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Aryl and cycloalkyl analogues of AMPA: synthetic, pharmacological and stereochemical aspects

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Abstract

We have previously shown that (*RS*)-2-amino-3-(3-hydroxy-5-phenylisoxazol-4-yl)propionic acid (APPA, **2**) is a functional partial agonist at the (*RS*)-2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionic acid (AMPA) subtype of excitatory amino acid receptors, reflecting that (*S*)-APPA is a full agonist and (*R*)-APPA a competitive antagonist at AMPA receptors. We have now synthesized and pharmacologically characterized (*RS*)-2-amino-3-[3-hydroxy-5-(2-fluorophenyl)isoxazol-4-yl]propionic acid (2-F-APPA, **5a**), 3-F-APPA (**5b**), 4-F-APPA (**5c**), (*S*)-4-F-APPA (**6**), (*R*)-4-F-APPA (**7**), and the fully and partially, respectively, saturated APPA (**2**) analogues, (*RS*)-2-amino-3-(3-hydroxy-5-cyclohexylisoxazol-4-yl)propionic acid (**5d**) and compound **5e** containing a 1-cyclohexenyl ring. The absolute stereochemistry of **6** and **7** was established on the basis of comparative circular dichroism studies on **6**, **7**, and (*S*)- and (*R*)-APPA. 4-F-APPA (**5c**), (*S*)-4-F-APPA (**6**), **5d**, and **5e** were shown to selectively inhibit [³H]AMPA binding and to activate AMPA receptors. Whereas (*S*)-4-F-APPA (**6**) showed full AMPA receptor agonism, (*R*)-4-F-APPA (**7**) was an AMPA receptor antagonist. Co-administration of (*S*)- and (*R*)-4-F-APPA to the rat cortical wedge preparation produced functional partial AMPA receptor agonism. Semi empirical calculations showed that the magnitude of the torsional angle of the bond connecting the two rings in the series of nonannulated bicyclic AMPA analogues appears to be of importance for the potency and efficacy of these compounds. © 1998 Elsevier Science Ltd. All rights reserved.

Key words: Excitatory amino acid receptor, AMPA agonist, AMPA antagonist, functional partial agonism, conformational analysis.

1. Introduction

(*S*)-Glutamic acid [(*S*)-Glu, Fig. 1], which is the main excitatory neurotransmitter in the central nervous system (CNS), and other excitatory amino acids (EAAs) operate through four different classes of receptors. In addition to the three heterogeneous classes of ionotropic EAA receptors (iGluRs), named *N*-methyl-*D*-aspartic acid (NMDA), (*RS*)-2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionic acid (AMPA), and kainic acid receptors [1–4], a heterogeneous class of metabotropic

receptors (mGluRs) has been shown to have important functions in neurotransmission processes in the CNS [5]. It is now generally agreed that all subtypes of these receptors are potential targets for therapeutic intervention in a number of CNS diseases [6,7].

EAA receptors are involved in the mechanisms of long-term potentiation, which is believed to play an important role in learning and memory functions, and the deficits of these functions in Alzheimer patients may, to some extent, be caused by hypoactivity at iGluRs and/or mGluRs in the brain [8–11]. There also is growing evidence of an implication of EAA receptors in schizophrenia [12,13]. As in Alzheimer's disease (AD), the role of these receptors in the etiology and the clinical

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manifestations of schizophrenia is still very incompletely understood, but there is evidence to suggest that hypoactivity at EAA receptors also is a factor of importance in the latter CNS disorder [13–15]. Thus, in AD as well as schizophrenia, compounds capable of activating EAA receptors as agonists or, perhaps more likely, partial or functional partial agonists may have therapeutic interest [15].

During the past years, a number of agonist and antagonist ligands for pharmacological characterization of subtypes of EAA receptors have been developed [16,17]. Whereas tritiated NMDA, the classical NMDA receptor agonist, is not useful for radioligand receptor binding [18], the tritiated form of glycine, which is a co-agonist at the NMDA receptor [19], the competitive antagonist [^3H]-(*RS*)-[3-(2-carboxypiperazin-4-yl)propyl]-phosphonic acid [^3H]CPP [20], and the noncompetitive antagonist [^3H]-(*RS*)-5,10-epimino-5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cycloheptene [^3H]MK-801 [21], have been extensively used as tools for the pharmaco-

logical characterization of the NMDA receptors. [^3H]Kainic acid is the standard ligand for studies of kainic acid receptors [22], and [^3H]AMPA [23] and [^3H]-6-cyano-7-nitro-quinoxaline-2,3-dione ([^3H]CNQX) [24] are effective agonist and antagonist ligands, respectively, for AMPA receptor characterization.

