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Original article

Ionotropic excitatory amino acid receptor ligands. Synthesis and pharmacology of a new amino acid AMPA antagonist

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Abstract – We have previously described the potent and selective (*RS*)-2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid (AMPA) receptor agonist, (*RS*)-2-amino-3-(3-carboxy-5-methyl-4-isoxazolyl)propionic acid (ACPA), and the AMPA receptor antagonist (*RS*)-2-amino-3-[3-(carboxymethoxy)-5-methyl-4-isoxazolyl]propionic acid (AMOA). Using these AMPA receptor ligands as leads, a series of compounds have been developed as tools for further elucidation of the structural requirements for activation and blockade of AMPA receptors. The synthesized compounds have been tested for activity at ionotropic excitatory amino acid (EAA) receptors using receptor binding and electrophysiological techniques, and for activity at metabotropic EAA receptors using second messenger assays. Compounds **1** and **4** were essentially inactive. (*RS*)-2-Amino-3-[3-(2-carboxyethyl)-5-methyl-4-isoxazolyl]propionic acid (ACMP, **2**), on the other hand, was shown to be a selective AMPA receptor antagonist (IC_{50} = 73 μM), more potent in electrophysiological experiments than AMOA (IC_{50} = 320 μM). The isomeric analogue of **2**, compound **5**, did not show AMPA antagonist effects, but was a weak NMDA receptor antagonist (IC_{50} = 540 μM). Finally, compound **3**, which is an isomer of ACPA, turned out to be a very weak NMDA antagonist, and an AMPA receptor agonist approximately 1 000 times weaker than ACPA. None of the compounds showed agonist or antagonist effects at metabotropic EAA receptors.

excitatory amino acids / AMPA antagonist / structure–activity relationships / isoxazole amino acids

1. Introduction

(*S*)-Glutamic acid (Glu) is the major excitatory neurotransmitter in the central nervous system (CNS) and is implicated in the development of a number of CNS disorders of acute as well as chronic nature, e.g. ischaemic neuronal damage, epilepsy, Alzheimer's disease and schizophrenia [1–3]. Thus, ligands for the excitatory amino acid (EAA) receptors are of great pharmacological and therapeutic interest. These receptors, operated by Glu, are divided into two main classes, ionotropic and metabotropic receptors, each of which are further subdivided into three groups [3–5]. Based primarily on studies using selective agonists and antagonists, ionotropic receptors have been divided into *N*-methyl-D-aspartic acid (NMDA), (*RS*)-2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid (AMPA) and kainic acid (Kain) receptors. This classification has subsequently been supported by the results of sequence homology studies of cloned receptor subunits [3, 6]. The metabotropic Glu receptors (mGluRs) have been subdivided into group I (mGluR 1 and 5), group II (mGluR 2 and 3) and group III (mGluR 4 and 6–8) receptors based on pharmacology, second messenger coupling and sequence homology [2, 5]. Both ionotropic and metabotropic receptors are po-

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Abbreviations: ACMP: (RS)-2-amino-3-[3-(2-carboxyethyl)-5-methyl-4-isoxazolyl]propionic acid; ACPA: (RS)-2-amino-3-(3-carboxy-5-methyl-4-isoxazolyl)propionic acid; AMOA: (RS)-2-amino-3-[3-(carboxymethoxy)-5-methyl-4-isoxazolyl]propionic acid; AMPA: (RS)-2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl) propionic acid; CPP: (RS)-3-(2-carboxy-4-piperazinyl)propyl-1-phosphonic acid; EAA: excitatory amino acid; Glu: (S)-glutamic acid; Kain: kainic acid; NBQX: 6-nitro-7-sulfamoylbenzo[f] quinoxaline-2,3-dione; NMDA: N-methyl-D-aspartic acid.

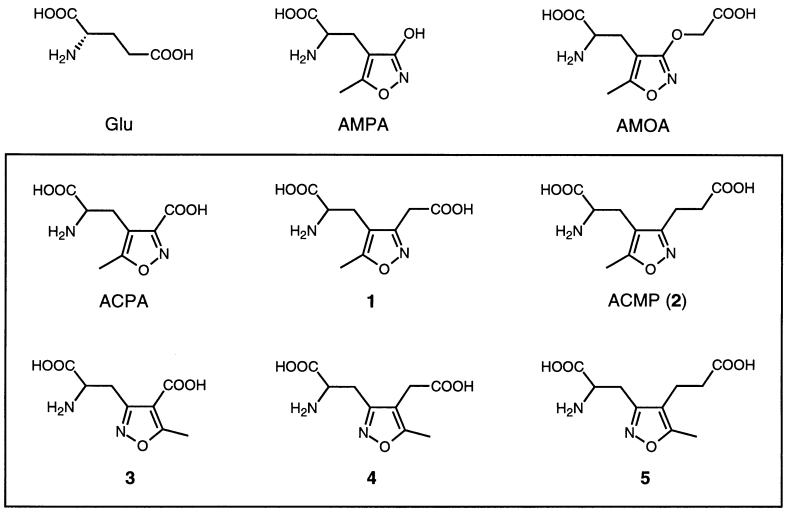

Figure 1. Structures of Glu, the AMPA receptor agonists AMPA and ACPA, the AMPA receptor antagonist AMOA, and the synthesized compounds **1–5**.

tential therapeutic targets in the CNS disorders mentioned above [1, 2].

