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ENZYMATIC RESOLUTION OF AMPA BY USE OF α-CHYMOTRYPSIN

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Abstract: The enantiomers of AMPA and its diethyl derivative were prepared by an enzymatic resolution using α-chymotrypsin in solution. Electrophysiological studies in vivo and in vitro showed the excitatory effects of AMPA to reside mainly in the L-enantiomer in agreement with the IC_{50} values of 0.02 μM and 76 μM observed for the L- and D-enantiomer, respectively, in [^{3}H]AMPA binding assays.

Introduction

(*RS*)-2-Amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propanoic acid (AMPA) was designed and synthesized as a structural analogue of the excitatory neurotransmitter (*S*)-glutamic acid (L-Glu) in which the γ-carboxyl group of L-Glu is replaced by an acidic 3-hydroxyisoxazole moiety.[1] This heterocyclic group is also found in the naturally occurring excitatory amino acid (EAA) ibotenic acid (IBO), but AMPA, in contrast to IBO, is a selective and potent agonist[2] at the EAA receptor subtype now termed AMPA receptors. The wide utility of this type of bioisosteric replacement has since been demonstrated by a large number of 3-hydroxyisoxazole amino acids with selective and potent excitatory action at either AMPA or *N*-methyl-(*R*)-aspartic acid (NMDA) receptors.[3]

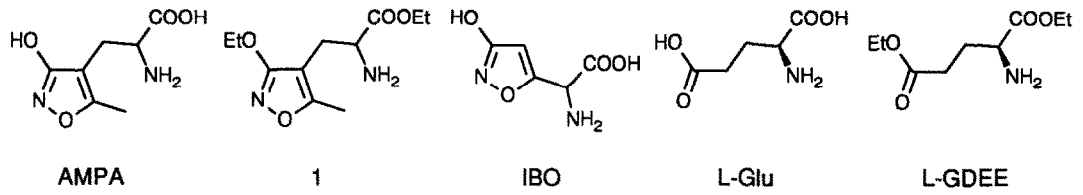

Figure 1. Structures (uncharged) of compounds mentioned in the text.

AMPA has previously been resolved using immobilized amino acylase,[4] and the absolute configuration of the enantiomers was empirically assigned from the well documented stereoselectivity of this enzyme.[5] The stereochemical assignment has now been supported by the resolution of AMPA using

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α-chymotrypsin (EC 3.4.21.1), another hydrolytic enzyme with a well-defined, albeit complex, stereoselectivity.[6] The α-chymotrypsin-catalyzed procedure proceeds much faster than the resolution with amino acylase and employs a solution of a cheaper and more readily available enzyme. In addition, the enantiomers of ethyl 2-amino-3-(3-ethoxy-5-methylisoxazol-4-yl)propanoate (**1**; Figure 1) were prepared as possible bioisosteres of L-GDEE, the diethyl ester of L-Glu, which has been used as a weak but selective antagonist at AMPA receptors.[2]

Results and Discussion

The substrate for the α-chymotrypsin-catalyzed resolution (Figure 2), ethyl (*RS*)-2-acetylamino-3-(3-ethoxy-5-methylisoxazol-4-yl)propanoate (**4**), was synthesized by monodecarbethoxylation[7] of the previously prepared[8] diethyl acetylamino[(3-ethoxy-5-methylisoxazol-4-yl)methyl]malonate (**5**).

