic acid (*N*-Ac-*O*-Me-Br-HIBO) (**4**), which served as substrate for the enzymic hydrolysis. The acetylation was performed with acetyl chloride in pyridine or by using acetyl chloride

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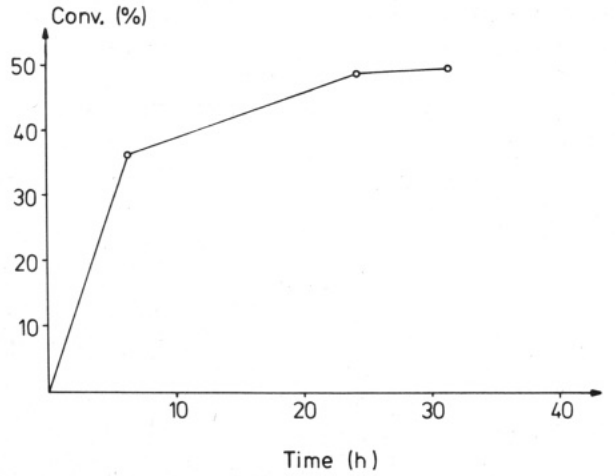

Figure 1. Progression of the enzymic hydrolysis of *N*-Ac-*O*-Me-Br-HIBO.

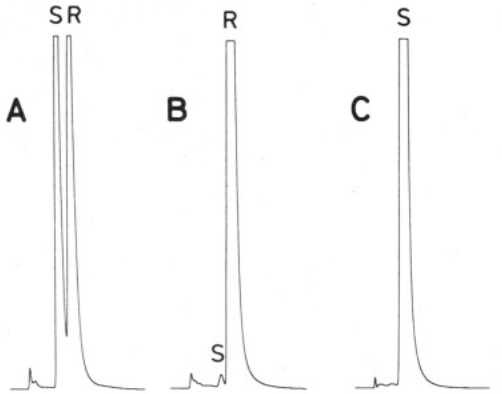

Figure 2. Chiral stationary phase HPLC of (A) (*RS*)-, (B) (*R*)-, and (C) (*S*)-Br-HIBO.

in a mixture of ethyl acetate and acetic acid with added triethylamine, following a published general procedure.[19] Both of these methods gave better yields of **4** than was obtained by following an acetylation procedure[13] using acetic anhydride in acetic acid.

The enzymic hydrolysis was performed with the commercially available enzyme aminoacylase (*N*-acylamino-acid amidohydrolase, EC 3.5.1.14) in an immobilized form. Preliminary experiments using soluble enzyme showed, in agreement with earlier observations,[20] that low concentrations of Co^{2+} caused a large increase in the hydrolysis rate. The preparative resolution using immobilized enzyme was therefore performed in the presence of 1 mM $CoCl_2$, and the progression of the hydrolysis was followed by monitoring the formation of free amino acid via its reaction with ninhydrin. The reaction progressed until ca. 50% conversion of the racemic starting material **4** (Figure 1), and after workup by ion-exchange chromatography, the hydrolysis product (*S*)-**3** and the unreacted starting material (*R*)-**4** were isolated in optically active forms. The immobilized enzyme, which was recovered from the reaction mixture by filtration, still possessed 80% of its original activity, after washing and lyophilization. Deprotection of (*S*)-**3** and (*R*)-**4** by using hydrogen bromide in glacial acetic acid and aqueous hydrobromic acid, respectively, furnished the two enantiomers of **1**.

Enantiomeric Purity. The optical purity of the products was determined by ligand-exchange HPLC

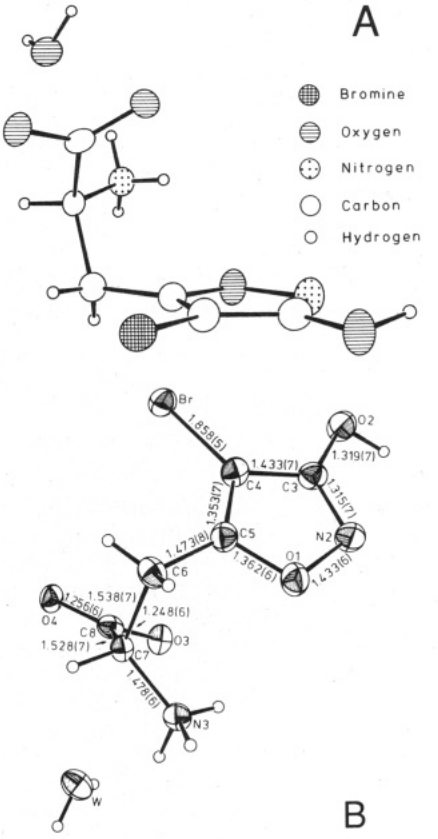

Figure 3. Perspective drawings from two different views of the molecular structure of (*S*)-Br-HIBO in the crystalline state, showing atom numbering and bond distances (Å) between non-hydrogen atoms. The intramolecular distances N3···O1, N3···O2, N3···N2, C8···O1, C8···O2, and C8···N2 are 3.012 (5), 6.137 (6), 4.285 (6), 3.672 (6), 5.560 (6), and 4.585 (7) Å, respectively. Thermal ellipsoids for non-hydrogen atoms are scaled to 50% probability; hydrogen atoms are represented as spheres of arbitrary radius.