The availability of selective ligands is a prerequisite for pharmacological characterization of the receptors and subsequent development of potential therapeutics on a systematic or rational basis. AMPA [7] and (*RS*)-2-amino-3-(3-carboxy-5-methyl-4-isoxazolyl)propionic acid (ACPA) [8] have been developed as potent and selective agonists for AMPA receptors, whereas (*RS*)-2-amino-3-[3-(carboxymethoxy)-5-methyl-4-isoxazolyl]-propionic acid (AMOA) [9] is a selective but relatively weak antagonist for AMPA receptors (*figure 1*). The knowledge about the structural requirements of decisive importance for agonist or antagonist activity at AMPA receptors is, however, limited. In this paper, the synthesis and pharmacological characterization of a series of compounds (**1–5**), designed and synthesized using ACPA and AMOA as lead structures, is described. The distal acidic group of these acidic isoxazole amino acids is introduced in side chains of different chain length. The position of ring substituents has been varied to give the following three pairs of isomeric compounds: ACPA and **3**, compounds **1** and **4**, and ACMP (**2**) and **5** (*figure 1*). The new compounds have been tested in vitro at ionotropic EAA receptors using receptor binding assays and electrophysiological techniques, and at metabotropic EAA receptors based on second messenger measurements.

2. Chemistry

Compounds **1**, **2**, **4** and **5** were synthesized via 3,4-di(bromomethyl)-5-methylisoxazole (**10**) (*figure 2*). Attempts were made to obtain this key intermediate directly from the starting material 3-(hydroxymethyl)-5-methylisoxazole (**6**) [10] by reaction with 1,3,5-trioxane in 62% aqueous HBr in order to obtain simultaneous bromomethylation in the 4-position of the isoxazole ring and replacement of the hydroxy group by a bromo atom. This reaction turned out to give a complex mixture of compounds, which after separation by column chromatography gave the three compounds **8**, **9** and **10**, in 3%, 27% and 27% yields, respectively. These three oily compounds were analysed by ^{1}H-NMR spectroscopy, for compound **9** supported by ^{13}C- and DEPT-NMR spectra.

In another approach, reaction of compound **6** with thionyl chloride afforded 3-(chloromethyl)-5-methylisoxazole (**7**) [11], which underwent bromomethylation and halogen exchange when treated with 1,3,5-trioxane in 62% aqueous HBr to give compound **10** in 89% yield. Compound **10** could be selectively substituted at the 4-bromomethyl group by treatment with the sodium salt of dimethyl acetamidomalonate, the sodium salt of dimethyl malonate, or sodium cyanide to give compounds **11**, **12**, or **13**, respectively. The selective substitution at the 4-bromomethyl group was proven by NMR spectros-