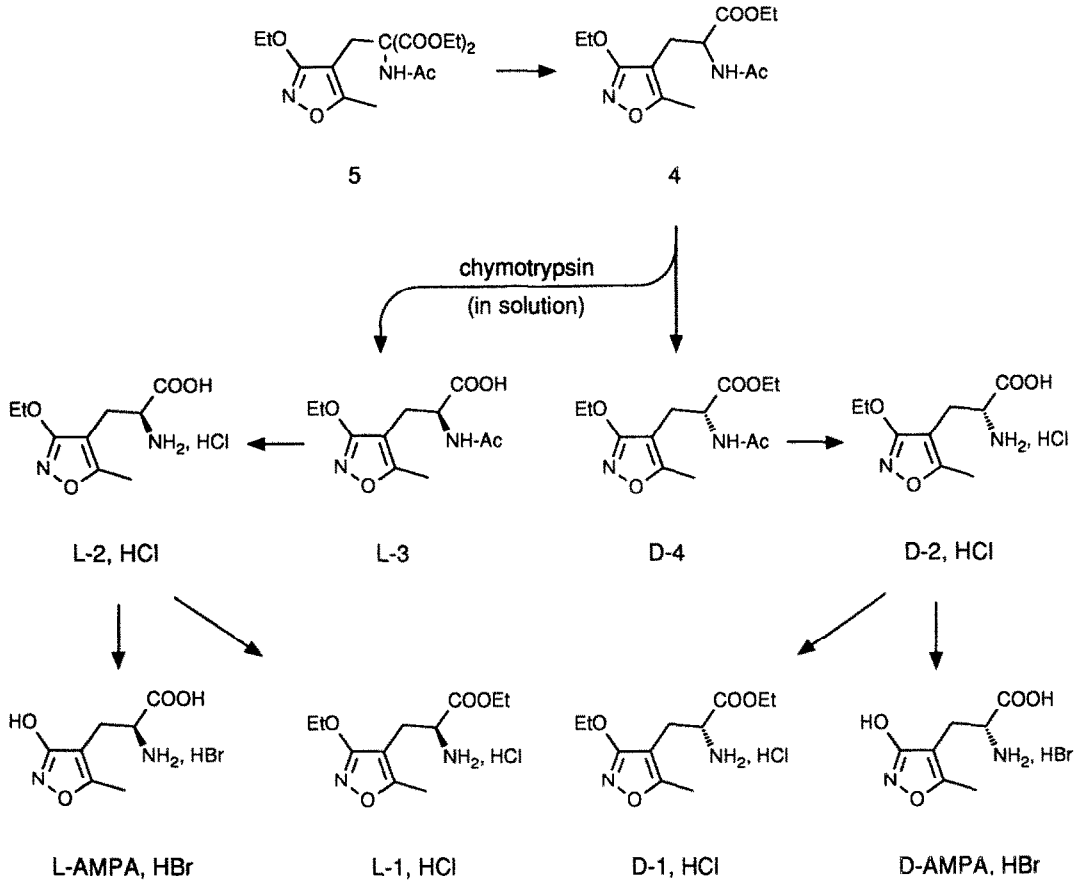

Figure 2. Resolution of AMPA and its diethyl derivative **1** by use of α-chymotrypsin.

The enzymatic reaction, utilizing the esterase activity of the enzyme, was performed in a pH-stat, where the pH was maintained at 7.8 by continuous addition of base to neutralize the carboxylic acid formed. No spontaneous hydrolysis of the substrate was detected in the absence of enzyme, and the consumption of base thus allowed a direct monitoring of the progression of the enzymatic hydrolysis. The base consumption ceased after 50% conversion of the racemic substrate (Figure 3), and after separation of the hydrolyzed product, 2-acetylamino-3-(3-ethoxy-5-methylisoxazol-4-yl)propanoic acid (L-**3**), from unreacted starting material (D-**4**) by extraction, the enantiomers of the *O*-ethyl derivative (**2**) of AMPA were obtained as their hydrochloride salts after deprotection in hydrochloric acid. Further deprotection (Figure 2), using 48% hydrobromic acid, furnished the enantiomers of AMPA, isolated as their hydrobromides, while esterification of the enantiomers of **2** gave the enantiomers of the diethyl derivative **1**, isolated as their hydrochlorides. No hydrolysis of the 3-ethoxyisoxazole moiety of **4** was observed in the enzymatic step, indicating that this potential bioisostere of a carboxyl ester is not a substrate for α-chymotrypsin.

