Synthesis and Single Cell Pharmacology of Potential Heterocyclic Bioisosteres of the Excitatory Amino Acid Antagonist Glutamic Acid Diethyl Ester

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A series of heterocyclic analogues of glutamic acid diethyl ester (GDEE), an antagonist at central excitatory amino acid receptors, have been synthesized and tested biologically. (*RS*)-Ethyl α-amino-α-(3-ethoxyisoxazol-5-yl)acetate (**7**), (*RS*)-ethyl 2-amino-3-(3-ethoxy-5-methylisoxazol-4-yl)propionate (**16**) and closely related analogues were synthesized. Compound **7**, a diethyl derivative of the naturally occurring excitatory amino acid ibotenic acid (IBO), was synthesized from 3-hydroxy-5-methylisoxazole (**1**) via 3-ethoxyisoxazol-5-ylacetic acid (**5**) and its ethyl ester. Nitrosation of this ester followed by catalytic reduction gave **7**. The ethyl ester of IBO, **9**, was synthesized in a similar manner from 3-benzyloxyisoxazol-5-ylacetic acid (**8**). Ethyl derivatives of the synthetic excitatory amino acid 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionic acid (AMPA) were synthesized from 3-hydroxy-4,5-dimethylisoxazole (**10**) through a diethyl acetylaminomalonate derivative, which upon deprotection gave the 3-ethoxy derivative of AMPA (**15**). Esterification of **15** gave the diethyl derivative **16** and the ethyl ester of AMPA (**18**) as well as *N*-ethylated derivatives of AMPA, **21** and **22** were synthesized. The final products were tested microelectrophoretically. The derivatives **7**, **9**, **15**, **16** and **18** were weak and non-selective excitatory amino acid antagonists, whereas **21** and **22** were found to be inactive.

Glutamic acid (GLU) is the major excitatory amino acid (EAA) neurotransmitter in the central nervous system.[1-3] Much pharmacological and therapeutic interest is focused on antagonists for central EAA synapses in particular, due to the possible involvement of hyperactivity at these synapses in the development of certain neurodegenerative diseases.[4-6]

At present, receptors for the EAAs are subdivided into three main classes:[1,7-9] (1) NMDA receptors, at which *N*-methyl-D-aspartic acid (NMDA) is a selective agonist; (2) QUIS/AMPA receptors, at which quisqualic acid (QUIS) is a potent but non-selective agonist, whereas 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionic acid (AMPA) is a potent and selective agonist; and (3) KA receptors, at which kainic acid (KA) is a potent agonist (Fig. 1).

The pharmacology of the NMDA receptor complex has been quite extensively elucidated,[10] largely due to the availability of potent and selective antagonists such as 2-amino-5-phosphonovaleric acid and (*RS*)-3-(2-carboxypiperazin-4-yl)propylphosphonic acid.[1,10-12] In contrast, selective antagonists for AMPA or KA receptors have not yet been described. Glutamic acid diethyl ester (GDEE) is an antagonist for QUIS/AMPA receptors,[7,13,14] but low potency, lack of specificity and chemical instability limit the utility of GDEE. During the past years GDEE has, however, been used to characterize the *in vivo* pharmacology of a number of QUIS/AMPA receptor agonists.[15-17]

Using GDEE as a lead structure a series of compounds has been designed and synthesized as potential antagonists at EAA receptors. Thus, two GLU analogues, ibotenic acid (IBO) and AMPA have been converted into mono- and di-ethyl derivatives (Fig. 1). IBO is a naturally occurring amino acid, which primarily acts as an NMDA agonist and to a lesser extent activates QUIS/AMPA and KA receptors.[17-19] As mentioned above, AMPA is a highly selective synthetic agonist for the QUIS/AMPA receptors.[8,20-22] IBO and AMPA are GLU bioisosteres, in which the ω-carboxy group of GLU has been replaced by 3-hydroxyisoxazole moieties. An analogous ester bioisosteric approach has now been designed, and we have synthesized isoxazole ester analogues of GDEE, namely (*RS*)-ethyl α-amino-α-(3-ethoxyisoxazol-5-yl)acetate (**7**), (*RS*)-ethyl 2-amino-3-(3-ethoxy-5-methylisoxazol-4-yl)propionate (**16**), (*RS*)-ethyl 2-amino-3-(2-ethyl-5-methyl-3-oxoisoxazolin-4-yl)propionate (**22**) and other closely related analogues. Pharmacological investigations of these compounds using microelectrophoretic techniques are also described.