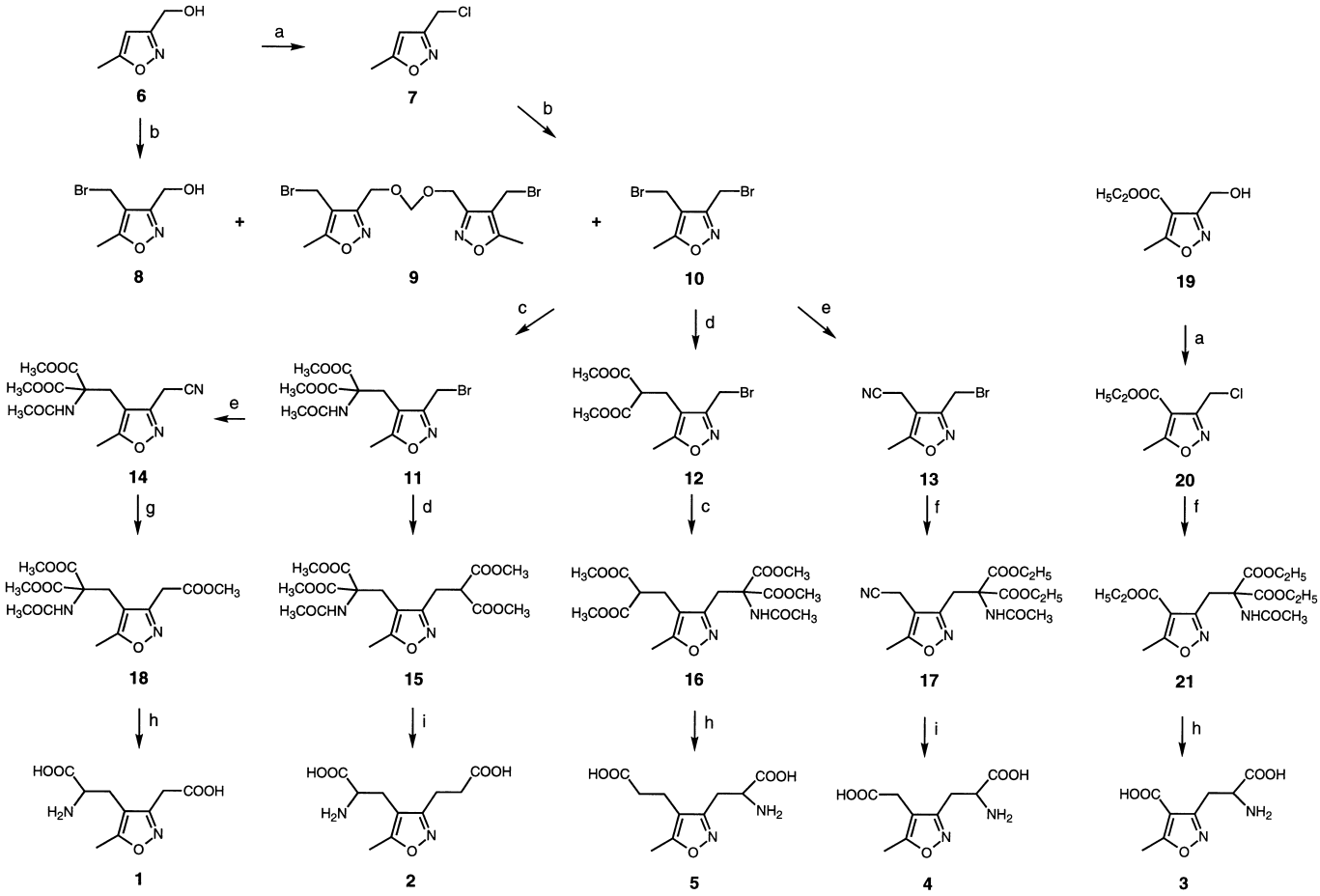

Figure 2. (a) $SOCl_2$; (b) 1,3,5-trioxane, 62% aqueous HBr; (c) $H_3CCONHCNa(COOCH_3)_2$; (d) $NaCH(COOCH_3)_2$; (e) NaCN; (f) $H_3CCONHCNa(COOC_2H_5)_2$; (g) HCl, CH_3OH; (h) 1 M HCl; (i) 48% aqueous HBr.

copy. A NOESY spectrum of compound **11** showed positive NOE between the 4-methylene group (δ 3.54) and the 5-methyl group (δ 2.28), whereas no NOE was observed between the 3-bromomethyl group (δ 4.26) and the 5-methyl group. Cyanide or malonate could be substituted for the bromo atom of the substituent at C-3 of compound **11** to give compounds **14** and **15**, respectively, and compounds **16** and **17** were obtained from compounds **12** and **13**, respectively, by reaction with the sodium salts of acetamidomalonates. Attempts to transform **14** into target compound **1** in one step by treatment with aqueous HBr gave a complex reaction mixture. In a two-step sequence via compound **18**, **14** was converted into **1** in 54% yield. Compounds **15**, **16** and **17** could be fully deprotected and hydrolysed in one step by reflux in Br or HCl to give the final products **2**, **5** and **4**, respectively. Compound **3** was obtained from the starting material **19** [12] through the chloromethyl derivative **20** and the diethyl acetamidomalonate derivative **21**, which was deprotected to give **3** in one step.

3. In vitro pharmacology

The synthesized compounds were tested for affinity at AMPA, Kain and NMDA receptors using [^{3}H]AMPA [13], [^{3}H]Kain [14] and [^{3}H](*RS*)-3-(2-carboxy-4-piperazinyl)propyl-1-phosphonic acid ([^{3}H]CPP) [15], respectively. Low affinity for AMPA receptors was observed for compounds **1**, **2** and **3**, whereas compounds **3**, **4**, and **5** showed low affinity for NMDA receptors (*table I*). No significant affinity was observed for Kain receptors for any of the five compounds. Electrophysiological testing of the five compounds, using the rat cortical slice

Table I. Receptor binding and electrophysiological data.

Compound	IC_{50} (μM)			EC_{50} (μM)	IC_{50} (μM)
	[^{3}H]AMPA	[^{3}H]Kain	[^{3}H]CPP	Electrophysiology	
AMPA	0.04 ± 0.015	> 100	> 100	3.5 ± 0.2	
ACPA	0.02 ± 0.012	6.3 ± 1.3	> 100	1.0 ± 0.1	
AMOA	90 ± 14	> 100	> 100		320 ± 25[a]
1	89 ± 8	> 100	> 100	> 1 000[b]	
ACMP (**2**)	17 ± 3	> 100	> 100		73 ± 8[a]
3	65 ± 13	> 100	22 ± 2	1 000[c]	500[d]
4	> 100	> 100	63 ± 2	> 1 000	> 1 000
5	> 100	> 100	34 ± 9		540 ± 90[e]

[a]Antagonism of 5 μM AMPA. [b]Weak (ca. 25% of maximal AMPA response) NBQX sensitive reponse at 1 000 μM. [c]Estimated EC_{50}, NBQX sensitive response. [d]Estimated IC_{50}, antagonism of 10 μM NMDA. [e]Antagonism of 10 μM NMDA.