α-Chymotrypsin shows considerable stereoselectivity for esters of *N*-acetylated α-amino acids, preferentially hydrolyzing, like amino acylase, the L-form of conformationally flexible substrates.[6] The AMPA derivative hydrolyzed by α-chymotrypsin and the AMPA derivative (*N*-acetyl *O*-methyl AMPA) hydrolyzed by amino acylase both gave the same enantiomer of AMPA after deprotection, supporting the assignment of the L-configuration to this enantiomer.[4]

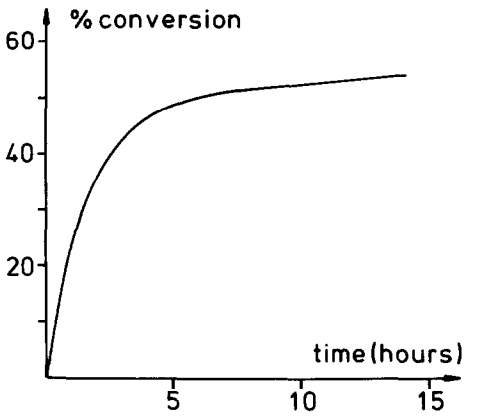

Figure 3. Progression of the α-chymotrypsin-catalyzed hydrolysis of the racemic ester **4**.

No resolution is complete without a determination of the enantiomeric purity of the products. In the α-chymotrypsin-catalyzed resolution of AMPA this was obtained by chiral ligand-exchange HPLC using optically active proline immobilized[9] on silica and chelated with Cu^{2+}. By use of a stationary phase containing L-proline for determination of the enantiomeric purity of L-AMPA and a D-proline-based material for the D-enantiomer of AMPA, this procedure allowed the elution of the enantiomeric impurity in front of the main peak, thereby avoiding that the enantiomeric impurity was obscured by tailing from the dominant peak. The enantiomeric purity of the isolated L- and D-enantiomers of AMPA was 99.9% and 99.8% e.e., respectively, and the observed elution order was in agreement with that previously reported for these chiral stationary phases, where the D-forms of most proteinogenic amino acids elute before their corresponding L-enantiomers on silica-based materials containing L-proline.[9]

The optical activity of several of the compounds is noteworthy. In ethanol the hydrobromide of AMPA shows the opposite sign of rotation (positive for the L-form) to that found in water for both the hydrobromide (unpublished data) and amfoion.[4] Further, in water the enantiomers of **2**, HCl display a modest rotation, which is numerically smaller at 365 nm than at 589 nm, but a considerable rotation of the opposite sign is observed in methanol. Similarly, D-**4** shows very little optical activity in ethanol but a sizable negative rotation in ethyl acetate.

[^{3}H]AMPA binding studies, performed in the presence of 100 mM chaotropic thiocyanate ions, demonstrated a pronounced stereoselectivity of AMPA receptors with IC_{50} values of 0.02 μM and 76 μM for L- and D-AMPA, respectively. These values are consistent with the IC_{50} of 0.04 μM reported for the racemate,[10] and the eudismic ratio of 3800 is considerably higher than that found by using an older and less sensitive version of the [^{3}H]AMPA binding assay without added thiocyanate.[4]

The stereoselectivity of AMPA receptors was confirmed by electrophysiological investigations of the AMPA enantiomers. By in vivo studies on cat spinal neurones the excitatory activity of AMPA was found to reside mainly in the L-enantiomer (Figure 4), in agreement with earlier studies.[11] Similar results were obtained by in vitro studies in the rat cortical wedge preparation, where the dose-response curves (Figure 5) gave EC_{50} values of 3.8 μM and 580 μM for L- and D-AMPA, respectively.