A large number of agonists, including (*S*)-AMPA (1), and antagonists for AMPA receptors have been described [1,16,17], but so far only one compound, (*RS*)-2-amino-3-(3-hydroxy-5-phenylisoxazol-4-yl)propionic acid (APPA, 2) (Fig. 1), shows partial AMPA receptor agonism [25]. This effect is, however, only apparent and reflects that (*S*)-APPA is a full AMPA agonist and (*R*)-APPA a competitive antagonist of comparable potencies [26]. This interaction between full agonist and competitive antagonist underlies the pharmacological concept, functional partial agonism [27,28], which may have therapeutic interest [29].

In agreement with the findings for (*S*)- and (*R*)-APPA, the (*S*)-form of (*RS*)-2-amino-3-[3-hydroxy-5-(2-pyridyl)isoxazol-4-yl]propionic acid (2-Py-AMPA, 3), which is an AMPA agonist, and the competitive AMPA antagonist, (*R*)-2-Py-AMPA, also interact in a functional partial agonistic manner, whereas 3- and 4-Py-AMPA (4), do not interact significantly with AMPA receptors [30]. In order to further explore this pharmacological concept, we now report the synthesis and pharmacological characterization of the (*S*)-form (6) and (*R*)-form (7) of (*RS*)-2-amino-3-[5-(4-fluorophenyl)-3-hydroxyisoxazol-4-yl]propionic acid (4-F-APPA, 5c), the 2-fluorophenyl (5a) and 3-fluorophenyl (5b) isomers and the fully (5d) and partially (5e) saturated analogues of APPA (2) (Fig. 1).