model [16], revealed that the observed affinities in some cases reflected agonist activities, and for other compounds antagonist activity at the respective receptors. Compounds **1** and **3** showed very weak agonist activity, which could be antagonized by the AMPA receptor antagonist 6-nitro-7-sulfamoylbenzo[*f*]quinoxaline-2,3-dione (NBQX) [17]. The weak affinity for the [^{3}H]CPP binding site observed for compound **3** was further investigated using the cortical slice model. In an experiment performed in the presence of 5 μM NBQX (to antagonize the AMPA agonist activity of compound **3**), 500 μM **3** produced an approximately 50% reduction of the depolarization evoked by 10 μM NMDA. Thus, the affinity of **3** for the CPP binding site reflects an NMDA receptor antagonist effect. ACMP (**2**) showed an AMPA receptor antagonist effect with an IC_{50} value of 73 ± 8 μM towards AMPA (5 μM) evoked responses indicating that **2** is 4–5 times more potent than AMOA (IC_{50} = 320 ± 25 μM), in agreement with the relative AMPA receptor affinities observed for these two compounds. Finally, compound **5** was shown to be a very weak NMDA receptor antagonist, IC_{50} = 540 ± 90 μM towards NMDA (10 μM) evoked responses.

Compounds **1–5** were also tested for activity at metabotropic EAA receptors. None of the compounds (1 000 μM) showed agonist or antagonist effects at neither mGluR 1α, mGluR 2 nor at mGluR 4a, used as representatives of group I, II and III metabotropic EAA receptors, respectively.

4. Discussion

A series of compounds (**1–5**) structurally related to the AMPA receptor agonist ACPA and the AMPA receptor antagonist AMOA (*figure 1*) has been synthesized and pharmacologically characterized. ACPA and compound **3** are homologues of AMPA in terms of the carbon backbone connecting the α-amino acid moiety and the distal acidic group (*figure 1*). The carbon backbones of compounds **1** and **4** are one carbon atom longer than that of ACPA. Compounds **2** and **5** are the most distant homologues and thus are analogues of AMOA. The respective members of the two series of compounds, ACPA, **1**, **2** and compounds **3**, **4**, **5**, have similar chain lengths, though with different orientations of the substitutents. The location of substituents in compound **3** entailed almost complete loss of activity, compound **3** having 1 000 times weaker agonist activity than ACPA at AMPA receptors in electrophysiological experiments. Furthermore, compound **3** showed a very weak NMDA antagonist effect. Thus, the orientation of substituents at the isoxazole ring is of utmost importance both for potency at and selectivity for AMPA receptors. Compound **1** and **4** have intermediate chain lengths compared to the two parent compounds ACPA and AMOA. Both compounds show very weak effects, compound **1** as an AMPA agonist, whereas the weak affinity of compound **4** for the [^{3}H]CPP binding site did not detectably manifest itself in electrophysiological experiments. Obviously, the chain length and the orientation of substituents represented by these two compounds are unfavourable for potent interaction with EAA receptors. ACMP (**2**), the 'carbon analogue' of AMOA, proved to have interesting pharmacology, being 4–5 times more potent than AMOA as an AMPA receptor antagonist, and with similarly high selectivity for AMPA receptors. In contrast to this, the isomeric analogue **5** proved to be a very weak NMDA antagonist, but with no detectable effect at AMPA receptors.

In conclusion, structure–activity studies on the new compounds **1**–**5** have emphasized that the chain length is of importance for the effects at EAA receptors. Like ACPA, compound **3** is an agonist, though markedly

weaker than ACPA, whereas AMOA, ACMP (**2**) and **5** are antagonists. The orientation of substituents at the isoxazole ring of these acidic isoxazole amino acid analogues is a factor of key importance for the observed potency as well as for receptor selectivity. ACMP (**2**) was shown to be a selective AMPA receptor antagonist more potent than AMOA. Thus, ACMP (**2**) may be a useful pharmacological tool, since AMOA has previously been shown to possess, neuroprotective properties [18].

5. Experimental protocols

5.1. General chemistry

Column chromatography (CC) was performed on silica gel C60-H (230–400 mesh, Rhône-Poulenc). Solvents were dried using molecular sieves. Compounds containing amino groups were visualized on TLC plates (Merck silica gel 60 F_{254}) using a ninhydrin spray reagent, and all other compounds were visualized using UV light and/or a $KMnO_4$ spray reagent. ^{1}H-NMR and ^{13}C-NMR spectra were recorded and NOESY experiments carried out on a Bruker AC-200 MHz spectrometer or a Varian Gemini 2000 BB 300 MHz spectrometer. Chemical shifts (δ) are in parts per million with respect to TMS or 1,4-dioxane for compounds dissolved in organic solvents or D_2O, respectively. Melting points were determined in capillary tubes and are uncorrected. Elemental analyses were performed by the Analytical Department, H. Lundbeck A/S Denmark, and were within ± 0.4% of the calculated values unless otherwise stated.