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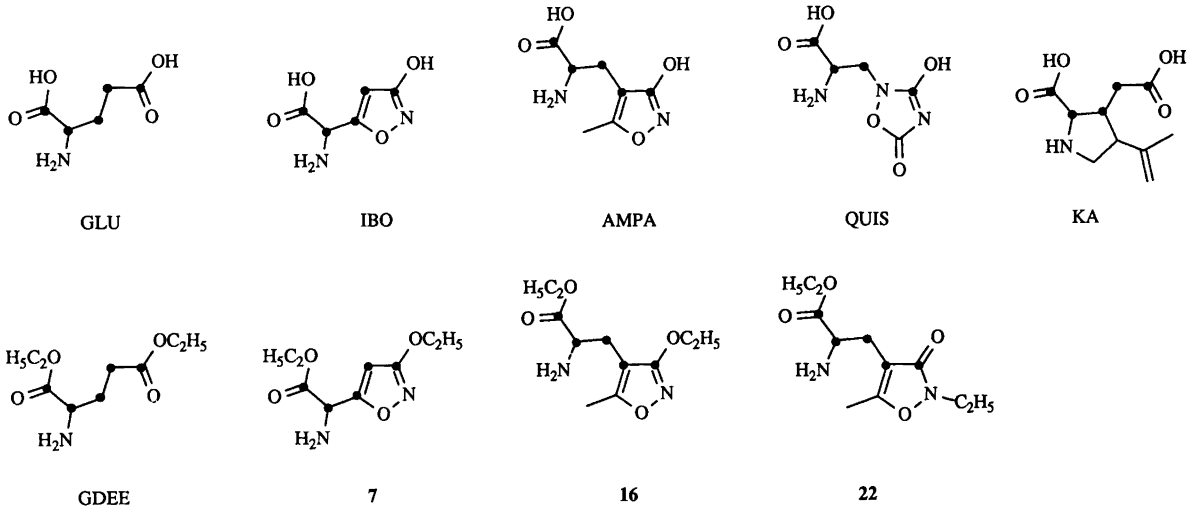
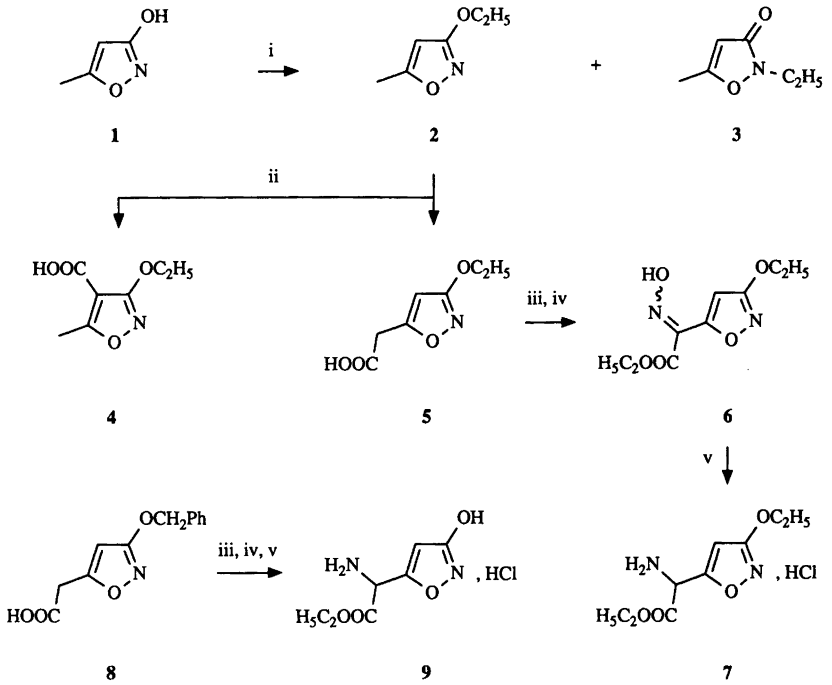


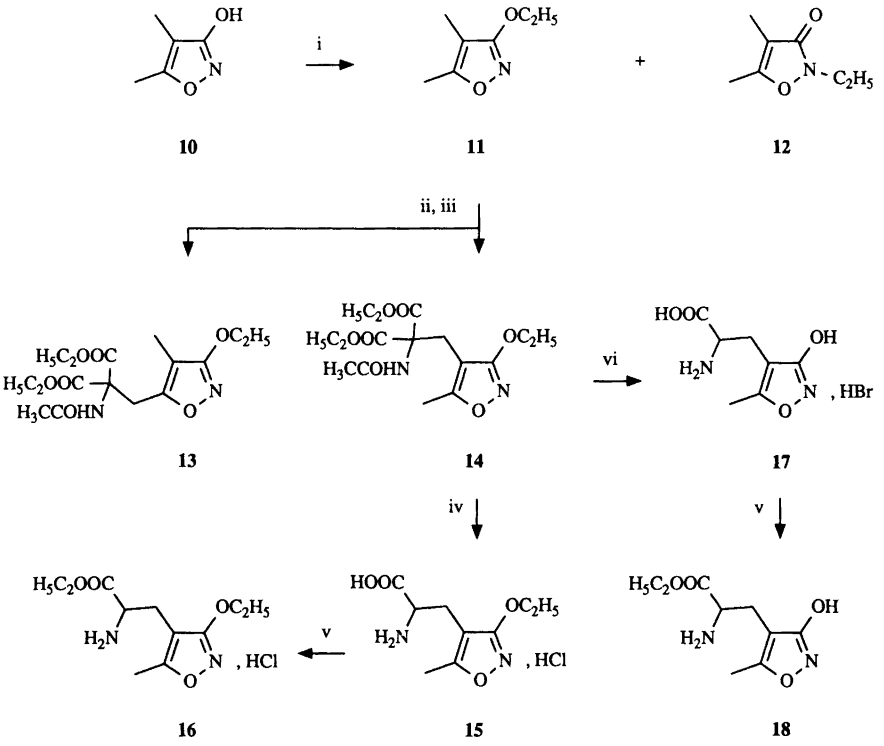
Fig. 1.