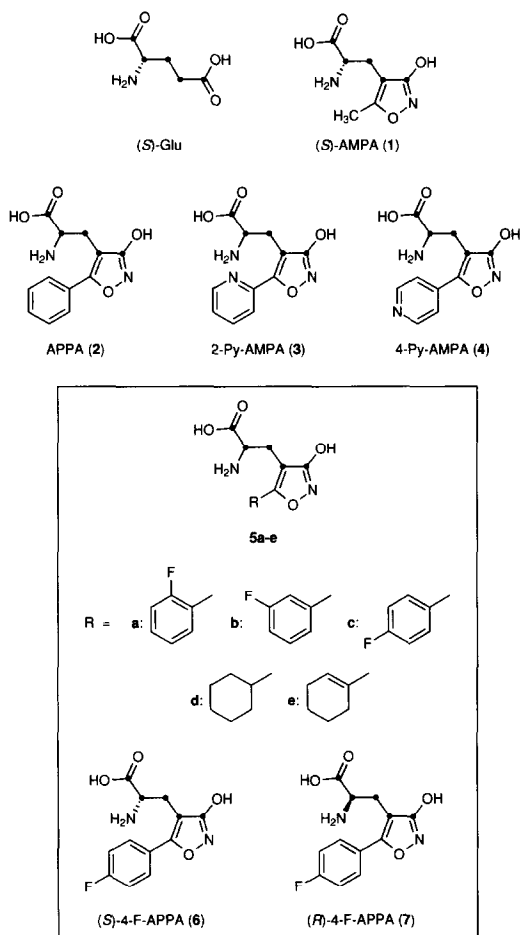


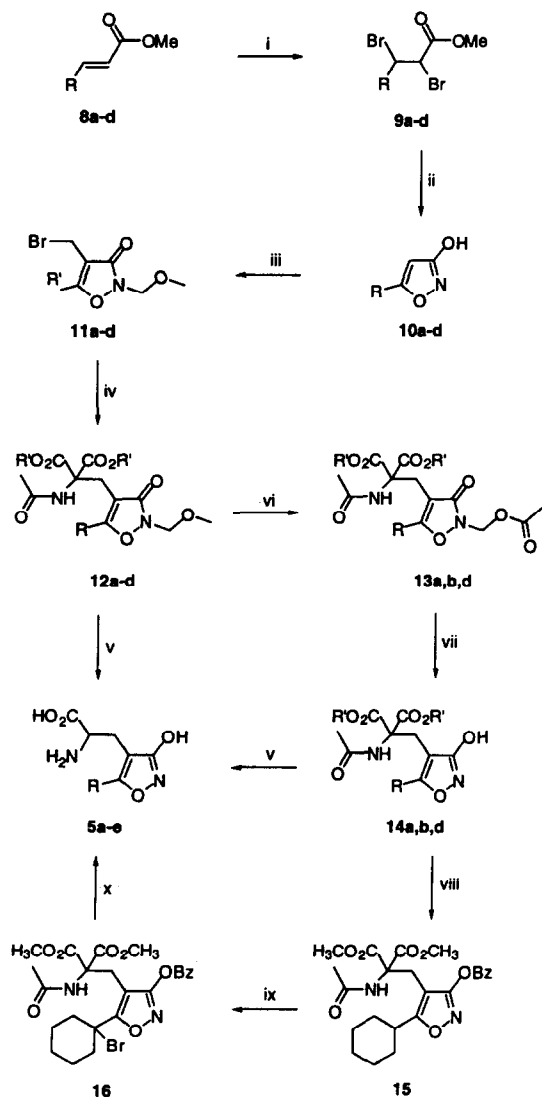
Fig. 1. Structures of (*S*)-glutamic acid [(*S*)-Glu], (*S*)-AMPA and a number of nonannulated bicyclic AMPA analogues.

2. Synthesis

2.1 Chemistry

The 3-substituted 2,3-dibromopropanoates (9a–d), were easily obtained by bromination of the corresponding propanoates (8a–d) (Scheme 1). The 5-aryl-3-isoxazolols (10a–c) were prepared in high yields by treatment of 9a–c with hydroxylamine in alkaline media using an improved version of a previously reported method [31]. Under similar conditions 9d gave a complex mixture from which 10d was isolated in a very low yield. By substituting *N*-hydroxyurea for hydroxylamine, compound 10d was obtained in a yield of 61%. We have previously shown that 3-aryl-2,3-dibromopropanoates easily undergo base-catalyzed dehydrobromination to give the corresponding 2-bromopropanoates [32]. It is assumed that the reaction of these intermediates with anionic *N*-hydroxyurea is initiated by a conjugated addition reaction followed by cyclization and dehydrobromination reactions and, finally,

elimination of the carboxamide group, from N-2 of the oxazoline ring formed, as an isocyanate ion [32,33]. Reaction of **10a–d** with 1,3,5-trioxane in aqueous hydrobromic acid (62%) and subsequent treatment of the intermediate 2,4-bis(bromomethyl)isoxazolin-3-ones with methanol under previously described conditions [34] gave **11a–d** (Scheme 1). A Sorensen reaction



- a: R = 2-F-phenyl, R' = Me
 b: R = 3-F-phenyl, R' = Me
 c: R = 4-F-phenyl, R' = Et
 d: R = cyclohexyl, R' = Me
 e: R = 1-cyclohexenyl, R' = Me

Scheme 1. (i) Br_2 ; (ii) NH_2OH or $\text{H}_2\text{NCONHOH}$, NaOH ; (iii) $(\text{CH}_2\text{O})_3$, 62% aq HBr , MeOH ; (iv) $\text{AcNHCH}(\text{COOR}')_2$, NaH ; (v) $\text{CF}_3\text{CO}_2\text{H}$ (1 M); (vi) $\text{BF}_3\cdot\text{OEt}_2$, $(\text{Ac})_2\text{O}$; (vii) MeONa ; (viii) BzCl ; (ix) NBS ; (x) HBr (1 M).