5.2. Synthetic procedures

5.2.1. 4-(Bromomethyl)-3-(hydroxymethyl)-5-methylisoxazole **8**, *bis[(4-(bromomethyl)-5-methyl-3-isoxazolyl) methyl] methyl ether* **9** *and 3,4-di(bromomethyl)-5-methylisoxazole* **10**

3-(Hydroxymethyl)-5-methylisoxazole (**6**) [10] (1.13 g; 10 mmol) and 1,3,5-trioxane (1.35 g; 15 mmol) were dissolved in concentrated hydrobromic acid (62%, 10 mL) and heated at 60 °C for 18 h in a closed pressure vessel. After cooling to room temperature the reaction mixture was extracted with CH_2Cl_2 and the organic phase dried ($MgSO_4$) and evaporated. CC (Tol./EtOAc, 6:1) afforded crude **10** (70 mg; 2.6%) as an oil. ^{1}H-NMR (200 MHz, $CDCl_3$): δ 2.42 (3H, s), 4.40 (2H, s), 4.49 (2H, s). Further elution gave crude **9** (580 mg; 27%) as an oil. ^{1}H-NMR (200 MHz, $CDCl_3$): δ 2.42 (6H, s), 4.38 (4H, s), 4.79 (4H, s), 4.86 (2H, s). ^{13}C-NMR (200 MHz, $CDCl_3$): δ 10.9, 20.0, 60.4, 94.5, 111.7, 158.9, 168.2. ^{13}C-DEPT-NMR (200 MHz, $CDCl_3$): δ 10.9, (CH_3), 20 ($CH)_2$, 60.4 (CH_2) 94.5 (CH_2). Further elution gave crude **8** (550 mg; 27%) as an oil. ^{1}H-NMR (200 MHz, $CDCl_3$): δ 2.43 (3H, s), 3.85 (1H, broad s), 4.39 (2H, s), 4.80 (2H, s).

5.2.2. 3,4-Di(bromomethyl)-5-methylisoxazole **10**

3-(Chloromethyl)-5-methylisoxazole (**7**) [11] (5.98 g; 45.6 mmol) and 1,3,5-trioxane (6.25 g; 68.4 mmol) were dissolved in concentrated hydrobromic acid (62%, 46 mL) and heated at 60 °C for 18 h in a closed pressure vessel. After cooling to room temperature the reaction mixture was extracted with CH_2Cl_2 and the organic phase dried ($MgSO_4$) and evaporated. CC (Tol.) afforded crude **10** (10.9 g; 89%) as an oil. ^{1}H-NMR as described above.

5.2.3. Methyl 2-acetamido-2-(methoxycarbonyl)-3-[3-(bromomethyl)-5-methyl-4-isoxazolyl]propionate **11**

Dimethyl acetamidomalonate (1.35 g; 7.1 mmol) was dissolved in dry DMF (25 mL) and sodium hydride (350 mg, 60% suspension in mineral oil; 8.8 mmol) was added. The reaction mixture was stirred for 30 min and then added dropwise over a period of 30 min to a solution of compound **10** (1.93 g; 7.2 mmol) dissolved in dry DMF (5 mL). After stirring at room temperature overnight the reaction mixture was quenched with AcOH (8 drops) and evaporated. To the residue was added water and the mixture was extracted with EtOAc. The organic phase was dried ($MgSO_4$), evaporated and subjected to CC (CH_2Cl_2/EtOAc, 6:1), which afforded compound **11** after recrystallization (EtOAc/light petroleum) as colourless crystals (1.04 g; 38%): m.p. 135–138 °C. ^{1}H-NMR (200 MHz, $CDCl_3$): δ 2.06 (3H, s), 2.28 (3H, s), 3.54 (2H, s), 3.81 (6H, s), 4.26 (2H, s), 6.91 (1H, broad s). Anal. $C_{13}H_{17}N_2O_6Br$ (C, H, N).

5.2.4. Methyl 2-(methoxycarbonyl)-3-[3-(bromomethyl)-5-methyl-4-isoxazolyl]propionate **12**

Dimethyl malonate (984 mg; 7.45 mmol) was dissolved in dry DMF (10 mL) and sodium hydride (240 mg, 60% suspension in mineral oil; 6.0 mmol) was added. The reaction mixture was stirred for 30 min and then added to a solution of compound **10** (2.0 g; 7.4 mmol) in dry DMF (10 mL) and stirred overnight at room temperature. After evaporation, H_2O was added to the residue and the mixture was extracted with EtOAc. The organic phase was dried ($MgSO_4$), evaporated and CC (Tol./EtOAc, 6:1) afforded crude **12** (1.9 g; 80%) as an oil which was used in the next step without further purification. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 2.37 (3H, s), 3.06 (2H, d, J = 8 Hz), 3.72 (1H, t, J = 8 Hz), 3.73 (6H, s), 4.44 (2H, s).