Results and discussion

3-Hydroxy-5-methylisoxazole[23] (**1**) was ethylated with ethyl bromide, which gave an easily separable mixture of **2** and **3**, the *O*-ethylated compound **2** being the major product (Scheme 1). Lithiation of **2** followed by reaction with carbon dioxide gave the two carboxylic acids **4** and **5** in 4.4 and 42 % yield, respectively. Esterification of **5** was accomplished by reaction with ethyl chloroformate and triethylamine dissolved in dichloromethane to give the desired ester in practically quantitative yield. This mixed anhydride synthesis has been described as requiring *N,N*-dimethylaminopyridine (DMAP) as a catalyst,[24] but this and other syntheses[25] have shown no need for DMAP as a catalyst. Nitrosation of the obtained ester by reaction with NaH and immediate addition of butyl nitrite gave the oxime **6**, which could be reduced to the final product **7** by low-pressure hydrogenation with Pd-on-C as a catalyst. The ethyl ester of IBO (**9**) was synthesized by analogy with **7** except for the protecting group of the 3-hydroxyisoxazole. Thus, 3-benzyloxyisoxazol-5-ylacetic acid[25] (**8**) was esterified and converted into an oxime by mixed anhydride esterification and



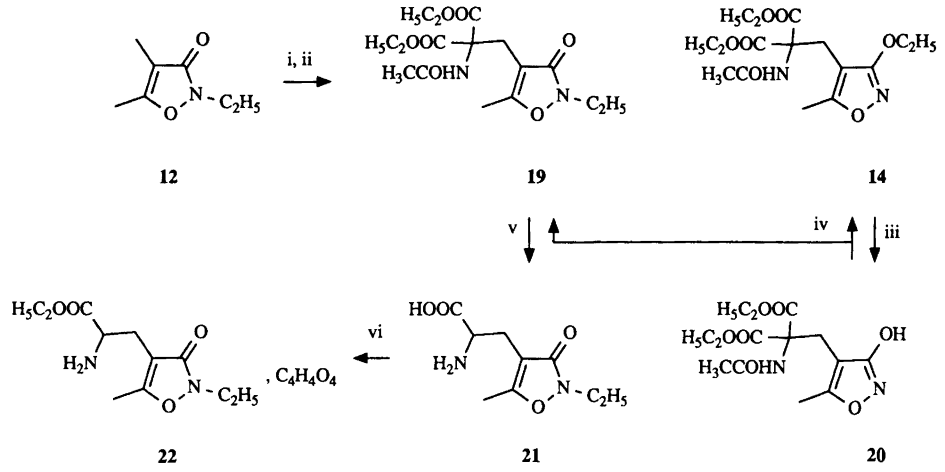
Scheme 1. (i) C_2H_5Br, K_2CO_3 (ii) 1) BuLi 2) CO_2 (iii) Cl-$COOC_2H_5$, TEA (iv) 1) NaH 2) BuONO (v) H_2/Pd, HCl



Scheme 2. (i) C_2H_5Br, K_2CO_3 (ii) NBS (iii) C_2H_5ONa, AAME (iv) 1 M HCl (v) C_2H_5OH/H^+ (vi) HBr/H_2O

subsequent nitrosation. The final compound **9** was obtained by simultaneous reduction of the oxime and reductive cleavage of the benzyloxy group.

The preparation of ethylated derivatives of AMPA are shown in Scheme 2. Ethylation of 3-hydroxy-4,5-dimethylisoxazole[26] (**10**) gave **11** as the major product, easily separable from the *N*-ethylated product **12**. NBS bromination of **11** and subsequent reaction with the sodium salt of diethyl acetamidomalonate (AAME) gave **13** and **14**. Deprotection of compound **14** with 1 M HCl gave **15**, which, by conventional esterification with ethanolic HCl, led to **16**. Reflux of **14** in aqueous HBr (48 %) gave AMPA hydrobromide (**17**). This synthetic route to AMPA is slightly different from previously published procedures for the



Scheme 3. (i) NBS (ii) C_2H_5ONa, AAME (iii) HBr/AcOH (iv) C_2H_5Br, K_2CO_3 (v) 1 M HCl (vi) C_2H_5OH/H^+

preparation of AMPA.[27-28] Conventional esterification of **17** gave **18**.

The syntheses of *N*-ethylated derivatives of AMPA are shown in Scheme 3. NBS bromination of **12** and subsequent reaction with the sodium salt of AAME gave **19** and probably also a 5-methyl substituted isomer, which was not isolated. The structure of **19** was established by its preparation by an alternative route. Compound **14** was selectively de-ethylated to **20** by treatment with HBr in glacial acetic acid (33 %) and re-ethylated to give a mixture of **14** and **19**. Compound **19** prepared from **12** was identical with the sample of **19** synthesized from **14** via **20**, and the structure of **14** was confirmed by its conversion into AMPA hydrobromide (**17**) (Scheme 2). Deprotection of **19** with 1 M HCl gave **21**, which by conventional esterification gave **22**.

The pharmacological effects of compounds **7**, **9**, **15**, **16**, **18**, **21** and **22** on cat spinal neurones were tested using microelectrophoretic techniques.[15-17,29,30] The 3-ethoxyisoxazole isosteres of GDEE, compounds **7** and **16**, were shown to antagonize reversibly excitations induced by NMDA, QUIS or KA (results not shown). On most cells studied these EAA antagonist effects were weaker than those of GDEE. Thus, although the 3-ethoxyisoxazole nucleus has been shown to act as a bioisostere for the ω ester group of GDEE, this bioisosteric substitution has not led to EAA antagonists superior to GDEE in terms of potency or receptor selectivity.

