# Enzymic Resolution and Binding to Rat Brain Membranes of the Glutamic Acid Agonist α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid

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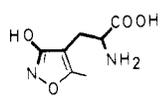
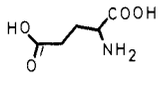
The enantiomers of the glutamic acid central nervous system receptor agonist α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) were prepared via kinetic resolution of the racemic N-acetylated 3-methoxy derivative by reusable, immobilized aminoacylase. L-AMPA was more effective ($IC_{50}$ = 0.6 μM) than D-AMPA ($IC_{50}$ = 4.8 μM) in displacing racemic [$^3$H]AMPA from binding sites on rat brain synaptic membranes in agreement with their relative in vivo excitatory potencies.

(*RS*)-α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA, 1), synthesized first by Hansen and Krogsgaard-Larsen,[1] is a potent and selective agonist of the putative, excitatory neurotransmitter L-glutamic acid (L-Glu).[2] Central nervous system (CNS) receptors for excitatory amino acids have been tentatively subdivided into three groups, *N*-methyl-D-aspartic acid (NMDA) receptors, quisqualic acid receptors, and kainic acid receptors, based on their relative sensitivity to various agonists and antagonists.[3] AMPA (1) does not interfere with binding sites for kainic acid in vitro, and AMPA-induced neuronal excitation is not significantly affected by NMDA antagonists.[2] Quisqualic acid and 1 are equally effective in displacing racemic [$^3$H]AMPA from binding sites on rat brain synaptic membranes that may represent a population of physiologically relevant L-Glu receptors.[4] These and other studies of 1 with respect to uptake,[5,6] inhibition of L-[$^3$H]Glu binding,[7] pharmacology,[8-10] and X-ray crystallography[11] so far have been performed with the racemic compound. In view of the remarkable potency and selectivity of 1 at CNS receptors, we decided to resolve 1, preferably by a procedure that would yield both of the enantiomers, with the object of studying the stereoselectivity of L-Glu receptors in vivo and in vitro.



AMPA (1)



GLU

Aminoacylase (*N*-acylamino-acid amidohydrolase, EC 3.5.1.14) is an enzyme that catalyzes the hydrolysis of acylated amino acids with a pronounced specificity for the L form,[12] and a number of amino acids have been resolved by using a soluble form of this enzyme.[13] Enzyme immobilization gives advantages in enzyme recovery, product isolation, and, sometimes, enzyme stability, and immobilized aminoacylase is used for the industrial production of L-methionine.[14] However, only a few examples are reported on the use of immobilized aminoacylase for the preparative resolution of unnatural amino acids.[15,16]

This paper describes the application of a commercially available, immobilized aminoacylase for the preparation of both enantiomers of AMPA (1). These were then evaluated for their ability to compete with racemic [$^3$H]-AMPA for binding to rat brain synaptic membranes.

## Results and Discussion

**Resolution.** Preliminary experiments showed that *N*-Ac-*O*-Me-AMPA [(*RS*)-α-(acetylamino)-3-methoxy-5-methyl-4-isoxazolepropionic acid (2)] was a better substrate than *N*-Ac-AMPA [(*RS*)-α-(acetylamino)-3-hydroxy-5-methyl-4-isoxazolepropionic acid[17]] for the immobilized

Scheme I

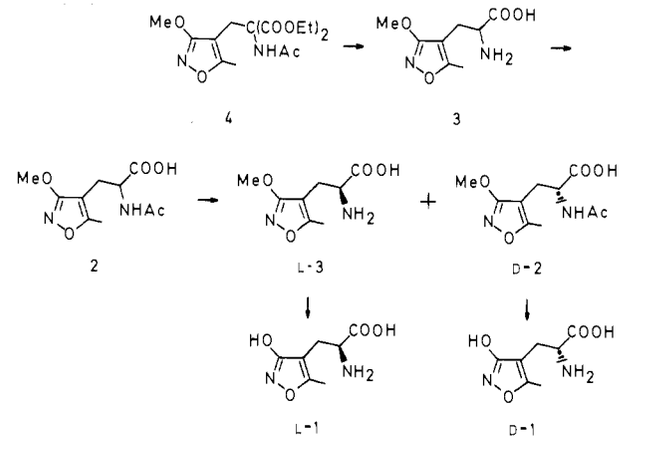



Table I. Inhibition of Specific DL-[$^3$H]AMPA Binding to Rat Brain Membranes

| compd | $IC_{50}$ ± SEM,[a] μM |
|---|---|
| DL-AMPA (1) | 0.8 ± 0.3 |
| L-AMPA (L-1) | 0.6 ± 0.1 |
| D-AMPA (D-1) | 4.8 ± 0.8 |
| *O*-Me-DL-AMPA (3) | >100 |
| L-Glu[b] | 1.3 ± 0.6 |
| D-Glu[b] | ~100 |

[a] See Experimental Section for details. [b] From ref 4.