5.2.5. [2-(Bromomethyl)-5-methyl-4-isoxazolyl]-acetonitrile **13**

Compound **10** (2.0 g; 7.4 mmol) was dissolved in acetonitrile (100 mL) containing benzyltrimethylammonium chloride (60 mg), and a solution of sodium cyanide (400 mg; 8.2 mmol) in water (60 mL) was added. The reaction mixture was stirred for 5 days, water was added and the mixture was extracted with CH_2Cl_2. The organic phase was dried ($MgSO_4$), evaporated, and CC (Tol./EtOAc, 3:1) afforded starting material **10** (690 mg; 35%) and crude **13** (710 mg; 44%) as an oil, which was used in the next step without further purification. ^{1}H-NMR (200 MHz, $CDCl_3$): δ 2.47 (3H, s), 3.58 (2H, s), 4.47 (2H, s).

5.2.6. Methyl 2-acetamido-2-(methoxycarbonyl)-3-[3-(cyanomethyl)-5-methyl-4-isoxazolyl]propionate **14**

Sodium cyanide (140 mg; 2.9 mmol) was dissolved in DMSO (10 mL) followed by addition of compound **11** (1.0 g; 2.65 mmol) and stirring overnight. H_2O was added and the mixture extracted with CH_2Cl_2. The organic phase was dried ($MgSO_4$) and evaporated, and recrystallization (CH_2Cl_2) of the residue afforded **14** (557 mg; 65%) as colourless crystals: m.p. 206–209 °C. ^{1}H-NMR (200 MHz, DMSO-d_6): δ 1.95 (3H, s), 2.26 (3H, s), 3.26 (2H, s), 3.69 (6H, s), 3.91 (2H, s). Anal. $C_{14}H_{17}N_3O_6$ (C, H, N).

5.2.7. Methyl 2-acetamido-2-(methoxycarbonyl)-3-{3-[2,2-(dimethoxycarbonyl)ethyl]-5-methyl-4-isoxazolyl}propionate **15**

Dimethyl malonate (176 mg; 1.33 mmol) was dissolved in dry DMF (5 mL) and sodium hydride (58.5 mg, 60% suspension in mineral oil; 1.46 mmol) was added. The reaction mixture was stirred for 15 min and to this was then dropwise added a solution of compound **11** (500 mg; 1.33 mmol) in dry DMF (5 mL). Stirring overnight at room temperature and then quenching with AcOH (5 drops). After evaporation of the reaction mixture, H_2O was added to the residue and the mixture was extracted with EtOAc. The organic phase was dried ($MgSO_4$) and evaporated, and recrystallization (Tol./light petroleum) of the residue afforded **15** (350 mg; 62%): m.p. 91–93 °C. ^{1}H-NMR (200 MHz, $CDCl_3$): δ 2.06 (3H, s), 2.21 (3H, s), 3.00 (2H, d, J = 7.4 Hz), 3.41 (2H, s), 3.72 (6H, s), 3.82 (6H, s), 4.09 (1H, t, J = 7.4 Hz), 6.95 (1H, broad s). Anal. $C_{18}H_{24}N_2O_{10}$ (C, H, N).

5.2.8. Methyl 2-acetamido-2-(methoxycarbonyl)-3-{4-[2,2-di(methoxycarbonyl)ethyl]-5-methyl-3-isoxazolyl}propionate **16**

Dimethyl acetamidomalonate (600 mg; 3.17 mmol) was dissolved in dry DMF (4 mL) and sodium hydride (127 mg, 60% suspension in mineral oil; 3.17 mmol) was added. The reaction mixture was stirred for 30 min, and to this mixture was dropwise added a solution of compound **12** (920 mg; 2.88 mmol) in dry DMF (5 mL). The reaction mixture was stirred overnight at room temperature and then quenched with AcOH (5 drops). After evaporation of the reaction mixture, ice-cold 1 M NaOH (20 mL) was added to the residue and the mixture was extracted with CH_2Cl_2. The organic phase was dried ($MgSO_4$), evaporated and CC (Tol./EtOAc, 1:1) afforded **16** (650 mg; 53%) after recrystallization (Tol.): m.p. 99–103 °C. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 2.01 (3H, s), 2.31 (3H, s), 2.87 (2H, d, J = 7.4 Hz), 3.50 (1H, t, J = 7.4 Hz), 3.70 (2H, s), 3.71 (6H, s), 3.82 (6H, s), 6.93 (1H, broad s). Anal. $C_{18}H_{24}N_2O_{10}\cdot 1/3C_7H_8$ (C, H, N).

5.2.9. Ethyl 2-acetamido-2-(ethoxycarbonyl)-3-[4-(cyanomethyl)-5-methyl-3-isoxazolyl]propionate **17**

Diethyl acetamidomalonate (1.32 g; 6.1 mmol) was dissolved in dry DMF (30 mL) and sodium hydride (290 mg, 60% suspension in mineral oil; 7.3 mmol) was added. The reaction mixture was stirred for 30 min, and to this mixture was dropwise added a solution of compound **13** (1.31 mg; 6.1 mmol) in dry DMF (20 mL). The reaction mixture was stirred overnight at room temperature and then evaporated. Water was added to the residue and the mixture was extracted with EtOAc. The organic phase was dried ($MgSO_4$) and evaporated, and CC (Tol./EtOAc, 1:1) afforded **17** (530 mg; 25%) as colourless crystals after recrystallization (Tol.): m.p. 112–114 °C. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.28 (6H, t, J = 7.1 Hz), 2.03 (3H, s), 2.46 (3H, s), 3.39 (2H, s), 3.72 (2H, s), 4.30 (4H, q, J = 7.1 Hz), 6.87 (1H, broad s). Anal. $C_{16}H_{21}N_3O_6$ (C, H, N).