The mono-ethyl derivatives **9**, **15** and **18** showed EAA antagonist profiles similar to those of **7** and **16**, but with most cells studied these three compounds were generally weaker than GDEE and the GDEE isosteres **7** and **16**. The *N*-ethylated compounds **21** and **22** did not significantly reduce excitations induced by NMDA, QUIS or KA on cat spinal neurones. Thus, although the 2-ethylisoxazolin-3-one nucleus in **21** and **22** can be considered as an isostere of the ethyl ester group, this heterocyclic unit does not act as an effective ester bioisostere at the EAA receptors. This lack of effect may reflect the structural and electronic differences between the ethyl ester group and the 2-ethylisoxazolin-3-one group or, alternatively, that this latter group in **21** or **22**, cannot easily adopt a conformation that reflects the active conformation of the ω ethyl ester group of GDEE at EAA receptors, notably the QUIS/AMPA receptor subtype.

Experimental

Melting points are corrected and were determined in capillary tubes. Elemental analysis were performed by Mr. G. Cornali, Microanalytical Laboratories, Leo Pharmaceutical Products, Denmark or Mr. P. Hansen, Department of General and Organic Chemistry, University of Copenhagen. The 60 MHz ^{1}H NMR spectra were recorded on a Varian EM 360L spectrometer and the 90 MHz ^{1}H NMR spectra on a Jeol FX 90Q spectrometer with compounds dissolved in $CDCl_3$ using TMS as a reference unless otherwise stated. IR spectra, obtained on a Perkin-Elmer 781 Infrared spectrophotometer, were recorded in KBr pellets or as liquid films between NaCl discs. A Waters PrepLC-system 500A instrument was used for the preparative high-pressure liquid chromatography (HPLC) using silica gel columns (PrepPAK®-500/Silica). Thin layer chromatography (TLC) and gravity column chromatography were performed using silica gel F_{254} plates (Merck) and silica gel (Woelm, 0.063–0.200 mm), respectively. Compounds containing the isoxazol-3-ol unit were visualized on TLC plates using UV light and an $FeCl_3$ spraying reagent (yellow color). Compounds containing amino groups were visualized using a ninhydrin spraying reagent, and all compounds under study were detected on TLC plates using a $KMnO_4$ spraying reagent. All evaporations were performed at ca. 15 mmHg using a rotary evaporator.

3-Ethoxy-5-methylisoxazole (**2**) *and 2-ethyl-5-methylisoxazolin-3-one* (**3**). To a solution of **1**[23] (20 g, 0.2 mol) in acetone (500 ml) was added K_2CO_3 (56 g, 0.4 mol) and the mixture was stirred at 60 °C for 0.5 h. Ethyl bromide (23 ml, 0.3 mol) was added dropwise and the mixture was left to stir at 60 °C overnight. After cooling, filtration and evaporation, preparative HPLC (ethyl acetate–dichloromethane 5:1) of the product gave **2** (16.7 g, 65 %) as a colourless oil. Anal. $C_6H_9NO_2$: C, H, N. ^{1}H NMR: δ 5.5 (s, 1 H), 4.2 (q, *J* 7 Hz, 2 H), 2.3 (s, 3 H), 1.35 (t, *J* 7 Hz, 3 H). IR: 2975 (m), 2925 (w), 1620 (s), 1505 (s), 1455 (s) cm^{-1}. Further elution (ethyl acetate) gave (**3**) (6.5 g, 25 %) as a colourless oil. Anal. $C_6H_9NO_2$: H, N; Found, C 56.06; Calc., C 56.68. ^{1}H NMR: δ 5.35 (s, 1 H), 3.8 (q, *J* 7 Hz, 2 H), 2.2 (s, 3 H), 1.25 (t, *J* 7 Hz, 3 H). IR: 2975 (m), 2925 (w), 1775 (s), 1625 (m), 1440 (m) cm^{-1}.

3-Ethoxy-5-methylisoxazol-4-ylcarboxylic acid (**4**) *and 3-ethoxyisoxazol-5-ylacetic acid* (**5**). To a solution of butyllithium (50 ml, 1.1 M in hexane; 55 mmol) in dry tetrahydrofuran (THF) (100 ml) cooled to −78 °C was added slowly a solution of **2** in dry THF (50 ml). After being stirred for 15 min at −78 °C the brown–black solution was poured into a slurry of crushed carbon dioxide (ca. 250 g) in dry ether (200 ml). The excess of carbon dioxide evaporated overnight and the reaction mixture was evaporated, and treated with water (75 ml), which was then acidified with 4 M HCl and extracted with dichloromethane (3×100 ml). The combined dichloromethane extracts were dried ($MgSO_4$) and evaporated and the residue gave, by preparative HPLC (toluene–ethyl acetate 9:1 with 1 % glacial acetic acid), compound **4** (385 mg, 4.4 %) after recrystallization (ethyl acetate–light petroleum), m.p. 162–164 °C. Anal. $C_7H_9NO_4$: C, H, N. ^{1}H NMR: δ 11.3 (s, 1 H), 4.1 (q, *J* 7 Hz, 2 H), 2.2 (s, 3 H), 1.2 (t, *J* 7 Hz, 3 H). IR: 3300–2350 (w–m, several bands), 1685 (s), 1620 (s), 1515 (s), 1470 (m) cm^{-1}. Further elution gave **5** (3.7 g, 42 %) after recrystallization (ethyl acetate–light petroleum), m.p. 69–70 °C. Anal. $C_7H_9NO_4$: C, H, N. ^{1}H NMR: δ 10.8 (s, 1 H), 5.7 (s, 1 H), 3.85 (q, *J* 7 Hz, 2 H), 3.2 (s, 2 H), 1.3 (t, *J* 7

Hz, 3 H). IR: 3300–2350 (w–m, several bands), 1730 (s), 1625 (s), 1615 (s), 1515 (s), 1465 (s) cm^{-1}.