converted these compounds into **12a–d**. Compounds **12c** and **12d** were deprotected to give **5c** and **5d** using aqueous trifluoroacetic acid. Deprotection of **12a** and **12b** under the same reaction conditions resulted in low yields of **5a** and **5b**. Therefore, **12a** and **12b** were transformed into the 2-acetoxymethyl compounds **13a** and **13b** by treatment with acetic anhydride in the presence of boron trifluoride etherate [34]. Selective deprotection with sodium methoxide gave the 3-isoxazols **14a** and **14b**, which were converted into the isoxazole amino acids **5a** and **5b** by treatment with aqueous trifluoroacetic acid. An attempt to introduce bromine at C-1 of the cyclohexyl group of **12d** was unsuccessful. Thus, **12d** was also selectively deprotected to give **14d** via **13d** using the reaction conditions described for the transformation of **12a,b** into **14a,b**. *O*-Benzoylation of **14d** followed by bromination of intermediate **15** using NBS gave **16**, which was dehydrohalogenated, deprotected, and decarboxylated by reflux in aqueous hydrobromic acid (1 M) to give **5e**. Compound **5e** was isolated as the zwitterion.

3. Resolution and stereochemistry

3.1 Resolution of 4-F-APPA (**5c**)

The chemical resolution of zwitterionic 4-F-APPA (**5c**) to give (*S*)-(+)-4-F-APPA (**6**) and (*R*)-(–)-4-F-APPA (**7**) was achieved via diastereomeric salt formation using the (*S*)-(+)- and (*R*)-(–)-forms of 1-phenylethylamine (PEA), respectively. Compounds **6** and **7** were obtained with enantiomeric excess (*ee*) of 99.8% and 99.6%, respectively. The enantiomeric purity of the enantiomer **7** was determined by using a chiral crown ether column [Crownpak CR(–)]. The stereochemical purity of the enantiomer **6** was determined by ligand exchange HPLC on a chiral stationary phase consisting of (*S*)-pipecolic acid bound to silica and chelated with Cu^{++} [35]. In agreement with earlier observations using these types of columns for the separation of α -amino acids of known absolute stereochemistry [26,36,37], (*S*)-(+)-4-F-APPA (**6**) eluted before the corresponding (*R*)-form (**7**) using the crown ether column, and after the (*R*)-form (**7**) using (*S*)-pipecolic acid derivatized column material.

The CD spectrum of the (*S*)-(+)-form of APPA (**2**) (Fig. 2), the configuration of which previously has been determined by an X-ray analysis [26], shows a positive Cotton effect at 210 nm ($\Delta\epsilon = +0.3 \text{ m}^2/\text{mol}$) and a negative band at about 240 nm. As expected, the CD spectrum of the (*R*)-(–)-form of APPA (**2**) is a mirror image of the (*S*)-(+)-enantiomer in the $\Delta\epsilon = 0 \text{ m}^2/\text{mol}$ axis. The positive Cotton effect of (*S*)-(+)-APPA at 210 nm is in agreement with the empirical correlation between the absolute configuration and the CD spectra

of (*S*)-amino acids in acidic solution, stating that (*S*)-amino acids show a positive Cotton effect near 220 nm, most likely due to a forbidden $n \rightarrow \pi^*$ transition of the α -carboxylic acid.

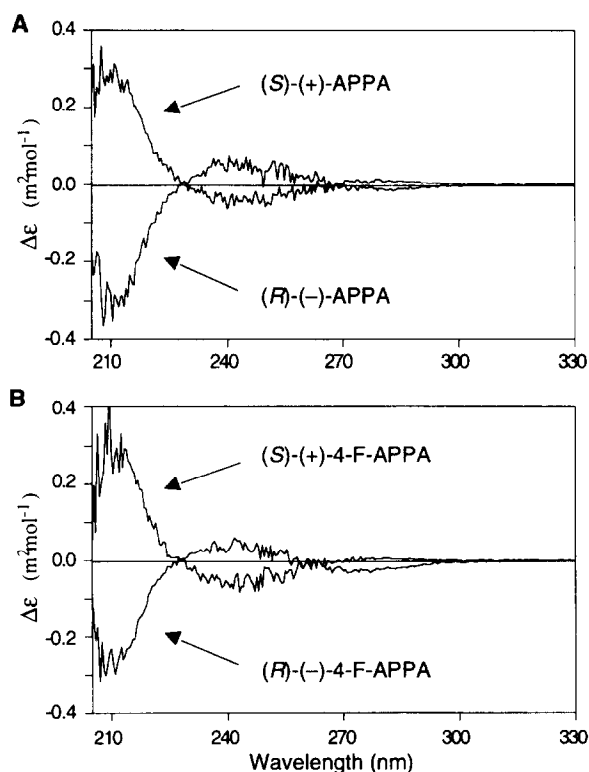


Fig. 2. Circular dichroism spectra of (*S*)-(+)-APPA and *R*-(-)-APPA (A) and (*S*)-(+)-4-F-APPA (6) and (*R*)-(-)-4-F-APPA (7) (B).