5.2.10. Methyl 2-acetamido-2-(methoxycarbonyl)-3-[3-(methoxycarbonyl)methyl-5-methyl-4-isoxazolyl]propionate **18**

Acetyl chloride (25 mL) was slowly added to ice-cold MeOH (100 mL), and **14** (970 mg; 3.0 mmol) was then added to the solution. After stirring for 2 days, water (50 mL) was added and the MeOH evaporated. Saturated sodium bicarbonate was added until neutral pH, followed by extraction with CH_2Cl_2. The organic phase was dried ($MgSO_4$), evaporated and recrystallized (Tol.) to give **18** (960 mg; 90%): m.p. 168–179 °C (decomp.). ^{1}H-NMR (300 MHz, $CDCl_3$): δ 2.03 (3H, s), 2.27 (3H, s), 3.45 (2H, s), 3.60 (2H, s), 3.73 (3H, s), 3.79 (6H, s), 6.72 (1H, broad s). Anal. $C_{15}H_{20}N_2O_8$ (C, H), N: calcd: 7.86; found: 8.48.

5.2.11. Ethyl 3-(chloromethyl)-5-methyl-4-isoxazolecarboxylate **20**

Thionyl chloride (50 mL) was slowly added to ethyl 3-hydroxymethyl-5-methyl-4-isoxazolecarboxylate (**19**) [12] (3.0 g; 16.2 mmol) followed by addition of DMF (5 drops). The reaction mixture was refluxed for 90 min, cooled to room temperature and evaporated. H_2O was added and the mixture extracted with CH_2Cl_2. The organic phase was dried ($MgSO_4$) and evaporated, and CC (Tol.) afforded crude **20** (3.1 g; 94%) as an oil, which was used in the next step without further purification. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.39 (3H, t, *J* = 7.2 Hz), 2.69 (3H, s), 4.36 (2H, q, *J* = 7.2 Hz), 4.77 (2H, s).

5.2.12. Ethyl 2-acetamido-2-(ethoxycarbonyl)-3-[4-(ethoxycarbonyl)-5-methyl-3-isoxazolyl]propionate **21**

A solution of diethyl acetamidomalonate (3.2 g; 14.7 mmol) in dry DMF (5 mL) was added to a suspension of sodium hydride (590 mg, 60% suspension in mineral oil; 14.7 mmol) in dry DMF (8 mL), and then a solution of **20** (2.56 g; 12.6 mmol) in dry DMF (20 mL) was added. After stirring overnight at room temperature AcOH (1.5 mL) was added and the reaction mixture was evaporated. Ice-cold 1 M NaOH was added to the residue, and the mixture was extracted with CH_2Cl_2. The organic phase was dried ($MgSO_4$), evaporated and CC (Tol./EtOAc, 3:1) afforded **21** (2.6 g; 54%) after recrystallization (Tol.): m.p. 110–111 °C. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.26 (6H, t, *J* = 7.2 Hz), 1.38 (3H, t, *J* = 7.2 Hz), 1.98 (3H, s), 2.64 (3H, s), 4.01 (2H, s), 4.28 (2H + 4H, m), 6.80 (1H, broad s). Anal. $C_{17}H_{24}N_2O_8$ (C, H, N).

5.2.13. (RS)-2-Amino-3-[3-(carboxymethyl)-5-methyl-4-isoxazolyl]propionic acid **1**

Compound **18** (500 mg; 1.4 mmol) was refluxed in 1 M HCl (50 mL) for 16 h, and then the solution was evaporated. The residue was dissolved in H_2O, re-evaporated and then dissolved in 70% EtOH (10 mL), and to the solution was added propylenoxide (1 mL). The reaction mixture was kept at 5 °C overnight, evaporated, and the residue recrystallized (H_2O/EtOH) to give **1** (192 mg; 60%): m.p. 137–138 °C. ^{1}H-NMR (300 MHz, D_2O): δ 2.20 (3H, s), 2.69 (1H, dd, *J* = 15.5 and 8.0 Hz), 2.85 (1H, dd, *J* = 15.5 and 6.8 Hz), 3.43 (2H, s), 3.62 (1H, dd, *J* = 8.0 and 6.8 Hz). Anal. $C_9H_{12}N_2O_5 \cdot 1.5H_2O$ (C, H, N).