(EZ)-*Ethyl α-hydroxyimino-α-(3-ethoxyisoxazol-5-yl)acetate* (**6**). To a solution of **5** (3.4 g, 20 mmol) and triethylamine (3 ml, 22 mmol) in dichloromethane (50 ml) cooled to 0 °C, was added slowly ethyl chloroformate (2.3 ml, 24 mmol). After being stirred for 5 min the reaction mixture was extracted with 1 M HCl (50 ml) and semi-saturated $NaHCO_3$ (50 ml). The dried ($MgSO_4$) dichloromethane phase was evaporated and subjected to Kugelrohr destillation (0.2 mmHg, 150 °C), which gave ethyl 3-ethoxyisoxazol-5-ylacetate (3.7 g, 93 %) as a colourless oil. Anal. $C_9H_{13}NO_4$: C, H, N. ^1H NMR: δ 5.8 (s, 1 H), 4.25 (q, *J* 7 Hz, 2 H), 4.15 (q, *J* 7 Hz, 2 H), 3.65 (s, 2 H), 1.35 (t, *J* 7 Hz, 3 H), 1.25 (t, *J* 7 Hz, 3 H). IR: 3140 (w), 2980 (s), 2935 (m), 1740 (s), 1620 (s), 1510 (s) cm^{-1}. NaH (100 mg, 80 % dispersion in white oil; 3.3 mmol) was added to a solution of ethyl 3-ethoxyisoxazol-5-ylacetate (600 mg, 3 mmol) in dry THF (10 ml) cooled to 0 °C and immediately after was added butyl nitrite (500 µl, 5 mmol). After being stirred for 5 min the reaction was quenched with glacial acetic acid (200 µl). Water (20 ml) was then added and the mixture was extracted with dichloromethane (3×20 ml). The combined dichloromethane extracts gave, after being dried ($MgSO_4$), evaporated and column chromatographed (dichloromethane–ethyl acetate 9:1), compound **6** (415 mg, 60 %). An analytical sample was recrystallized (toluene–light petroleum), m.p. 126–127 °C. Anal. $C_9H_{12}N_2O$: C, H, N. ^1H NMR: δ 6.7 (s, 1 H), 4.4 (q, *J* 7 Hz, 2 H), 4.3 (q, *J* 7 Hz, 2 H), 1.45 (t, *J* 7 Hz, 3 H), 1.4 (t, *J* 7 Hz, 3 H). IR: 3240 (s, broad), 3150 (s), 2985 (m), 2975 (m), 1725 (s), 1565 (s) cm^{-1}.

(RS)-*Ethyl α-amino-α-(3-ethoxyisoxazol-5-yl)acetate hydrochloride* (**7**). A stream of hydrogen was passed through a solution of **6** (25 mg, 0.11 mmol) and acetyl chloride (25 µl, ca. 0.4 mmol) in ethanol (5 ml) containing Pd-on-C (10 mg, 10 %) for 3 h. After filtration of the reaction mixture through Celite and evaporation of the filtrate, recrystallization (ethanol–ether) gave **7** (19 mg, 69 %), m.p. 172–174 °C (decomp.). Anal. $C_9H_{15}ClN_2O_4$: H, N; Found, C 42.25, Cl 14.75; Calc., C 43.10, Cl 14.14. ^1H NMR (90 MHz, D_2O): δ 6.4 (s, 1 H), 4.35 (q, *J* 7 Hz, 2 H), 4.25 (q, *J* 7 Hz, 2 H), 1.35 (t, *J* 7 Hz, 3 H), 1.25 (t, *J* 7 Hz, 3 H). IR: 3200–2500 (m–w, several bands), 1995 (w), 1745 (s), 1620 (s), 1570 (w), 1510 (s) cm^{-1}.

(RS)-*Ethyl α-amino-α-(3-hydroxyisoxazol-5-yl)acetate hydrochloride* (**9**). To a solution of **8**[25] (3 g, 12.9 mmol) and triethylamine (2 ml, 14.3 mmol) in dichloromethane (40 ml) cooled to 0 °C was added ethyl chloroformate (1.5 ml, 15.7 mmol). The reaction mixture was stirred for 5 min and extracted with 1 M HCl (40 ml) and semi-saturated $NaHCO_3$ (40 ml). After drying ($MgSO_4$) and evaporation of the dichloromethane phase, Kugelrohr destillation of the residue (0.02 mmHg, 250 °C) gave ethyl 3-benzyloxyisoxazol-5-ylacetate (2.9 g, 86 %) as a colorless oil, which slowly crystallized at room temperature, m.p. 35–36 °C. Anal. $C_{14}H_{16}NO_4$: C, H, N. ^1H NMR: δ 7.35 (s, 5 H), 5.9 (s, 1 H), 5.2 (s, 2 H), 4.2 (q, *J* 7 Hz, 2 H), 3.7 (s, 2 H), 1.25 (t, *J* 7 Hz, 3 H). IR: 3140 (w), 3060 (m), 3030 (m), 2975 (s), 2935 (s), 1740 (s), 1615 (s) cm^{-1}. To a solution of ethyl 3-benzyloxyisoxazol-5-ylacetate (785 mg, 3 mmol) in dry THF (10 ml) cooled to 0 °C was added NaH (100 mg, 80 % dispersion in white oil; 3.3 mmol) and followed immediately by butyl nitrite (500 µl, 5 mmol). After being stirred for 5 min the reaction was quenched with glacial acetic acid (200 µl) and treated with water (15 ml). Extraction with dichloromethane (3×15 ml), drying and evaporation of the combined dichloromethane extracts, and column chromatography (dichloromethane containing 5–11 % ethyl acetate) of the residue gave (*EZ*)-ethyl α-hydroxyimino-α-(3-benzyloxyisoxazol-5-yl)acetate (455 mg, 52 %). An analytical sample was recrystallized (toluene–light petroleum), m.p. 107–109 °C. Anal. $C_{14}H_{14}N_2O_5$: C, H, N. ^1H NMR: δ 10.6–10.0 (broad, 1 H), 7.4 (s, 5 H), 6.8 (s, 1 H), 5.3 (s, 2 H), 4.4 (q, *J* 7 Hz, 2 H), 1.35 (t, *J* 7 Hz, 3 H). IR: 3280 (s), 3030 (w), 2975 (w), 1735 (s), 1570 (s) cm^{-1}.