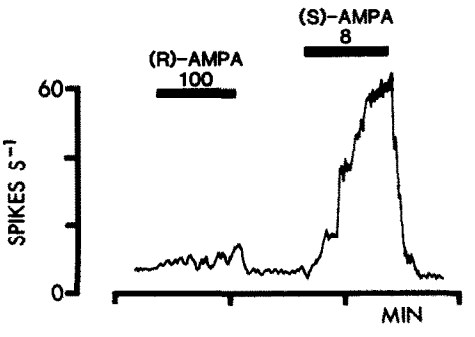

Figure 4. In vivo excitation of a cat spinal dorsal horn interneurone by the D- [(*R*)-] and L- [(*S*)-] enantiomers of AMPA administered microelectrophoretically with currents (nA) and for times indicated by the numbers and horizontal bars. Ordinate: firing rate (spikes per second); abscissae: time (min).

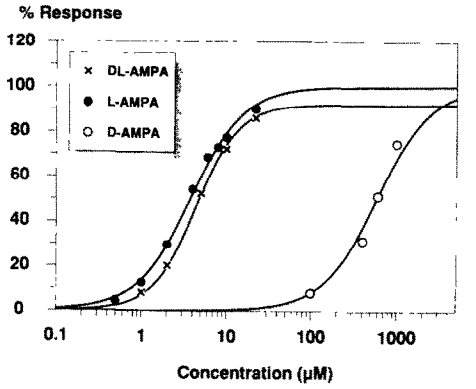

Figure 5. Dose-response curves for the L- and D-enantiomers and racemic AMPA in the rat cortical wedge preparation. Each data point is the mean of three determinations with SEM less than 10%.

Consistent with earlier studies on the racemate,[10] the enantiomers of AMPA showed little or no inhibitory effect in binding studies using L-[^{3}H]Glu in the presence of 2.5 mM $CaCl_2$. Additionally, no potentiation of the excitatory effects of L-AMPA by its D-enantiomer was seen in electrophysiological

studies, unlike what had been observed with the enantiomers of 4-bromohomoibotenic acid,[12] another 3-hydroxyisoxazole amino acid with potent excitatory activity at AMPA receptors.

The racemic form of the diethyl derivative **1** of AMPA was previously found to be a weak and non-selective EAA antagonist by electrophysiological studies in vivo,[8] and racemic **1**, like L-GDEE, shows little or no inhibitory effect (IC_{50} >100 μM) in binding studies using [^{3}H]AMPA or L-[^{3}H]Glu. Similarly, although L-**1** is both a structural and stereochemical analogue of L-GDEE, the enantiomers of **1** showed no useful antagonist properties by electrophysiological studies in vivo and in vitro.

Experimental Section

Ethyl (*RS*)-2-Acetylamino-3-(3-ethoxy-5-methylisoxazol-4-yl)propanoate (4). To a solution of 0.52 g (1.5 mmol) diethyl acetylamino[(3-ethoxy-5-methylisoxazol-4-yl)methyl]malonate[8] (**5**) in dimethylsulfoxide (1.5 mL) was added sodium chloride (0.094 g, 1.7 mmol) and water (0.053 g, 2.9 mmol). The mixture was heated to 160 °C for 7 h, and, after cooling, the darkly coloured reaction mixture was mixed with ethyl acetate (3 mL) and chromatographed on a 50 x 1.6 cm column of silica gel (63-200 μm, 50 g) eluted with ethyl acetate. The main product was recrystallized (ethyl acetate-hexane) to give **4** (0.33 g, 78%) with mp 98-99 °C; ^{1}H NMR ($CDCl_3$) δ 6.25 (1 H, br d, 7 Hz), 4.67 (1 H, dt, 6 and 7 Hz), 4.25 (2 H, q, 7 Hz), 4.15 (2 H, q, 7 Hz), 2.75 (2 H, d, 6 Hz), 2.20 (3 H, s), 1.97 (3 H, s), 1.37 (3 H, t, 7 Hz), 1.20 (3 H, t, 7 Hz); IR (KBr) 3300 (s), 3075 (w), 2980 (m), 2920 (w), 1740 (s), 1730 (s), 1645 (s), 1550 (m), 1515 (s), 1470 (s), 1375 (m), 1350 (m), 1230 (s), 1210 (s), 1200 (s) cm^{-1}. Elemental analysis ($C_{13}H_{20}N_2O_5$) C, H, N.