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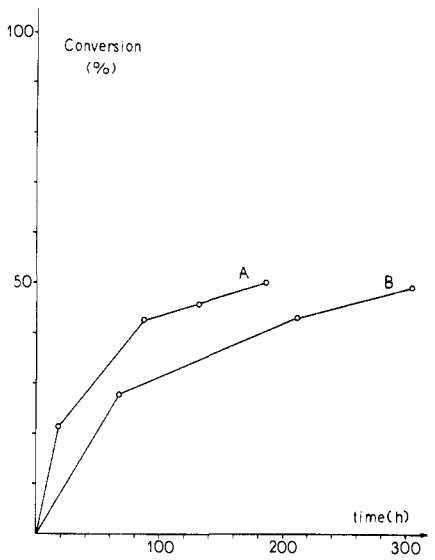


**Figure 1.** Aminoacylase-catalyzed hydrolysis of *N*-Ac-*O*-Me-DL-AMPA (**2**): (A) first batch; (B) second batch with recovered enzyme.

aminoacylase. Thus, **2**, which was synthesized from diethyl acetamido[(3-methoxy-5-methylisoxazol-4-yl)methyl]-malonate[1,11] (**4**) by hydrolysis and decarboxylation, followed by acetylation (Scheme I), was used for the preparative resolution. This was performed on a relatively low concentration (6 mM) of **2** in order to prevent precipitation of free amino acid and because the degree of conversion at equilibrium increases with decreasing substrate concentration. Further, the preparative resolution was carried out in two successive batches by using the recovered enzyme from the first batch as the catalyst in the second batch, demonstrating reuse of the immobilized enzyme. After isolation of the products, deprotection of the optically active isomers of **2** and *O*-Me-AMPA [α-amino-3-methoxy-5-methyl-4-isoxazolepropionic acid (**3**)] with aqueous hydrobromic acid and hydrogen bromide in acetic acid, respectively, furnished the enantiomers of **1** (Scheme I).

An enantiomeric resolution should preferably also provide information on the absolute configuration and the optical purity of the products. The progression of the enzyme-catalyzed hydrolysis of **2** (Figure 1) is consistent with the selective hydrolysis of one enantiomer. This enantiomer is assigned the L configuration based on the well-documented specificity of the enzyme.[13] This assignment is further supported by application of the Clough–Lutz–Jirgensons rule[13,18] on the observed optical rotations of the free amino acids and their hydrobromides. The evidence is less conclusive, however, with respect to the optical purity of the enantiomers. Aminoacylase reportedly catalyzes with a high degree of stereoselectivity, the hydrolysis rate of the L form being more than 10 000 times as fast as that of the corresponding D form,[12] but this is not necessarily so for **2**. Furthermore, some degree of racemization may occur during the strongly acidic deprotection conditions, although only a few percent racemization was observed after treatment of L-phenylalanine with 48% aqueous hydrobromic acid under similar conditions. Nevertheless, the identical numerical rotations obtained for the enantiomeric preparations of the free amino acids, as well as of their hydrobromides, are taken to indicate a high optical purity, but no independent determination of this was carried out.

**Binding Affinity.** The order of potency for AMPA (**1**) and a number of structurally related, racemic isoxazole amino acids as inhibitors of L-[$^3$H]Glu binding to rat cerebellar membranes was different from their relative potency as excitants of cat spinal neurons in vivo, suggesting that the L-[$^3$H]Glu binding sites were different from the physiological L-Glu receptors.[7] On the other hand, the inhibition of [$^3$H]AMPA binding to a preparation of rat brain synaptic membranes roughly paralleled the in vivo excitatory potency for the above-mentioned isoxazole amino acids preferentially antagonized[19] by the selective L-Glu antagonist, diethyl L-glutamate.[4] L-Aspartic acid and isoxazole amino acids sensitive[19] to the NMDA antagonist, α-D-aminoadipic acid, were inactive as inhibitors of [$^3$H]AMPA binding.[4]