(Figure 2) on a chiral stationary phase[21] containing (*R*)-proline as described in the Experimental Section. The enantiomeric excess was found to be 98.8% and 99.8% for (*R*)-**1** and (*S*)-**1**, respectively. The chromatogram shown in Figure 2B allows quantification of the amount of (*S*)-**1** present in the preparation of (*R*)-**1**, but the presence of 0.1% enantiomeric impurity in the preparation of (*S*)-**1** cannot be determined from the chromatogram shown in Figure 2C, because the small peak due to (*R*)-**1** is obscured by the tail of the major peak. However, when a similarly prepared column containing (*S*)-proline instead of (*R*)-proline is used, the small peak due to (*R*)-**1** now elutes before the main peak (chromatogram not shown), and quantification can be performed as in Figure 2B.

X-ray Crystallography. The absolute configuration of the products was established by an X-ray crystallographic analysis of that enantiomer of Br-HIBO which was assumed from the progression of the enzymic reaction to be of the *S* configuration. Perspective drawings of the molecule with atom numbering and bond lengths between non-hydrogen atoms are shown in Figure 3. Valency angles between non-hydrogen atoms are listed in Table I.

The molecule crystallizes as a monohydrate in a zwitterionic form with the hydroxy group of the isoxazole nucleus un-ionized. The isoxazole ring is planar within the limits of experimental error. The exocyclic atoms Br, C6, O2, and the hydrogen atom (H21) of the 3-hydroxy group are at distances −0.09, −0.03, 0.03, and −0.18 Å, respectively,

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Table I. Valency and Torsion Angles of (*S*)-Br-HIBO Monohydrate[a]

	Valency Angles (deg)		
C5–O1–N2	109.1 (4)	C4–C5–C6	133.7 (5)
O1–N2–C3	105.1 (4)	O1–C5–C6	117.4 (4)
N2–C3–C4	111.3 (5)	C5–C6–C7	114.4 (4)
N2–C3–O2	124.6 (5)	C6–C7–C8	110.2 (4)
C4–C3–O2	124.1 (5)	C6–C7–N3	112.0 (4)
C3–C4–C5	105.6 (4)	C8–C7–N3	110.0 (4)
C3–C4–Br	127.1 (4)	C7–C8–O3	117.5 (4)
C5–C4–Br	127.2 (4)	C7–C8–O4	116.9 (4)
C4–C5–O1	109.1 (5)	O3–C8–O4	125.6 (5)
	Torsion Angles (deg)		
C5–O1–N2–C3	0.4 (5)	C5–C6–C7–N3	67.3 (6)
O1–N2–C3–C4	–0.1 (5)	C5–C6–C7–C8	–55.6 (5)
N2–C3–C4–C5	–0.2 (6)	N3–C7–C8–O3	–29.0 (5)
C3–C4–C5–O1	0.4 (5)	C6–C7–C8–O4	–84.5 (5)
C4–C5–O1–N2	–0.5 (5)	N2–C3–O2–H21	15 (5)
C4–C5–C6–C7	109.4 (6)	O2–C3–C4–Br	5.5 (7)
O1–C5–C6–C7	–69.5 (6)	C6–C5–C4–Br	–2.5 (8)

[a] Estimated standard deviations are given in parentheses.

Table II. Hydrogen-Bond Distances (Å) and Angles (deg) and Other Close Contacts of (*S*)-Br-HIBO Monohydrate[a]

D–H···A[b]	D–H	D···A	H···A	∠DHA
N3–H31···O1^{i}	0.79 (8)	3.011 (9)	2.53 (8)	121 (7)
N3–H31···O3^{i}	0.79 (8)	2.698 (6)	2.5 (1)	93 (7)
N3–H31···O3^{ii}	0.79 (8)	2.925 (5)	2.22 (8)	149 (7)
N3–H32···OW^{i}	0.92 (8)	2.764 (6)	1.97 (9)	143 (7)
N3–H33···O3^{iii}	1.00 (9)	2.799 (6)	1.83 (9)	161 (8)
O2–H21···O4^{iv}	0.95 (8)	2.563 (5)	1.70 (8)	149 (8)
OW–HW1···N2^{v}	1.0 (1)	2.913 (6)	2.0 (1)	178 (7)
OW–HW2···O4^{vi}	0.83 (9)	2.813 (5)	1.99 (9)	175 (9)
	Other Close Contacts			
O2·····Br^{vii}		3.004 (4)		
C7·····OW^{i}		3.047 (6)		
C8·····OW^{i}		2.941 (6)		