Enzymatic Resolution. A solution of **4** (121 mg, 0.426 mmol) in 80 mL water was adjusted to pH 7.8 by addition of 0.099 M aqueous sodium hydroxide and combined with a solution, similarly adjusted to pH 7.8, of α-chymotrypsin (Sigma type II, 58.2 mg, corresponding to 2850 IU[13]) in 2.5 mL water. The reaction mixture was blanketed by a gentle stream of nitrogen to prevent absorption of carbon dioxide from the air and mechanically stirred at room temperature while the pH was kept constant at 7.8 by continuous addition of 0.099 M sodium hydroxide from the autoburette of an RTS822 pH-stat (Radiometer, Copenhagen). After the addition of 2.30 mL base, corresponding to 53% conversion, obtained in 14 h, the reaction mixture was stored frozen until it was combined with a similarly prepared solution obtained from 155 mg (0.546 mmol) of **4** hydrolyzed for 13 h at pH 7.8 in the presence of 75 mg (3670 IU) α-chymotrypsin.

The combined reaction mixtures were adjusted to pH 8.5 by addition of 0.5 M aqueous sodium carbonate and extracted with ethyl acetate (6 x 75 mL). Additional sodium carbonate solution was added to the aqueous phase after each extraction to maintain the pH at ca. 8.5. The combined organic phases were dried (sodium sulfate), evaporated and twice recrystallized (ethyl acetate-hexane) to give D-**4** (110 mg, 80%), pure by TLC, with mp 66.5-67.5 °C and $[\alpha]_D^{24}$ -38° ± 1°, $[\alpha]_{365}^{24}$ -145° ± 2° (*c* 0.45, AcOEt); $[\alpha]_D^{26}$ +0.5° ± 0.7°, $[\alpha]_{365}^{26}$ -2.2° ± 0.7° (*c* 0.45, EtOH). The ^{1}H NMR ($CDCl_3$) spectrum was identical with that of racemic **4**, while IR (KBr) 3320 (s), 2980 (m), 2930 (w), 1735 (s), 1645 (s), 1515 (s), 1470 (m), 1380 (m), 1355 (m), 1225 (m), 1210 (m) cm^{-1} was similar to, but not identical with, that of the racemate.

The aqueous phase from the above extractions was acidified to pH 2 by addition of 1 M hydrochloric acid and extracted with ethyl acetate (3 x 100 mL) and chloroform (3 x 100 mL). The combined organic phases were dried (sodium sulfate), evaporated and twice recrystallized (ethyl acetate-hexane) to give 103 mg (83%) of L-**3**, pure by TLC, with mp 126-127 °C and $[\alpha]_D^{25}$ +25.1° ± 0.7°, $[\alpha]_{365}^{25}$ +103° ± 2° (*c* 0.46, EtOH); ^{1}H NMR ($CDCl_3$) δ 6.52 (1 H, br d, 8 Hz), ca. 4.7 (1 H, m), 4.33 (2 H, q, 7 Hz), 2.86 (2 H, d, 6 Hz), 2.26 (3 H, s), 2.03 (3 H, s), 1.43 (3 H, t, 7 Hz), COOH and H_2O at δ 4.85 (br s); IR (KBr) 3330 (m), 3100-2900 (several bands, m), 1745 (s), 1730 (s), 1605 (s), 1515 (s), 1465 (s), 1390 (m), 1265 (m), 1195 (s) cm^{-1}.