A stream of hydrogen was passed through a solution of (*EZ*)-ethyl α-hydroxyimino-α-(3-benzyloxyisoxazol-5-yl) acetate (100 mg, 0.34 mmol) and acetyl chloride (100 µl; ca. 1.5 mmol) in ethanol (10 ml) containing Pd-on-C (40 mg, 10 %) for 3 h. After filtration of the reaction mixture through Celite and evaporation of the filtrate, recrystallization (ethanol–ether) gave **9** (60 mg, 78 %), m.p. 184–186 °C (decomp.). Anal. $C_7H_{11}ClN_2O_4$: H, Cl; Found, C 36.12, N 11.93; Calc., C 37.75, N 12.63. ^1H NMR (D_2O): δ 6.35 (s, 1 H), 5.55 (s, ca. 1/4 H, partially exchanged), 4.3 (q, *J* 7 Hz, 2 H), 1.25 (t, *J* 7 Hz, 3 H). IR: 3300–2400 (m–s, several bands), 2050 (w), 1745 (s), 1625 (s), 1535 (s) cm^{-1}.

3-Ethoxy-4,5-dimethylisoxazole (**11**) and *2-ethyl-4,5-dimethylisoxazolin-3-one* (**12**). To a solution of **10**[26] (7.7. g, 68 mmol) in acetone (300 ml) was added K_2CO_3 (18.8 g, 136 mmol), and the mixture was stirred at 60 °C for 1 h. Ethyl bromide (10.4 ml, 136 mmol) was slowly added and the reaction mixture was stirred overnight at 60 °C. After cooling and filtration of the reaction mixture and evaporation of the filtrate, column chromatography (dichloromethane) gave **11** (4.7 g, 48 %) as a colourless oil. Anal. $C_7H_{11}NO_2$: C, H, N. ^1H NMR: δ 4.1 (q, *J* 7 Hz, 2 H), 2.2 (s, 3 H), 1.75 (s, 3 H), 1.4 (t, *J* 7 Hz, 3 H). IR: 2980 (m), 2930 (m), 1665 (m), 1515 (s), 1470 (s) cm^{-1}. Further elution (dichloromethane–ethyl acetate 3:1) gave **12** (2.4 g, 25 %) as a yellow oil. Anal. $C_7H_{11}NO_2$: H, N; Found, C 58.99; Calc., C 59.55. ^1H NMR: δ 3.6 (q, *J* 7 Hz, 2 H), 2.1 (s, 3 H), 1.65 (s, 3 H), 1.2 (t, *J* 7 Hz, 3 H). IR: 2980 (m), 2930 (m), 1660 (s, broad), 1440 (s), 1415 (s) cm^{-1}.

Ethyl 2-acetamido-2-ethoxycarbonyl-3-(3-ethoxy-4-methylisoxazol-5-yl)propionate (**13**) *and ethyl 2-acetamido-2-ethoxycarbonyl-3-(3-ethoxy-5-methylisoxazol-4-yl)propionate* (**14**). A mixture of **11** (1.3 g, 9.2 mmol), *N*-bromosuc-

cinimide (NBS) (1.64 g, 9.2 mmol) and benzoyl peroxide (40 mg) in carbon tetrachloride was refluxed for 3 h. The NBS and benzoyl peroxide were added in four equal portions at intervals of 45 min. After being cooled and filtered, the reaction mixture was evaporated to give an oil, which was dissolved in ethanol (5 ml) and added to a solution of Na (212 mg, 9.2 mmol) and AAME (2 g, 9.2 mmol) in ethanol (15 ml). The reaction was refluxed for 4 h, cooled, filtered and evaporated. Water (25 ml) was added and the mixture was extracted with dichloromethane (3×75 ml). The combined organic phases were washed with ice-cold 1 M NaOH (50 ml), dried ($MgSO_4$) and evaporated and the residue was subjected to column chromatography (dichloromethane containing 10–50 % ethyl acetate), which gave **13** (23 mg, 0.7 %) and **14** (436 mg, 13.3 %) both after recrystallization (ethyl acetate–light petroleum).

13: m.p. 132–132.5 °C. Anal. $C_{16}H_{24}N_2O_7$: H, N; Found, C 52.46; Calc., C 53.92. ^{1}H NMR: δ 6.5 (s, 1 H), 4.15 (q, *J* 7 Hz, 2×2 H), 4.1 (q, *J* 7 Hz, 2 H), 3.6 (s, 2 H), 1.95 (s, 3 H), 1.7 (s, 3 H), 1.2 (t, *J* 7 Hz, 3×3 H). IR: 3420 (m, broad), 3250 (s), 2985 (s), 2935 (m), 1750 (s), 1640 (s), 1515 (s) cm^{-1}.