The CD spectra of (+)- and (-)-4-F-APPA (Fig. 2) clearly illustrates that the two compounds are enantiomers. Furthermore, the CD spectrum of (+)-4-F-APPA has almost the exact same location and magnitude of the positive (210 nm) as well as the negative (240 nm) Cotton effect as that of (*S*)-(+)-APPA, supporting the assignment of (+)-4-F-APPA (6) as having the (*S*)-configuration and (-)-4-F-APPA (7) as having the (*R*)-configuration.

4. In vitro pharmacology

Receptor binding studies were performed using [³H]AMPA, [³H]kainic acid, and [³H]CNQX in the presence or absence of KSCN, and the NMDA receptor complex ligands [³H]CPP, [³H]MK-801 and [³H]glycine. The tested compounds were only active in receptor binding assays evaluating AMPA receptor activities (Table 1). In [³H]AMPA binding, compounds 5c, 6, 5d, and 5e were rather weak inhibitors, whereas 7 was inactive ($IC_{50} > 100 \mu M$). In the rat cortical wedge, 4-F-APPA (5c) showed weak partial AMPA receptor agonism of approximately 65% relative efficacy and with an EC_{50} value of $310 \mu M$ (Fig. 3 and Table 1). The response to 5c ($100 \mu M$) could be reduced by the AMPA receptor antagonist, 6-nitro-7-sulfamoylbenzo-*f*quinoxaline-2,3-dione (NBQX) ($10 \mu M$) (data not shown). Compounds 5a and 5b were much weaker than 5c. Full dose response curves could not be obtained due to the limited solubility of the compounds (data not shown). Both compounds were tested at concentrations up to 4 mM. Assuming that 5a and 5b both produce dose response curves with a maximum response of 100% (relative to AMPA), EC_{50} values of $1300 \mu M$ and

Table 1

pK_a Values and in vitro radioligand binding and electrophysiological data (mean \pm SEM, $n = 3-5$)

Compound	pK_a Values	IC_{50} , μM			EC_{50} , μM	K_i , μM
		[³ H]AMPA	[³ H]CNQX	[³ H]CNQX + KSCN		
AMPA	2.1; 5.2; 10.1	0.04 ± 0.01	18 ± 5	0.4 ± 0.1	3.5 ± 0.5	
APPA	2.0; 4.9; 10.4	35 ± 10	190 ± 20^a	86 ± 10	390 ± 60	
(<i>R</i>)-APPA		> 100	66 ± 8	320 ± 40^a		290 ± 24^b
(<i>S</i>)-APPA		6 ± 2	310 ± 20^a	84 ± 15	230 ± 12	
2-F-APPA (5a)	2.1; 4.6; 9.9	93 ± 12	> 100	> 100	> 1000 (1300)	
3-F-APPA (5b)	1.6; 4.6; 9.8	> 100	> 100	> 100	> 1000 (2000)	
4-F-APPA (5c)	1.5; 5.0; 10.1	34 ± 17	> 100	> 100	310 ± 120	
(<i>S</i>)-4-F-APPA (6)		16 ± 6	> 100	> 100	150 ± 18	
(<i>R</i>)-4-F-APPA (7)		> 100	> 100	> 100		340 ± 23^b
5d	2.2; 5.1; 10.0	26 ± 14	100 ± 19	108 ± 33	640 ± 40	
5e	< 2 ; 4.8; > 10	6.4 ± 0.4	68 ± 11	76 ± 7	44 ± 4	

^a IC_{50} values above $100 \mu M$ in binding assays are calculated using the equation: % Inhibition = $100 \times [\text{Inhibitor}] / IC_{50} + [\text{Inhibitor}]$, assuming competitive interaction and one binding site.