[a] Estimated standard deviations are given in parentheses. [b] Symmetry code: (i) x, y, z; (ii) $^1/_2 + x, ^1/_2 - y, 1 - z$; (iii) $x + 1, y, z$; (iv) $x, y - 1, z$; (v) $x - ^1/_2, ^1/_2 - y, 1 - z$; (vi) $^1/_2 + x, 1^1/_2 - y, 1 - z$; (vii) $-x, ^1/_2 + y, ^1/_2 - z$.

from the least-squares plane through the isoxazole ring atoms. The conformation of the side chain is described by the selected torsion angles listed in Table I. The absolute configuration of the compound was found to be *S*, confirming that the well-established[20] stereoselectivity of the enzyme is maintained also for this xenobiotic amino acid. The crystal structure is stabilized by hydrogen bonds. All hydrogen atoms covalently bonded to nitrogen or oxygen atoms are utilized in the formation of hydrogen bonds (Table II). One of the hydrogen atoms (H31) of the ammonium group may be involved in four-centered ("trifurcated") hydrogen bonding. The dimensions of these bonds are within published limits.[22] The hydrogen atom positions have not been "normalized".[22]

Binding Affinity. The receptor affinities in vitro of the two Br-HIBO enantiomers were investigated in ligand binding assays using purified synaptic membranes from rat brain (Table III). All assays showed stereoselective inhibition by the enantiomers of Glu. The [^{3}H]AMPA binding site, assumed to represent the physiological QUIS/AMPA receptor, showed a marked stereoselectivity toward the enantiomers of Br-HIBO, the *S* form being 2 orders of magnitude more potent than the *R* form. Neither (*S*)- nor (*R*)-Br-HIBO showed any significant affinity for [^{3}H]KAIN binding sites or [^{3}H]-(*R*)-Asp binding sites, the latter of which is assumed to represent a high-affinity

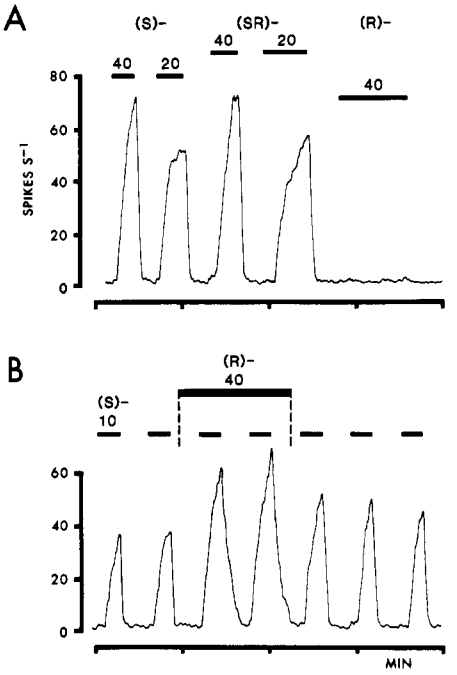

Figure 4. Comparison of the excitation of a spinal interneurone by (*S*)-, (*R*)-, and (*RS*)-Br-HIBO, all ejected microelectrophoretically from 0.1 M solutions (pH 8, NaOH) within different barrels of a seven-barrel micropipette using currents (nA) and ejection times indicated by the numbers and the horizontal bars. The ordinates represent the cell-firing rate (spikes per second). The abscissae represent time (minutes).

neuronal (*S*)-Glu uptake carrier.[23] However, the two Br-HIBO enantiomers were approximately equipotent as inhibitors of [^{3}H]-(*S*)-Glu binding in the presence of $CaCl_2$.

Electrophysiology. The results from in vivo microelectrophoretic studies on cat spinal neurones of the enantiomers and the racemic form of Br-HIBO are shown in Figure 4. (*S*)-Br-HIBO was a potent neuroexcitant, while (*R*)-Br-HIBO showed very weak excitatory activity (Figure 4A). Interestingly, on most cells studied, the racemate of Br-HIBO was significantly more potent than (*S*)-Br-HIBO in exciting cat spinal neurones, indicating some degree of cooperative effects of the enantiomers. This is also apparent from Figure 4B, which shows that the presence of (*R*)-Br-HIBO caused a significant increase in the excitatory response to (*S*)-Br-HIBO.