(*S*)-2-Amino-3-(3-ethoxy-5-methylisoxazol-4-yl)propanoic Acid Hydrochloride (L-2, HCl). The acetylated amino acid L-**3** (95.8 mg, 0.374 mmol) was refluxed in 1 M hydrochloric acid (15 mL) for 8 h, after which TLC showed no trace of remaining starting material. The colorless solution was evaporated and three times dissolved in water (5 mL) and reevaporated. The residue, after drying in vacuum, was gently (to avoid esterification) recrystallized in methanol-ethyl acetate to give L-**2**, HCl (85.1 mg, 91%) with mp 216 °C (decomp.) and $[\alpha]_D^{21}$ +12.7° ± 0.5°, $[\alpha]_{365}^{21}$ +58° ± 1° (*c* 0.61, MeOH); $[\alpha]_D^{20}$ -4.1° ± 0.5°, $[\alpha]_{365}^{20}$ 0° ± 0.5° (*c* 0.61, H_2O); ^{1}H NMR (D_2O) δ 4.12 (1 H, t, 6 Hz), 4.12 (2 H, q, 7 Hz), 2.90 (2 H, d, 6 Hz), 2.21 (3 H, s), 1.35 (3 H, t, 7 Hz); IR (KBr) 3000-2400 (several bands, m-s), 1730 (s), 1640 (m), 1585 (m), 1520 (s), 1470 (s), 1390 (m), 1310 (m), 1240 (s) cm^{-1}.

(*R*)-2-Amino-3-(3-ethoxy-5-methylisoxazol-4-yl)propanoic Acid Hydrochloride (D-2, HCl). The ester D-**4** (103 mg, 0.364 mmol) was hydrolyzed in 1 M hydrochloric acid as described for the acetylated amino acid L-**3**. Recrystallization (ethanol-ether) gave D-**2**, HCl (73.8 mg, 81%) with mp 213 °C (decomp.) and $[\alpha]_D^{20}$ -12.7° ± 0.6°, $[\alpha]_{365}^{20}$ -55° ± 1° (*c* 0.53, MeOH); $[\alpha]_D^{20}$ +4.4° ± 0.6°, $[\alpha]_{365}^{20}$ +1.5° ± 0.6° (*c* 0.52, H_2O); chiral HPLC showed the enantiomeric purity to be 98.2% e.e. The IR (KBr) and ^{1}H NMR (D_2O) spectra were identical with those of the L-enantiomer. Elemental analysis ($C_9H_{15}N_2O_4Cl$) C, H, N, Cl.

(*S*)-2-Amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propanoic Acid Hydrobromide (L-AMPA, HBr). A solution of L-**2**, HCl (33 mg, 0.13 mmol) in 48% hydrobromic acid (5.0 mL) was blanketed with nitrogen and heated at 140 °C for 15 min. The colorless solution was rapidly cooled and evaporated, then three times dissolved in water (2 mL) and reevaporated. Drying in vacuum and recrystallization (ethanol-ethyl acetate) gave L-AMPA, HBr (28 mg, 79%), pure by TLC, with mp 193-197 °C (decomp.) and $[\alpha]_D^{24}$ +15.5° ± 0.7°, $[\alpha]_{365}^{24}$ +61° ± 1° (*c* 0.46, EtOH) (litt.[4] $[\alpha]_D^{25}$ +14.6 ± 0.6° (*c* 0.62, EtOH)). The enantiomeric purity, determined by chiral HPLC, was 99.6% e.e., and an additional recrystallization increased this to 99.9% e.e. with mp 201-203 °C; IR (KBr) 3100-2400 (several bands, w-s), 1725 (s), 1650 (s), 1530 (s), 1480 (s), 1475 (s), 1305 (m), 1260 (s) cm^{-1}.

(*R*)-2-Amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propanoic Acid Hydrobromide (D-AMPA, HBr). Deprotection of D-**2**, HCl (20 mg, 0.08 mmol) in 48% hydrobromic acid was performed as described for the L-enantiomer. Recrystallization in ethanol-ether followed by recrystallization in ethanol-ethyl acetate gave D-AMPA, HBr (9.0 mg, 42%), pure by TLC, with mp 207-9 °C (decomp.) and $[\alpha]_D^{24}$ -15.3° ± 0.8°, $[\alpha]_{365}^{24}$

-58° ± 1° (*c* 0.41, EtOH) (litt.[4] $[\alpha]_D^{25}$ -14.2 ± 0.5° (*c* 0.62, EtOH)); the enantiomeric purity, determined by chiral HPLC, was 99.8% e.e. The IR (KBr) spectrum was identical with that of the L-enantiomer.