^bAntagonist, K_i value against AMPA. All compounds were inactive ($IC_{50} > 100 \mu M$) in [³H]MK-801 (baseline and fully stimulated), [³H]CPP, [³H]kainic acid, and [³H]glycine binding assays.

2000 μM , respectively, could be estimated. Compound **5d** was only slightly more potent than compound **5a** with an EC_{50} value of 640 μM . However, compound **5e** turned out to be a fairly potent and full agonist with an EC_{50} value of 44 μM . The responses to **5a**, **5b**, **5d**, and **5e** could all be antagonized by NBQX (5 μM or 10 μM), whereas CPP (5 μM) did not affect the responses (data not shown). Thus, in accordance with the binding assays (Table 1), **5a**, **5b** and **5d** are weak, but selective, AMPA receptor agonists, whereas **5e** is a selective and fairly potent AMPA receptor agonist.

Compound **6** is a full AMPA receptor agonist (Fig. 3). Analysis of the dose response curves resulted in an EC_{50} value of 150 μM , and a Hill slope close to 2. The response to **6** could be completely antagonized by co-application of 5 μM NBQX, whereas 5 μM CPP was unable to antagonize the response to **6**.

Since (*S*)-4-F-APPA (**6**) shows the characteristics of a full AMPA receptor agonist and since (*R*)-4-F-APPA (**7**) does not provoke any excitatory effect even when administered at a concentration of 1 mM, the partial agonism observed for the racemate, **5c** (Fig. 3), obviously only is apparent. The effects of **7** on responses by **6** were examined using a fixed concentration of **6** (200 μM) and varying concentrations of **7** (1 to 2000 μM). An analysis of these data using the method of Lazareno and Birdsall [38] disclosed that **7** acts as a competitive AMPA receptor antagonist with a K_i value of 340 μM . Co-administration of **6** and **7** at fixed ratios produced excitatory effects of different relative efficacies as exemplified in Fig. 4. Thus, co-administration of this pair of enantiomers at a 1:1 ratio gave a dose–response curve indistinguishable from that of **5c**, whereas co-administration of **6** and **7** at a 1:5 ratio produced excitatory effects of approximately 10% relative efficacy (Fig. 4). These dose–response relationships

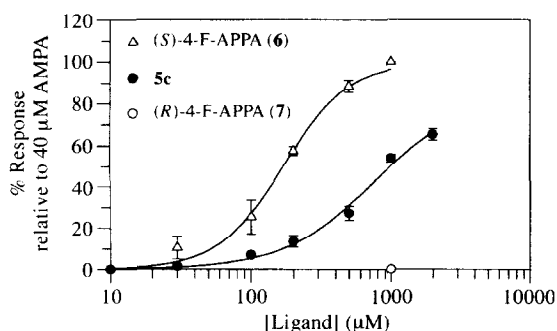


Fig. 3. Dose–response curves from the rat cortical wedge preparation. (*S*)-4-F-APPA (**6**) (Δ), 4-F-APPA (**5c**) (\bullet) and (*R*)-4-F-APPA (**7**) (\circ). The percentage response values are mean values \pm SEM relative to the maximum AMPA response and are plotted as a function of the concentration of the ligand (at least three experiments).

indicate that **6** and **7** show interactive effects at the AMPA receptor previously named functional partial agonism [26–28].

5. Conformational analysis

The torsional drive calculation was performed on model compounds relevant to the bicyclic AMPA analogues **2–4** and **5a–e**. The energy barrier ($E(\omega)$) for a 360° rotation of the substituent in the 5-position of the isoxazole ring was performed on the 5-substituted 4-methyl-3-isoxazolol moiety in 20° steps, which generated 18 low-energy conformations for each structure (Table 2). The low-energy conformation for each of these structures was further energy minimized using the AM1 force field in the Spartan package. For all of the structures shown in Table 2, the lowest-energy conformation turned out to prefer a rather co-planar conformation between the two ring systems, except **5d**, which preferred a conformation, which has a dihedral angle of approximately 90°. Performing the torsional drive for compound **5d**, the saturated ring is in a chair conformation with the isoxazole ring in an equatorial position.