Discussion

By using IBO as a lead structure we have previously synthesized and biologically tested a number of isoxazole amino acids as analogues or homologues of (*S*)-Glu. These isoxazole amino acids were all prepared as racemic compounds via multistep synthetic procedures and have provided information about the structural and conformational requirements for activation of different subtypes of central (*S*)-Glu receptors.[12] However, such structure–activity studies on racemic compounds are of limited value unless followed by stereostructure–activity studies on the individual stereoisomers. The aim of the present study was to resolve the central (*S*)-Glu receptor agonist Br-HIBO and to investigate the pharmacological profile of its enantiomers. Racemic Br-HIBO was previously found to be a potent neuroexcitant, which was effectively antagonized in vivo by (*S*)-GDEE but not by the NMDA receptor antagonist αAA.[5] From these and other studies, including

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Table III. In Vitro Binding Assays on Purified Synaptic Membranes from Rat Brain

	[^{3}H]AMPA binding: IC_{50}, μM	[^{3}H]KAIN binding: IC_{50}, μM	[^{3}H]-(*S*)-Glu binding: IC_{50}, μM	[^{3}H]-(*R*)-Asp binding: IC_{50}, μM
(*S*)-Br-HIBO [(*S*)-1]	0.34	>100	0.22	>100
(*R*)-Br-HIBO [(*R*)-1]	32	>100	0.15	>100
(*RS*)-Br-HIBO (1)	0.60	>100	0.20	>100
(*S*)-Glu	0.50	0.27	0.17	7.5
(*R*)-Glu	47	78	2.5	>100

in vitro binding data,[17] it was concluded that Br-HIBO is a selective agonist at the QUIS/AMPA subtype of central (*S*)-Glu receptors. The present study confirms and extends these results and shows that the effect of Br-HIBO on QUIS/AMPA receptors primarily is due to the *S* enantiomer and that neither of the enantiomers are substrates for Na^+-dependent neuronal uptake as reflected by their lack of inhibition of [^{3}H]-(*R*)-Asp binding. On the other hand, (*R*)-Br-HIBO is at least as potent as (*S*)-Br-HIBO as an inhibitor of [^{3}H]-(*S*)-Glu binding in the presence of $CaCl_2$. The physiological relevance of this $CaCl_2$-dependent (*S*)-Glu binding is largely unknown,[24] but a recent report has suggested that it may represent binding to glial transport sites.[25] If this is the case, then both enantiomers of Br-HIBO are inhibitors of and, perhaps, substrates for a glial uptake mechanism. This, in turn, could provide an explanation for the apparent paradox that the presence of the "inactive" *R* form increases the excitatory potency of the *S* enantiomer, because (*R*)-Br-HIBO would then be expected to inhibit glial uptake of the excitatory (*S*)-Br-HIBO, thus prolonging its presence near receptors and resulting in an increased excitatory response. The rapid offset of excitation, especially observed after application of (*S*)-Br-HIBO alone, may support the presence of an uptake mechanism, but the above interpretation is still quite speculative, and other explanations are possible.

Experimental Section

Chemistry. General Procedures. Melting points, determined in capillary tubes, are uncorrected. Elemental analyses were performed by P. Hansen at Chemical Laboratory II, University of Copenhagen. ^{1}H NMR spectra were recorded at 90 MHz on a JEOL FX 90Q spectrometer or, at 60 MHz, on a Varian EM 360L instrument; chemical shifts are recorded as δ values with tetramethylsilane or acetonitrile as internal standards. IR spectra, listed as ν_{max}, were recorded from KBr disks on a Perkin-Elmer grating infrared spectrophotometer, Model 781. Optical rotations were measured in thermostated microcuvettes on a Perkin-Elmer 241 polarimeter. Ninhydrin-photometric determinations were performed in 1-cm cuvettes at 570 nm on a modified Beckman spectrophotometer. TLC analyses were performed on silica gel 60 F_{254} precoated on aluminum sheets (Merck) and eluted with 1-butanol–acetic acid–water (4:1:1). Compounds were visualized on the TLC plates by UV light or by spraying with potassium permanganate (all compounds), ferric chloride (compounds containing 3-hydroxyisoxazole moieties), ninhydrin (compounds containing amino groups), or bromocresol green (acidic compounds). All evaporations were performed under vacuum on a rotary evaporator at temperatures below 40 °C.

(*RS*)-α-Amino-4-bromo-3-methoxy-5-isoxazolepropionic Acid Hemihydrate (*O*-Me-Br-HIBO, 3). Compound **3** was prepared from the previously[16] prepared compound **2** (219 mg, 0.54 mmol) by reflux in 1 M hydrochloric acid (30 mL) for 16 h. The resulting solution was evaporated and dried in vacuo over KOH and P_2O_5. The residue was dissolved in ethanol (5 mL), and triethylamine (a total of 57 μL) was added until the pH was 5–6 on moist pH-indicator paper. The precipitate was collected, dried in vacuo, and recrystallized from water. A total of 68 mg (46%) of recrystallized **3** was collected in two batches, both with mp 202–203 °C dec: ^{1}H NMR (D_2O) δ 3.32 (d, 2 H, J = 6 Hz), 3.97 (s, 3 H), 4.05 (t, 1 H, J = 6 Hz); IR 3440 (br, m), 3200–2400 (several bands, m), 1625 (s), 1540 (s), 1455 (m), 1415 (s), 1100 (m) cm^{-1}. Anal. ($C_7H_9N_2O_4Br \cdot {}^1/_2H_2O$) C, H, N.