14: m.p. 125.5–126.0 °C. Anal. $C_{16}H_{24}N_2O_7$: C, H, N. ^{1}H NMR: δ 6.5 (s, 1 H), 4.15 (3×q overlapping, *J* 7 Hz, 3×2 H), 3.25 (s, 2 H), 2.15 (s, 3 H), 1.95 (s, 3 H), 1.25 (t, *J* 7 Hz, 3×3 H). IR: 3420 (m, broad), 3250 (s), 2985 (s), 2935 (m), 1740 (s), 1640 (s), 1515 (s) cm⁻1.

(RS)-*2-Amino-3-(3-ethoxy-5-methylisoxazol-4-yl)propionic acid hydrochloride* (**15**). A solution of **14** (200 mg, 0.56 mmol) and 1 M HCl (5 ml) was refluxed for 20 h. The reaction mixture was extracted with ether (2×10 ml) and the aqueous phase was evaporated. Recrystallization (ethanol–ether) of the residue gave **15** (90 mg, 64 %), m.p. 199.5–200.5 °C (decomp.). Anal. $C_9H_{15}ClN_2O_4$: H, N, Cl; Found, C 42.65: Calc., C 43.40. ^{1}H NMR (D_2O): δ 4.2 (q, *J* 7 Hz, 2 H + 1 H), 2.9 (d, *J* 6 Hz, 2 H), 2.3 (s, 3 H), 1.35 (t, *J* 7 Hz, 3 H). IR: 3420 (m, broad), 3300–2400 (m–s, several bands), 1725 (s), 1640 (s), 1590 (s) cm^{-1}.

(RS)-*Ethyl 2-amino-3-(3-ethoxy-5-methylisoxazol-4-yl)propionate hydrochloride* (**16**). Compound **15** (105 mg, 0.42 mmol) was refluxed for 20 h in ethanol (10 ml) containing acetyl chloride (2 ml, ca. 25 mmol). The reaction mixture was evaporated, treated with 2 M ice-cold Na_2CO_3 (10 ml) and extracted with ether (3×10 ml). The combined organic phases were dried (K_2CO_3) and evaporated and the residue was dissolved in ethanol, to which acetyl chloride (2 ml) had been added, and re-evaporated. The residue was recrystallized (ethanol–ether), which gave **16** (83 mg, 71 %), m.p. 199–200 °C (decomp.). Anal. $C_{11}H_{19}ClN_2O_4$: C, H, N; Found, Cl 13.21; Calc., Cl 12.59. ^{1}H NMR (D_2O): δ 4.2 (q, *J* 7 Hz, 2×2 H + 1 H), 2.9 (d, *J* 6 Hz, 2 H), 2.25 (s, 3 H), 1.25 (t, *J* 7 Hz, 2×3 H). IR: 3450 (m), 2985 (s), 2850 (s), 2720 (m), 2640 (m), 1735 (s), 1645 (s) cm^{-1}.

(RS)-*Ethyl 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl) propionate zwitterion* (**18**). Compound **17**[27] (120 mg, 0.45 mmol) was refluxed in ethanol (10 ml), to which acetyl chloride (2 ml, ca. 25 mmol) had been added, for 4 h. The reaction mixture was evaporated and the residue was dissolved in water (ca. 1 ml) and ethanol (ca. 5 ml) and triethylamine (TEA) was added to pH ca. 6. The precipitated zwitterion was recrystallized twice from water to give **18** (25 mg, 26 %), m.p. 185–191 °C (decomp.). Anal. $C_9H_{14}N_2O_4$: C, H, N. ^{1}H NMR 90 MHz, D_2O: δ 4.25 (q, *J* 7.0 Hz, 2 H), 3.85 (t, *J* 6.1 Hz, 1 H), 2.8 (d, *J* 6.1 Hz, 2 H), 2.25 (s, 3 H), 1.35 (t, *J* 7.0 Hz, 3 H). IR: 3420 (m), 3300–2500 (m–s, several bands), 1660 (s), 1640 (s), 1585 (s), 1555 (s), 1515 (s), cm^{-1}.

Ethyl 2-acetamido-2-ethoxycarbonyl-3-(2-ethyl-5-methyl-3-oxoisoxazolin-4-yl)propionate (**19**). A mixture of **12** (1.5 g, 10.7 mmol), NBS (1.9 g, 10.7 mmol) and benzoyl peroxide (40 mg) in carbon tetrachloride (15 ml) was refluxed for 3 h. The NBS and benzoyl peroxide were added in four equal portions at intervals of 45 min. After being cooled and filtered the reaction mixture was evaporated to give an oil, which was dissolved in ethanol (10 ml) and added to a solution of Na (245 mg, 10.7 mmol) and AAME (2.3 g, 10.7 mmol) in ethanol (15 ml). The reaction mixture was refluxed for 4 h, evaporated, treated with water (20 ml) and extracted with dichloromethane (3×20 ml). The organic phases were washed with ice-cold 1 M NaOH (50 ml), dried ($MgSO_4$), evaporated and subjected to column chromatography (dichloromethane–ethyl acetate 1:1 containing 0–10 % methanol), which gave **19** (670 mg, 18 %). An analytical sample was recrystallized (ethyl acetate–light petroleum), m.p. 115–116 °C. Anal. $C_{16}H_{24}N_2O_7$: C, H, N. ^{1}H NMR (90 MHz): δ 4.25 (q, *J* 7.0 Hz, 2×2 H), 3.85 (q, *J* 7.0 Hz, 2 H), 3.24 (s, 2 H), 2.13 (s, 3 H), 2.00 (s, 3 H), 1.27 (t, *J* 7.0 Hz, 2×3 H), 1.24 (t, *J* 7.0 Hz, 3 H). IR: 3380 (s), 2990 (m), 2945 (w), 1765 (s), 1730 (s), 1660 (s, broad), 1510 (s) cm^{-1}. A spot on TLC (dichloromethane–ethyl acetate 1:1) of lower R_f-value (R_f 0.15) was discarded without characterization, but supposed to be the 5-methyl substituted isomer of **19**.