6. Discussion and conclusion

There is evidence to suggest that hypoactivity at central EAA receptors is a factor of importance in the clinical manifestations of AD [8–10] and schizophrenia [12–15]. Stimulation of EAA receptors, including AMPA receptors, by full agonists may, however, not be accomplished without concomitant excitotoxicity making therapeutic use of such compounds quite unlikely.

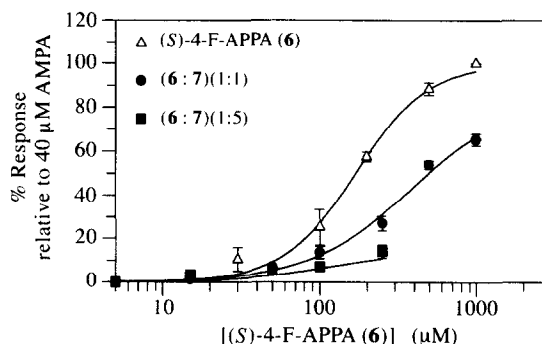
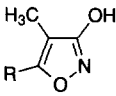




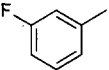
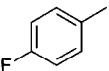




Fig. 4. Dose–response curves from the rat cortical wedge preparation. (*S*)-4-F-APPA (**6**) (Δ), and fixed molar ratios of (*S*)-4-F-APPA (**6**) and (*R*)-4-F-APPA (**7**) of (1:1) (\bullet) and (1:5) (\blacksquare). The percentage response values are mean values \pm SEM relative to the maximum AMPA response and are plotted as a function of the concentration of (*S*)-4-F-APPA (**6**) (at least three experiments).

Table 2

Calculated approximate energy barriers for rotation about the bond connecting the two rings of model compounds relevant to compounds 2–4, 5a–e

Compound		$E(\omega)$ kcal/mol
2		3.46
3		3.80
4		3.74
5a		2.08
5b		3.65
5c		3.56
5d		0.76
5e		3.17

Partial agonists showing an appropriately adjusted agonist/antagonist profile may, on the other hand, be capable of restoring, in a nontoxic manner, activity in brain areas suffering from glutamatergic hypoactivity.

With the exception of APPA (2) [26,27], compounds showing partial AMPA receptor agonism have not been described. The partial agonism of APPA (2) is, however, only apparent, since (*S*)-APPA turned out to be a full AMPA receptor agonist and (*R*)-APPA a competitive antagonist [26]. In continuation of these studies, it was demonstrated that partial agonism at any desired level of relative efficacy could be achieved by co-administration of (*S*)- and (*R*)-APPA at appropriate fixed ratios, and this pharmacological principle of potential therapeutic interest was termed functional partial agonism [27–29].

Within the series of fluoro-substituted analogues of APPA, compounds 5a–c (Fig. 1), only 4-F-APPA (5c) was active at AMPA receptors (Table 1), showing the characteristics of a partial agonist (Fig. 3). As in the case of APPA [26–28], this effect is only apparent, and

pharmacological studies on enantiomerically pure (*S*)-4-F-APPA (6) and (*R*)-4-F-APPA (7) revealed that this pair of enantiomers produces functional partial agonism (Fig. 4).

Prompted by these observations, we also synthesized the cyclohexyl and 1-cyclohexenyl analogues of APPA, compounds 5d and 5e, respectively (Fig. 1 and Scheme 1). Whereas 5d was some threefold weaker than (*S*)-APPA as an AMPA receptor agonist, 5e was markedly more potent (Table 1), and 5e was shown to be a full agonist at AMPA receptors. Thus, the potency of this class of AMPA agonists is strongly dependent on the structure of the 6-membered ring in the 5-position of the 3-isoxazolol unit.