(*RS*)-α-(Acetylamino)-4-bromo-3-methoxy-5-isoxazolepropionic Acid (*N*-Ac-*O*-Me-Br-HIBO, 4). A solution of **3** (89 mg, 0.34 mmol) in a mixture of glacial acetic acid (9 mL), ethyl acetate (9 mL), and triethylamine (100 μL, 0.71 mmol) was heated on a 50 °C warm oil bath, and acetyl chloride (28 μL, 0.40 mmol) was slowly added. The solution was evaporated after a 6-h reaction time and toluene (1 mL) was added three times and the mixture was reevaporated. To the residue, after drying in vacuo, was added ethyl acetate (5 mL), and the mixture was filtered to remove triethylamine hydrochloride. To the filtrate was added 135 μL of 14% ethanolic hydrogen chloride (0.27 mmol), and the mixture was evaporated to dryness. The residue was digested with water to dissolve the residual triethylamine hydrochloride, and the insoluble material was collected by filtration, dried in vacuo over P_2O_5, and recrystallized from acetone to yield TLC-pure **4** with mp 174–177 °C dec. The mother liquor was evaporated and recrystallized from water to yield a second crop of crystals: mp 174–176 °C dec (combined yield 80 mg, 77%); IR 3300 (s), 3200–2400 (several bands, m), 1710 (s), 1640 (s), 1620 (m), 1535 (s), 1450 (m), 1405 (s), 1260 (m) cm^{-1}. Anal. ($C_9H_{11}N_2O_5Br$) C, H, N, Br.

Another synthesis of **4** used an acetylation procedure in pyridine solution. To compound **3** (13 mg, 0.05 mmol), suspended in dry pyridine (2.5 mL), was added acetyl chloride (5 μL, 0.07 mmol). The reaction mixture was heated on a 45–50 °C warm oil bath, and after 16 h, an additional 5 μL of acetyl chloride was added, because TLC showed the presence of unreacted starting material. After an additional 4 h at 45–50 °C, the reaction mixture was evaporated, dissolved three times in water (1 mL), and reevaporated. The residue was dried in vacuo over P_2O_5 and dissolved in ethyl acetate (0.5 mL). A small amount of starting material was removed by filtration, and the filtrate was evaporated to dryness and recrystallized from water (0.3 mL) to give 7 mg (50%) of **4**, mp 174–176 °C, which by IR and TLC was identical with *N*-Ac-*O*-Me-Br-HIBO prepared above.

Enzymic Resolution. To an aqueous solution (24 mL) of **4** (73 mg, 0.24 mmol), neutralized with LiOH to pH 7.2, was added 240 μL of 0.1 M $CoCl_2$, which was adjusted to pH 7.1 with LiOH, and 134 mg (32 IU[26]) of Enzygel-immobilized aminoacylase (Boehringer). The reaction mixture was evacuated for a few minutes in order to expel air from the pores of the enzyme carrier, and the flask containing the reaction mixture was covered with Parafilm to prevent evaporation. The reaction mixture was then placed on a Griffin flask shaker at ambient temperature and gently shaken to keep the enzyme in suspension. At intervals the vibration was stopped, and after the enzyme had settled, an aliquot of the clear supernatant was removed and assayed for free amino acid by the ninhydrin method.[27] After 32 h, 49% of the starting material was converted into free amino acid (Figure 1), and the reaction was stopped by filtering off the enzyme. The enzyme was washed thoroughly with 4 × 10 mL of water, and the filtrate and washings were applied on an ion-exchange column (5 × 1 cm) containing ca. 4 mL of Amberlite IR-120 (H^+). The column was

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(26) Assayed at ca. 25 °C with 15 mM *N*-acetyl-(*S*)-methionine [*N*-Ac-(*S*)-Met] as substrate in 6.0 mL of 20 mM phosphate buffer, pH 7.0. After 30 or 60 min the assay was filtered and free amino acid was determined by the ninhydrin procedure of Moore and Stein.[27] One IU of the enzyme hydrolyzes *N*-Ac-(*S*)-Met at a rate of 1 μmol/min.
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eluted (ca. 0.5 mL/min) with water (50 mL) followed by 0.5 M (80 mL) and 1 M (150 mL) aqueous ammonia.