Ethyl 2-acetamido-2-ethoxycarbonyl-3-(2-ethyl-3-hydroxy-5-methylisoxazol-4-yl)propionate (**20**). A mixture of **14** (200 mg, 0.56 mmol) and a solution of HBr in glacial acetic acid (10 ml, 33 %) was refluxed for 1 h and evaporated. After re-evaporation from water, recrystallization (ethyl acetate–light petroleum) gave **20** (127 mg, 69 %), m.p. 155.5–156.5 °C. Anal. $C_{14}H_{20}N_2O_7$: H; Found, C 49.70, N 8.08; Calc., C 51.21, N 8.53. ^{1}H NMR: δ 8.35 (s, 1 H), 6.6 (s, 1 H), 4.15 (q, *J* 7 Hz, 2×2 H), 3.3 (s, 2 H), 2.1 (s, 3 H), 1.95 (s, 3 H), 1.25 (t, *J* 7 Hz, 2×3 H). IR: 3320 (m, broad), 2980 (m), 2930 (m), 1740 (s), 1650 (s), 1510 (s) cm^{-1}.

(RS)-*2-Amino-3-(2-ethyl-5-methyl-3-oxoisoxazolin-4-yl) propionic acid zwitterion* (**21**). A mixture of **19** (300 mg,

0.84 mmol) and 1 M HCl (30 ml) was refluxed for 16 h. After evaporation and re-evaporation from water, the residue was dissolved in water (2 ml) and loaded onto a basic ion-exchange column (IRA-400). Elution with 1 M acetic acid gave, after recrystallization (water–ethanol), **21** (98 mg, 54 %), m.p. 211.5–212 °C (decomp.). Anal. $C_9H_{14}N_2O_4$: H, N; Found, C 49.42; Calc., C 50.46. ^{1}H NMR (90 MHz, D_2O): δ 3.92 (q, *J* 7.0 Hz, 2 H), 3.86 (t, *J* 5.5 Hz, 1 H), 2.81 (d, *J* 5.5 Hz, 2 H), 2.21 (s, 3 H), 1.24 (t, *J* 7.0 Hz, 3 H). IR: 3400 (w, broad), 3300–2300 (m–s, several bands), 1640 (s), 1610 (s), 1435 (s) cm^{-1}.

(RS)-*Ethyl 2-amino-3-(2-ethyl-5-methyl-3-oxoisoxazolin-4-yl)propionate · fumaric acid* (**22**). A solution of **21** (50 mg, 0.23 mmol) in ethanol (5 ml) and acetyl chloride (1 ml, ca. 12 mmol) was refluxed for 24 h. The reaction mixture was evaporated, treated with 3 M $NaHCO_3$ (5 ml) and extracted with ether (3×10 ml). The combined organic phases were dried ($MgSO_4$) and evaporated. The residue was dissolved in ether (ca. 2 ml) and a solution of fumaric acid (27 mg, 0.23 mmol) in isopropyl alcohol (500 µl) was added. The precipitated product was recrystallized (ethanol–ether) to give **22** (41 mg, 49 %), m.p. 147–147.5 °C. Anal. $C_{11}H_{18}N_2O_4 \cdot C_4H_4O_4$; C, H, N. ^{1}H NMR (90 MHz, D_2O): δ 6.60 (s, 2 H), 4.30 (t, *J* 6.4 Hz, 1 H), 4.19 (q, *J* 7.0 Hz, 2 H), 3.90 (q, *J* 7.3 Hz, 2 H), 2.90 (d, *J* 6.4 Hz, 2 H), 2.21 (s, 3 H), 1.21 (t, *J* 7.0 Hz, 3 H), 1.17 (t, *J* 7.3 Hz, 3 H). IR: 3300–2300 (m–s, several bands), 1745 (s), 1640 (s), 1610 (s), 1560 (s), 1510 (s) cm^{-1}.

Microelectrophoretic studies. Experiments were performed on lumbar dorsal horn interneurones or Renshaw cells of cats anaesthetized with pentobarbitone sodium (35 mg kg^{-1} intraperitoneally initially, supplemented intraveneously when required). Extracellular action potentials were recorded by means of the central barrel of seven-barrel micropipettes, which contained 3.6 M NaCl. The compounds were administered electrophoretically from the outer barrels of the micropipettes,[29] which contained aqueous solutions of NMDA (0.05 M in 0.15 M NaCl, pH 7.6), QUIS (0.005 M in 0.15 M NaCl, pH 7.5), KA (0.005 M in 0.15 M NaCl, pH 7.5) and the test compounds 0.1 M, pH 3–3.4, ejected as cations). The excitatory amino acids were administered for periods of time sufficient to obtain maximal effects at the particular rate of ejection.

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