Ethyl (*S*)-2-Amino-3-(3-ethoxy-5-methylisoxazol-4-yl)propanoate Hydrochloride (L-1, HCl). To a solution of 5% (w/w) hydrogen chloride in ethanol (25 mL), prepared by adding acetyl chloride (2.0 mL) to ethanol (23 mL) at 0 °C, was added L-**2**, HCl (43.3 mg, 0.173 mmol). The reaction mixture was refluxed for 2.5 h and evaporated, then twice dissolved in ethanol (5 mL) and reevaporated. The residue, which by TLC contained a small amount of a more polar impurity, presumably the ethyl ester of AMPA (which would be a possible source of AMPA in the biological assays), was dissolved in 2 M aqueous potassium carbonate (10 mL) and extracted with dichloromethane (6 x 15 mL). The combined organic phases were dried (sodium sulfate), evaporated and dissolved in ethyl acetate (3 mL). Addition of a solution of 2.8 M hydrogen chloride in ethyl acetate (2 mL) gave a precipitate which by recrystallization (ethanol-ethyl acetate) yielded L-**1**, HCl (36.5 mg, 76%), pure by TLC, with mp 193-196 °C (decomp.) and $[\alpha]_D^{23}$ +28.7° ± 0.8°, $[\alpha]_{365}^{23}$ +105° ± 2° (*c* 0.45, EtOH); IR (KBr) 2990 (m), 2860 (m, br), 1740 (s), 1645 (m), 1520 (m), 1495 (m), 1465 (m), 1390 (m), 1300 (m), 1245 (s) cm^{-1}, identical with that of the racemate.[8] Elemental analysis ($C_{11}H_{19}N_2O_4Cl$) C, H, N, Cl.

Ethyl (*R*)-2-Amino-3-(3-ethoxy-5-methylisoxazol-4-yl)propanoate Hydrochloride (D-1, HCl). Compound D-**2**, HCl (28.3 mg, 0.113 mmol) was converted into D-**1**, HCl by esterification, extraction and precipitation by hydrogen chloride as described for the L-enantiomer. Recrystallization in ethanol and ethanol-ethyl acetate yielded D-**1**, HCl (25.7 mg, 82%), pure by TLC, with mp 192-195 °C (decomp.) and $[\alpha]_D^{23}$ -28.7° ± 0.8°, $[\alpha]_{365}^{23}$ -104° ± 2° (*c* 0.44, EtOH); ^1H NMR (CD_3OD) δ 4.07 (2 + 2 + 1 H, m), 2.79 (2 H, d, 7 Hz), 2.20 (3 H, s), 1.34 (3 H, t, 7 Hz), 1.20 (3 H, t, 7 Hz). The IR (KBr) spectrum was identical with that of the L-enantiomer

Determination of Enantiomeric Purity. Chiral ligand-exchange HPLC columns (120 x 4.6 mm) containing either L- or D-proline immobilized on silica (LiChrosorb SI-60, 5 μm) and chelated with Cu^{2+} were prepared according to directions in the literature.[9] Elution was performed with 1 mL/min of an aqueous solution of 50 mM potassium phosphate, pH 4.5 at 50 °C using conventional HPLC instrumentation and UV detection at 210 nm.

[^{3}H]AMPA and L-[^{3}H]Glu Binding Assays were performed as previously described.[10,12]

In Vivo Electrophysiology on cat spinal neurones was performed as previously described[12] using electrophoretic administration of compounds from aqueous solutions (100 mM, pH 7.3 with sodium hydroxide) contained in separate barrels of a seven-barrel micropipette.

In Vitro Electrophysiology was performed by using a modified version[14] of the rat cortical wedge preparation.[15]

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