In the [$^3$H]AMPA binding studies reported here (Table I), DL-AMPA (**1**) exhibited an $IC_{50}$ value of 0.8 μM (compare the earlier reported[4] value of 0.3 μM). The AMPA binding sites showed a marked stereoselectivity with respect to the enantiomers of **1**. L-AMPA (L-**1**) had the highest affinity toward the sites, with an $IC_{50}$ value 8 times lower than that of D-AMPA (D-**1**). Correspondingly, the in vivo excitatory potency of L-**1** was 4–6 times higher than that of D-**1** by microelectrophoretic techniques on cat spinal neurons.[20] AMPA (**1**) lost its affinity to the binding sites by O-methylation of the acidic 3-hydroxyisoxazole moiety (equivalent to the ω-carboxy group of Glu); *O*-Me-AMPA (**3**), correspondingly, was found to be inactive as an L-Glu agonist or antagonist on cat spinal neurons.[20]

An even more pronounced stereoselectivity of the AMPA binding sites was found[4] for the enantiomers of Glu, L-Glu having a 75-fold lower $IC_{50}$ value than D-Glu. Similarly, L-Glu showed a higher affinity than D-Glu toward L-[$^3$H]Glu binding sites, and $IC_{50}$ ratios of ~5 and ~40 have been reported for different membrane preparations.[21,22] This stereoselectivity was quite unexpected in view of the relatively small difference in in vivo excitatory potency for the Glu enantiomers.[23,24] However, part of the in vivo potency of D-Glu apparently is due to its activation of excitatory receptors, notably NMDA receptors,[25] with a small affinity toward L-Glu and the AMPA enantiomers.

## Experimental Section

Melting points, determined in capillary tubes, are corrected. Elemental analyses were performed by P. Hansen at Chemical Laboratory II, University of Copenhagen. $^1$H NMR spectra were recorded on a Bruker HXE-90 (FT mode) or a JEOL JMN-C-60HL (at 60 MHz) instrument; chemical shifts are recorded in δ units. IR spectra, listed as $\nu_{max}$, were recorded from KBr disks on Perkin-Elmer spectrophotometers, Models 157, 247, and 421. Optical rotations were measured in thermostated cuvettes on a Perkin-Elmer 141 polarimeter. Ninhydrin-photometric mea-

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surements were carried out in standardized test tubes with a Bausch & Lomb Spectronic 21 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, system 1), chloroform–methanol–25% aqueous ammonia (2:2:1, system 2), ethanol–25% aqueous ammonia (7:3, system 3), or ethyl acetate–methanol (9:1), added 1% formic acid (system 4). Compounds were visualized on the TLC plates by iodine vapor or by spraying with ninhydrin or bromcresol green.

**(*RS*)-α-Amino-3-methoxy-5-methyl-4-isoxazolepropionic Acid (*O*-Me-AMPA, 3).** Compound **3** was prepared from 4[1,11] (340 mg, 1.0 mmol) by hydrolysis with refluxing 1 M hydrochloric acid (20 mL) for 8 h. The solution was evaporated, twice dissolved in water, and reevaporated. The residue was dissolved in water (2 mL), and triethylamine (101 mg, 1.0 mmol) in ethanol (3 mL) was added until pH ~6. The precipitated amino acid was filtered and dried to afford **3** (50 mg, 25%): mp 224–225 °C dec; $^1$H NMR ($D_2O$–$CF_3COOD$) δ 2.29 (s, 3 H), 3.01 (d, 2 H), 3.97 (s, 3 H), 4.28 (t, 1 H); IR $\nu_{max}$ 2950 (br), 1660, 1510, 1475, 1405, 1345 cm$^{-1}$. Anal. ($C_8H_{12}N_2O_4$) C, H, N.

**(*RS*)-α-(Acetylamino)-3-methoxy-5-methyl-4-isoxazolepropionic Acid (*N*-Ac-*O*-Me-AMPA, 2).** Compound **2** was prepared by acetylation of **3** (100 mg, 0.5 mmol) with acetic anhydride (1.0 g, 9.8 mmol) in glacial acetic acid (2 mL) under reflux for 70 min. To the reaction mixture was added water (5 mL), and the solution was evaporated. Toluene (5 mL) was added and reevaporated twice. The residue was recrystallized from ethyl acetate to give **2** (55 mg, 45%): mp 161 °C; $^1$H NMR ($CD_3OD$; also used as internal standard ≡ 3.36) δ 1.99 (s, 3 H), 2.31 (s, 3 H), 2.84 (m, 2 H), 3.99 (s, 3 H), 4.63 (dd, 1 H); IR $\nu_{max}$ 3370, 2300–2950 (several bands), 1725, 1610, 1520, 1465, 1410, 1345, 1230, 1200, 1135 cm$^{-1}$. Anal. ($C_{10}H_{14}N_2O_5$) C, H, N.