5.2.14. (RS)-2-Amino-3-[3-(2-carboxyethyl)-5-methyl-4-isoxazolyl]propionic acid (ACMP) **2**

Compound **15** (200 mg; 0.47 mmol) was refluxed for 1 h in hydrobromic acid (48%, 20 mL). The reaction mixture was evaporated. The residue was re-dissolved in AcOH and re-evaporated three times. The residue was then dissolved in 70% EtOH (15 mL) and propylenoxide (250 μL) was added. The mixture was kept at 5 °C overnight giving **2** (70 mg; 62%) as a colourless precipitate: m.p. 212–213 °C (decomp.). ^{1}H-NMR (300 MHz, D_2O): δ 2.20 (3H, s), 2.65 (2H, t, *J* = 7 Hz), 2.75 (1H + 2H, m), 2.88 (1H, dd, *J* = 13.5 and 7 Hz), 3.65 (1H, t, *J* = 7 Hz). Anal. $C_{10}H_{14}N_2O_5 \cdot 1.75H_2O$ (C, H, N).

5.2.15. (RS)-2-Amino-3-(4-carboxy-5-methyl-3-isoxazolyl)propionic acid **3**

Compound **21** (500 mg; 1.3 mmol) was refluxed in 1 M HCl (50 mL) for 16 h, and then the solution was evaporated. The residue was dissolved in H_2O, re-evaporated and then dissolved in 70% EtOH (10 mL). To this solution was added propylenoxide (0.5 mL), whereafter **3** (180 mg; 65%) precipitated: m.p. 218–220 °C (decomp.). ^{1}H-NMR (300 MHz, D_2O): δ 2.25 (3H, s), 3.15 (1H, dd, *J* = 15.5 and 8.0 Hz), 3.40 (1H, dd, *J* = 15.5 and 5.0 Hz), 4.20 (1H, dd, *J* = 8.0 and 5.0 Hz). Anal. $C_8H_{10}N_2O_5$ (C, H, N).

5.2.16. (RS)-2-Amino-3-[4-(carboxymethyl)-5-methyl-3-isoxazolyl]propionic acid **4**

Compound **17** (220 mg; 0.63 mmol) was refluxed for 25 min in hydrobromic acid (48%, 20 mL). The reaction mixture was evaporated. The residue was re-dissolved in AcOH and re-evaporated three times. The residue was then dissolved in 70% EtOH (5 mL) and propylenoxide (250 μL) was added. The mixture was stirred for 2 h, evaporated and the residue recrystallized (H_2O/isopropanol) to give **4** (70 mg; 49%): m.p. 201–202 °C (decomp.). ^{1}H-NMR (300 MHz, D_2O): δ 2.20 (3H, s), 3.00 (1H, dd, *J* = 15.5 and 8.0 Hz), 3.15 (1H, dd, *J* = 15.5 and 5.0 Hz), 3.27 (2H, s), 3.85 (1H, dd, *J* = 8.0 and 5.0 Hz). Anal. $C_9H_{12}N_2O_5 \cdot ½H_2O$ (C, H), N: calcd.: 11.81; found: 12.26.

5.2.17. (RS)-2-Amino-3-[4-(2-carboxyethyl)-5-methyl-3-isoxazolyl]propionic acid **5**

Compound **16** (430 mg; 1.0 mmol) was refluxed in 1 M HCl (20 mL) for 16 h and then the solution was evaporated. The residue was dissolved in H_2O, the mixture was extracted with EtOAc, and then the aqueous phase was evaporated. The residue was dissolved in 70% EtOH (5 mL), and to the solution was added propylenoxide (0.5 mL), whereafter **5** (118 mg; 49%) precipitated: m.p. 216–219 °C (decomp.). ^{1}H-NMR (300 MHz, D_2O): δ 2.24 (3H, s), 2.45 (2H, t, *J* = 6.5 Hz), 2.58 (2H, t, *J* = 6.5 Hz), 3.10 (1H, dd, *J* = 15.5 and 8.1 Hz), 3.19 (1H, dd, *J* = 15.5 and 4.8 Hz), 4.00 (1H, dd, *J* = 8.1 and 4.8 Hz). Anal. $C_{10}H_{14}N_2O_5$ (C, H, N).

5.3. Receptor binding

Affinity for AMPA, Kain and NMDA receptors was determined using the ligands [^{3}H]AMPA, [^{3}H]Kain and [^{3}H]CPP, respectively [13–15]. The membrane preparations used in all the receptor binding experiments were prepared according to the method of Ransom and Stec [19].

5.4. In vitro electrophysiology

The rat cortical slice preparation for determination of EAA-evoked depolarizations described by Harrison and Simmonds [16] was used in a modified version [20]. Application of agonists were made for 90 s at each concentration tested. The receptor selectivity of agonists was tested using the AMPA receptor antagonist NBQX (5 μm) or the NMDA receptor antagonist CPP (10 μm). In antagonist experiments, the antagonists were applied alone for 90 s followed by co-application with agonists for another 90 s.

5.5. Effect at metabotropic EAA receptors

The metabotropic EAA receptor subtypes mGluR 1α, mGluR 2 and mGluR 4a were expressed in Chinese hamster ovary cell lines and used as representatives for group I, II and III metabotropic EAA receptors, respectively. All compounds were tested for agonist and antagonist activity at 1 000 μM concentrations by the method previously described [21].

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