The initially acidic and subsequently neutral eluate was collected, evaporated, dissolved in water (5 mL), and reevaporated. After drying in vacuo over P_2O_5, the solid residue was recrystallized from water (0.5 mL) to give 31 mg (85%) of *N*-Ac-*O*-Me-(*R*)-Br-HIBO [(*R*)-**4**], pure by TLC: mp 98–100 °C; $[\alpha]^{25}_D$ −23.3° ± 0.8° (*c* 0.44, H_2O) and $[\alpha]^{25}_{365}$ −97° ± 2° (*c* 0.44, H_2O); IR 3300 (s), 3200–2800 (several bands, m), 1730 (s), 1640 (s), 1625 (m), 1535 (s), 1450 (m), 1410 (m), 1255 (m) cm^{-1}.

The combined alkaline eluate (ca. 220 mL) was evaporated, dissolved in water (5 mL), and reevaporated. After drying in vacuo over P_2O_5, the solid residue was dissolved in water, treated with activated charcoal, evaporated, and recrystallized from water (0.4 mL) to give 24 mg (76%) of *O*-Me-(*S*)-Br-HIBO [(*S*)-**3**], pure by TLC: mp 182–4 °C dec; $[\alpha]^{25}_D$ +2.7° ± 0.7° (*c* 0.45, H_2O) and $[\alpha]^{25}_{365}$ +19° ± 1° (*c* 0.45, H_2O); IR 3200–2500 (several bands, m), 1610 (br, s), 1550 (m), 1515 (s), 1430 (m), 1390 (s), 1085 (m) cm^{-1}.

The immobilized enzyme used in the resolution was lyophilized for future reuse. The lyophilized enzyme showed a specific activity which was 80% of the activity before starting the resolution.

(*R*)-α-Amino-4-bromo-3-hydroxy-5-isoxazolepropionic Acid Monohydrate [(*R*)-Br-HIBO, (*R*)-1]. A solution of (*R*)-**4** (34 mg, 0.11 mmol) in 48% aqueous hydrobromic acid (5 mL) was covered with a nitrogen blanket and heated under reflux on a 140 °C warm oil bath for 17 min. The reaction mixture was quickly cooled under tap water, evaporated, dissolved three times in water (2 mL), and reevaporated. After drying in vacuo over KOH and P_2O_5, the residue was dissolved in ethanol (0.25 mL), and a solution of 20 vol % of triethylamine in ethanol was added until the pH was 3–4 on moist pH-indicator paper. The precipitated free amino acid was collected by filtration, dissolved in water, treated with charcoal, and evaporated before being recrystallized from water (1 mL) to give (*R*)-**1** (14.1 mg, 51%), isolated as the monohydrate. The mother liquor furnished an additional 2.1 mg of recrystallized product (combined yield 59%): mp 203 °C dec; $[\alpha]^{27}_D$ −10.8° ± 0.8° (*c* 0.39, 50 mM HCl) and $[\alpha]^{27}_{365}$ −43° ± 1° (*c* 0.39, 50 mM HCl); ^1H NMR (D_2O) δ 3.34 (m, AB part of ABX system), 4.09 (m, X part of ABX system); IR 3460 (br, s), 3240 (m), 3150–2300 (several bands, m), 1595 (s), 1530 (s), 1430 (m), 1340 (m), 1305 (m), 1280 (s), 1255 (m), 1095 (m) cm^{-1}. Anal. ($C_6H_7N_2O_4Br·H_2O$) C, H, N.

(*S*)-α-Amino-4-bromo-3-hydroxy-5-isoxazolepropionic Acid Monohydrate [(*S*)-Br-HIBO, (*S*)-1]. A solution of (*S*)-**3** (26.5 mg, 0.100 mmol) in 33% hydrogen bromide in glacial acetic acid (5 mL) was covered with a nitrogen blanket and placed in a stoppered flask at room temperature. After 17 h, the reaction mixture was evaporated, dissolved three times in water (2 mL), and reevaporated. After drying in vacuo over KOH and P_2O_5, the semicrystalline residue was dissolved in ethanol (0.5 mL), and a solution of 20 vol % of triethylamine in ethanol was added until the pH was 3–4 on moist pH-indicator paper. The precipitated free amino acid was collected by filtration, dissolved in water, treated with charcoal, and evaporated before being recrystallized from water to give (*S*)-**1** (14.4 mg, 58%), essentially pure by TLC. The mother liquor furnished a second crop of crystals (1.8 mg, combined yield 65%): mp 202 °C dec; $[\alpha]^{27}_D$ +10.3° ± 0.7° (*c* 0.43, 50 mM HCl) and $[\alpha]^{27}_{365}$ +45° ± 1° (*c* 0.43, 50 mM HCl); IR and ^1H NMR spectra identical with those of (*R*)-**1**.