**Enzymic Resolution.** Enzygel aminoacylase (Boehringer) (212 mg), containing ~40 IU,[26] was added to 19.7 mL of an aqueous solution of **2** (28.6 mg, 0.118 mmol) neutralized to pH 7.1 with lithium hydroxide. The enzyme was kept in suspension by gentle, magnetic stirring at 30 °C in a centrifuge tube covered with Parafilm to prevent evaporation. At intervals the reaction mixture was centrifuged, and a small portion of the supernatant was assayed for free amino acid by the ninhydrin method.[27] After 186 h, 50% of the starting material was converted into free amino acid (Figure 1, curve A). The reaction mixture was centrifuged, and the supernatant was applied on an ion-exchange column (0.7 × 10 cm) containing ~4 mL of Amberlite IR-120 ($H^+$). The immobilized enzyme was washed with 3 × 10 mL of water, and the washings were applied on the ion-exchanger, which then was eluted (~0.5 mL/min) with 30 mL of water, followed by 90 mL of 1 M aqueous ammonia.

The initial, weakly acidic and subsequently neutral eluate was collected, evaporated, suspended in methanol, and filtered, and the filtrate was evaporated. The oily residue was extracted with 3 × 5 mL of hot ethyl acetate and filtered, and the filtrate was evaporated to yield 11.7 mg (>82%) of oily *N*-Ac-*O*-Me-D-AMPA (D-**2**) (pure by TLC, system 4) with $[\alpha]^{20}_D$ −25.2 ± 0.9° (*c* 1.17, EtOH) and $[\alpha]^{20}_{436}$ −56 ± 2° (*c* 1.17, EtOH).

The basic eluate was collected, evaporated, twice dissolved in water, and reevaporated to 9.8 mg of a crystalline, white residue, which was recrystallized from water–ethanol to yield 9.3 mg (>79%) *O*-Me-L-AMPA (L-**3**) (pure and identical with racemic **3** by TLC, systems 1–3) with $[\alpha]^{20}_D$ −8.6 ± 0.7° (*c* 0.47, $H_2O$) and $[\alpha]^{20}_{436}$ −14.5 ± 0.8° (*c* 0.47, $H_2O$).

The immobilized enzyme, after washing and centrifuging, was reused by suspending it in 11.5 mL of a fresh, aqueous solution of **2** (15.7 mg, 0.065 mmol) neutralized to pH 7.3 with lithium hydroxide. As before, the suspension was magnetically stirred at 30 °C in a centrifuge tube covered with Parafilm, and the progression of the reaction was followed by the ninhydrin procedure. After 305 h, ~50% of the starting material was converted into free amino acid (Figure 1, curve B). The reaction mixture was centrifuged, and the supernatant was applied on the regenerated ion-exchange column and eluted first with water and then with 1 M aqueous ammonia. Workup as described above yielded 7.4 mg (>94%) of oily D-**2** (pure by TLC, system 4) [with $[\alpha]^{20}_D$ −23 ± 1° (*c* 0.74, EtOH) and $[\alpha]^{20}_{436}$ −51 ± 3° (*c* 0.74, EtOH)] and 5.5 mg (>85%) recrystallized L-**3** (pure by TLC, system 1) [with $[\alpha]^{20}_D$ −8.7 ± 0.8° (*c* 0.55, $H_2O$) and $[\alpha]^{20}_{436}$ −14 ± 1° (*c* 0.55, $H_2O$)].

**(*R*)-α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid Hydrate (D-AMPA, D-1).** The two preparations of D-**2** were pooled (19.1 mg, 0.079 mmol), and ~5 mL of 48% aqueous hydrobromic acid was added. The solution was covered with nitrogen and heated under reflux on an oil bath (140 °C) for 23 min. After the solution was cooled and evaporated, the residue was twice dissolved in water and reevaporated. Drying in vacuo over potassium hydroxide and phosphorus pentoxide yielded a crystalline residue, which was recrystallized from ethanol–petroleum ether (bp 40–60 °C) to give D-1·HBr (14.1 mg, 67%): $[\alpha]^{25}_D$ −14.2 ± 0.5° (*c* 0.71, EtOH); $[\alpha]^{25}_{436}$ −31.6 ± 0.7° (*c* 0.71, EtOH).