HPLC Analysis. The enantiomeric purities of the products were determined by HPLC on a 120 × 4.6 mm column, containing a silica-based packing material with immobilized (*R*)-proline and chelated Cu^{2+}, prepared according to directions in the literature.[21] The column was thermostated at 50 °C and eluted at 1.0 mL/min with 50 mM potassium phosphate, pH 4.6, with Waters instrumentation consisting of an M510 pump connected to a U6K injector and an M481 spectrometric UV detector set at 210 nm. The enantiomeric composition was determined from peak areas with a Hitachi Chromato-Integrator D-2000.

X-ray Crystallographic Analysis of (*S*)-α-Amino-4-bromo-3-hydroxy-5-isoxazolepropionic Acid Monohydrate [(*S*)-Br-HIBO, (*S*)-1]. The colorless crystals used for the X-ray examination were grown by slowly cooling a heated aqueous solution of the compound. Crystal data are as follows: $C_6H_7N_2O_4Br·H_2O$, M_r = 269.07, orthorhombic, space group $P2_12_12_1$ (No. 19), a = 5.416 (1) Å, b = 8.090 (2) Å, c = 21.105 (6) Å, V = 924.7 Å^3, Z = 4, D_c = 1.932 g cm^{-3}, μ(Cu Kα) = 61.8 cm^{-1}.

A single, plate-shaped crystal of the size 0.05 × 0.10 × 0.30 mm was used for the determination of the unit-cell parameters and for the collection of intensity data. The measurements were performed at 110 (±5) K on an Enraf-Nonius CAD-4 diffractometer. The crystal was cooled in a stream of nitrogen gas provided by an Enraf-Nonius low-temperature device. The temperature was kept constant within 0.5 K during the experiment. Graphite-monochromated Cu Kα radiation (λ = 1.5418 Å) was used. Intensities of 2762 reflections (θ < 78°, h 0 → 6, k 0 → 10, l −26 → 26 and θ < 60°, h −6 → −3, k −7 → −3 and l −19 → 17) were measured using the ω scan mode. Intensities of two reflections measured every 10^4 s showed a 10% linear decay, for which the data were corrected. The data were corrected for absorption effects by an empirical method of Walker and Stuart.[28] The 2762 reflections were averaged (R_{int} = 0.043 on intensities) according to the point-group symmetry 222, resulting in 1947 reflections of which the 1670 with $|F_o|^2 \geq 3\ \sigma(|F_o|^2)$, $\sigma(|F_o|^2) = [\sigma_c^2(|F_o|^2) + (0.06|F_o|^2)^2]^{1/2}$ were classified as observed reflections and used in the subsequent structure determination and refinement. The $\sigma_c(|F_o|^2)$ was calculated from counting statistics. The structure was solved with standard Patterson and difference Fourier methods and refined by full-matrix least-squares methods. The quantity minimized was $\sum w(|F_o| - k|F_c|)^2$, where weights were initially taken as unity. The positions of all of the hydrogen atoms were obtained from intermediate difference maps. In subsequent full-matrix least-squares calculations an overall scale factor, atomic coordinates for all atoms and anisotropic thermal parameters for the non-hydrogen atoms were refined. The thermal parameters for the hydrogen atoms were fixed at B_{iso} = 2.5 Å^2. The refinements converged at R = 0.036, R_w = 0.046, $w^{-1} = \sigma^2(|F_o|)$, where $\sigma(|F_o|) = \sigma(|F_o|^2)/2|F_o|$. In the final difference density map the maximum peak (1.1 e Å^{-3}) and hole (−0.9 e Å^{-3}), which are about 2.5 times the general noise level in the map, were found near the bromine atom. The opposite polarity of the crystal could be rejected at a significance level $\alpha < 10^{-10}$ ($\mathcal{R}$ = 1.19, $\mathcal{R}_w$ = 1.20) by Hamilton's R value test performed as described by Rogers.[29] Neutral atomic scattering factors including terms for anomalous dispersion were those incorporated in the program system. All calculations were carried out by using the Enraf-Nonius Structure Determination Package.[30]

[^{3}H]AMPA Receptor Binding. Membrane preparation and binding assays were performed essentially as previously described[17] using a modification[31] of the original binding assay.[32] The frozen homogenate of rat brain cortices was thawed on the day of assay and centrifuged at 48000*g* for 10 min followed by twice washing the pellet by homogenization in 30 mM Tris-HCl buffer, pH 7.1, containing 2.5 mM $CaCl_2$, and centrifugation at 48000*g* for 10 min. The final pellet was homogenized in 30 mM Tris-HCl buffer, pH 7.1, containing 100 mM KSCN and 2.5 mM $CaCl_2$, (50 volumes per g of original tissue) and used for binding assays. Aliquots of 0.5 mL were incubated in triplicate for 30 min at 0 °C with 5 nM [^{3}H]AMPA (New England Nuclear, 27.5 Ci/mmol) and varying concentrations of test compound (final volume 550 μL). Nonspecific binding was determined by using 1.0 mM (*S*)-Glu in place of test compound. Free and bound radioactivity were separated by filtration through Whatman GF/C glass-fiber filters followed by washing three times with 5 mL of ice-cold buffer. IC_{50} values were calculated from at least four different concentrations of test compound by computerized log–probit analysis.