The hydrobromide was dissolved in 0.3 mL of ethanol, and a solution of 10 vol % triethylamine in ethanol was added until pH 5–6 to cause precipitation of the free amino acid. Filtration and recrystallization from water–ethanol gave 6.7 mg of D-1. The mother liquor furnished an additional 2.6 mg of recrystallized D-1: no sharp melting point, but gradual decomposition above ~200 °C; $[\alpha]^{27}_D$ +19 ± 2° (*c* 0.18, $H_2O$); $^1$H NMR [$D_2O$, $CH_3CN$ (δ 2.02) added as internal standard] δ 2.21 (s, 3 H), 2.83 (d, 2 H), 3.88 (t, 1 H); IR $\nu_{max}$ (broad bands) 3420, 3000, 1625, 1490, 1400, 1335, 1245, 1200 cm$^{-1}$. Anal. (for the monohydrate $C_7H_{12}N_2O_5$) H, N; C: calcd, 41.18; found 41.79.

**(*S*)-α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid Hydrate (L-AMPA, L-1).** The two preparations of L-**3** were pooled (14.8 mg, 0.074 mmol) and blanketed with nitrogen. A solution of 43% hydrogen bromide in glacial acetic acid (5.0 mL) was added. The conversion was essentially completed (TLC, system 1) after 77 h at ambient temperature (22 °C), and the reaction mixture was evaporated, twice dissolved in water, and reevaporated with an intermittent charcoal treatment. After drying in vacuo over potassium hydroxide and phosphorus pentoxide, the crystalline residue was recrystallized from ethanol–petroleum ether (bp 40–60 °C) to yield L-1·HBr (12.3 mg, 62%): $[\alpha]^{25}_D$ +14.6 ± 0.6° (*c* 0.62, EtOH); $[\alpha]^{25}_{436}$ +31.5 ± 0.8° (*c* 0.62, EtOH).

The hydrobromide was dissolved in 0.2 mL of ethanol, and triethylamine (10 vol % in ethanol) was added until pH 5–6. Filtration and recrystallization from water–ethanol yielded 5.9 mg of L-1. The mother liquor furnished an additional 1.9 mg of recrystallized L-1: $[\alpha]^{28}_D$ −21 ± 2° (*c* 0.19, $H_2O$); mp, TLC (systems 1–3), NMR, and IR spectrum identical with that of the D form.

The remaining mother liquors from the D and the L forms were pooled, evaporated, and recrystallized from water–ethanol to yield 1–2 mg of the less soluble DL form with an IR spectrum identical with that previously reported.[1]

**Binding Studies.** Membrane preparation and binding assays were performed essentially as detailed previously[4] with racemic [$^3$H]AMPA (New England Nuclear, Boston, MA) as labeled ligand. The assays consisted of a 2-mL suspension of rat brain membranes (protein concentration 0.1–0.6 mg/mL) in 0.05 M Tris–citrate buffer (pH 7.4) incubated in triplicate at 2 °C for 30 min with 49 nM [$^3$H]AMPA (specific activity 15.3 Ci/mmol) and varying concentrations of the test compound. Specific [$^3$H]AMPA binding was defined as the difference between binding in the absence and in the presence of 1 mM L-Glu. $IC_{50}$ values (the concentration of test compound giving 50% inhibition of specific [$^3$H]AMPA binding) were estimated from at least four different concentrations of test compound by computerized log-probit analysis.

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**Registry No.** 1, 74341-63-2; D-1, 84799-49-5; D-1·HBr, 84799-50-8; L-1, 84799-51-9; L-1·HBr, 84799-52-0; **2**, 84751-72-4; D-**2**, 84799-53-1; **3**, 83643-90-7; L-**3**, 84799-54-2; **4**, 75989-23-0; aminoacylase, 9012-37-7.

(26) Assayed at ca. 30 °C with 15 mM *N*-acetyl-L-methionine (*N*-Ac-L-Met) as substrate in 6.0 mL of 20 mM phosphate buffer, pH 7.0. After 30 or 60 min, the assay mixture was centrifuged, and free amino acid was determined by the ninhydrin procedure of Moore and Stein.[27] One IU of the enzyme hydrolyzes *N*-Ac-L-Met at a rate of 1 μmol/min.

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