[^{3}H]KAIN Receptor Binding. The preparation of the membranes from rat brain cortices followed the same procedure as that used for AMPA binding.[31] The binding assay was performed as described earlier[33] on the basis of a published procedure.[34] The frozen homogenate was thawed on the day of assay

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and centrifuged at 48000*g* for 10 min followed by twice washing the pellet by homogenization in 50 mM Tris–citrate buffer, pH 7.1, and centrifugation at 48000*g* for 10 min. The final pellet was resuspended in 50 mM Tris–citrate buffer, pH 7.1 (50 volumes per g of original tissue), and 0.5-mL aliquots were incubated in triplicate for 60 min at 0 °C with 2 nM [^{3}H]KAIN (New England Nuclear, 60 Ci/mmol) and varying concentrations of test compound. Nonspecific binding was determined by using 1.0 mM (*S*)-Glu in place of test compound. Free and bound radioactivity were separated by filtration through Whatman GF/C glass-fiber filters followed by washing with 3 × 5 mL of ice-cold buffer. IC_{50} values were calculated as described above.

[^{3}H]-(*S*)-Glu Receptor Binding. The synaptic membranes fraction was prepared and the binding assay was performed as detailed elsewhere.[35] The synaptic membranes were prepared from rat cerebral cortex which was frozen overnight. The final pellet was resuspended in 30 mM Tris-HCl, pH 7.1, containing 2.5 mM $CaCl_2$, and aliquots of 2.5 mL (0.5 mg of protein) were incubated in triplicate for 20 min at 37 °C with 2 nM [^{3}H]-(*S*)-Glu (Amersham, 48 Ci/mmol) and varying concentrations of test compound. Nonspecific binding was determined by using 1.0 mM (*S*)-Glu in place of test compound. Free and bound radioactivity were separated by filtration through a Whatman GF/C glass-fiber filter followed by washing with 3 × 5 mL of ice-cold buffer. IC_{50} values were calculated as described for [^{3}H]AMPA binding.

[^{3}H]-(*R*)-Asp Binding. A crude synaptosomal preparation was obtained as described elsewhere.[36] The resulting P_2 pellet was suspended in 10 volumes of 0.32 M sucrose in 50 mM Tris-HCl, pH 7.4, by gentle use of a Whirlimixer until homogeneity was obtained. Aliquots (20 μL) of this synaptosomal preparation were preincubated for 2 min at 37 °C with 2.0 mL of Krebs medium containing the test compound. Then, [^{3}H]-(*R*)-Asp (Amersham, 25 Ci/mmol) was added to give a final concentration of 1 μM, and the incubation was continued for an additional 3 min. Free and bound radioactivity were separated by filtration through a Whatman GF/C glass-fiber filter followed by washing with 3 × 5 mL of ice-cold buffer. IC_{50} values were calculated as described for [^{3}H]AMPA binding.

Microelectrophoretic Studies. Experiments were performed on lumbar dorsal horn interneurones and on Renshaw cells of cats anesthetized with pentobarbitone sodium (initially 35 mg/kg intraperitoneally, supplemented intravenously when required). Extracellular action potentials were recorded by means of the center barrel of seven-barrel micropipettes, which contained 3.6 M NaCl. The compounds were administered electrophoretically from the outer barrels of the micropipettes,[37] which contained 0.1 M aqueous solutions (pH 8 with NaOH) of (*S*)-, (*R*)-, and (*RS*)-Br-HIBO. The compounds were administered for times sufficient to obtain maximal effect at the particular rate of ejection. The relative potencies of the compounds were determined from a comparison of the electrophoretic currents required to produce equal and submaximal excitation of the cells.

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Registry No. (*R*)-**1**, 121313-14-2; (*S*)-**1**, 121313-15-3; (*S*)-**1**·H_2O, 121313-18-6; (*RS*)-**1**, 71366-32-0; **2**, 71366-31-9; (*S*)-**3**, 121313-16-4; (*RS*)-**3**, 121253-50-7; (*R*)-**4**, 121313-17-5; (*RS*)-**4**, 121253-51-8; aminoacylase, 9012-37-7.

Supplementary Material Available: X-ray crystallographic data including tables listing final positional and equivalent isotropic or isotropic thermal parameters, intramolecular distances and angles with estimated standard deviations, and anisotropic thermal parameters of the non-hydrogen atoms (3 pages); a list of structure factors (20 pages). Ordering information is given on any current masthead page.

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