We have previously postulated that the agonist conformation of nonannulated bicyclic analogues of AMPA containing a heterocyclic ring in the 5-position of the 3-isoxazolol unit may be rather planar and stabilized by a hydrogen bond between the ammonium group of the amino acid and an "ortho" positioned heteroatom of the heterocyclic substituent [39]. Thus, 2-Py-AMPA was markedly more potent than 4-Py-AMPA (Fig. 1) as an AMPA agonist [30]. In order to shed some light on the factors of importance in the structure-activity relationships of the present compounds, we have calculated approximate energy barriers for rotation about the bond connecting the two rings of model compounds relevant to compounds 2–4 and 5a–e (Table 2). Whereas 2–4 and 5b,c,e were estimated to show comparable approximate rotational energy barriers, this barrier appeared to be lower for 5a and, in particular, 5d, and in contrast to all of the other compounds, 5d seemed, on the basis of the energy minimization, to prefer a nonplanar conformation. This latter observation may contribute to the very low agonist potency of 5d, whereas unfavourable steric effects of the fluorine atoms of 5a,b may contribute to the virtual inactivity of these compounds (Table 1). It is postulated that the coplanarity of the two rings and the possible interaction between the ammonium group and the double bond of the cyclohexenyl group may contribute to the high potency of 5e.

7. Experimental

7.1 Chemistry

7.1.1 General procedures

A number of synthetic steps required the use of anhydrous solvents. CH_2Cl_2 was dried over NaH. DMF was dried as described [40]. Organic phases were dried using MgSO_4 . Solvents were removed under reduced pressure by rotary evaporation. Flash and Column chromatography (CC) were performed on silica gel 60, 0.063–0.200 mm (Merck) following described procedures [41].

Preparative thin-layer chromatography (TLC) was performed using silica gel 60 PF₂₅₄. All new compounds were colourless, unless otherwise stated. Melting points were determined in capillary tubes and are uncorrected. Elemental analyses were performed by Mr G. Cornali, Microanalytical Laboratory, Leo Pharmaceutical Products, Denmark, Mr P. Hansen, Department of General and Organic Chemistry, University of Copenhagen, or the Analytical Research Department, H. Lundbeck A/S, Denmark, and are within $\pm 0.4\%$ of the calculated values unless otherwise stated. ¹H and ¹³C NMR spectra were recorded at 200 and 50.32 MHz, respectively, on a Bruker AC-200 F instrument, unless otherwise stated. ¹H NMR Spectra at 60 MHz were recorded on a Varian EM-360-L spectrometer. Signal positions for the ¹H NMR spectra are given as δ values relative to TMS, when CDCl₃ was used as a solvent, and relative to 1,4-dioxane (δ 3.70) when D₂O was used. ¹³C NMR signals are given as δ values using the CDCl₃ peak (δ 76.93) as an internal standard. Coupling constants are given in Hz. Optical rotations were measured in thermostated cuvettes on a Perkin–Elmer 241 polarimeter. CD spectra of the enantiomers of APPA (**2**) and of (*S*)-4-F-APPA (**6**) and (*R*)-4-F-APPA (**7**) (*c* = 0.136 mM, 0.1 M HCl) were recorded in 1.0 cm cuvettes at room temperature on a Jasco J-720 spectropolarimeter.

7.1.2 Determination of stereochemical purity

Chiral HPLC was performed on a 150×4 mm Crownpak CR(–) column (Daicel) thermostated at 38°C with a Hetofrig thermostat and eluted with 0.4 ml/min of aqueous HClO₄, pH 1.5. The instrumentation used consisted of a Jasco 880-PU pump, a Rheodyne 7125 injector, and a Waters 480 UV detector set at 200 nm connected to a Hitachi Chromato-Integrator D-2000. Ligand exchange chiral HPLC was performed on a 120×4.6 mm column, containing a silica-based packing material with immobilized (*S*)-pipecolic acid and chelated Cu⁺⁺, prepared according to directions in the literature [35]. The column was thermostated at 50°C with an LKB 2155 HPLC column oven and eluted with 1.0 ml/min of 50 mM potassium dihydrogen phosphate containing 0.07 mM CuSO₄, pH 4.6, with Waters instrumentation consisting of an M510 pump connected to a U6K injector and a Waters 990 Photodiode Array Detector. The enantiomeric purity was determined from peak areas.

7.1.3 Determination of stoichiometric pK_a values

pK_a value determinations were performed on an interconnected automatic TitriLab[®] titrator system consisting of a burette station ABU 93 Triburette, a controller unit VIT90 Video Titrator and a sample station SAM90 from the Analytical Instruments Division of Radiometer A/S, DK-2400 Copenhagen NV, Denmark, using the following Radiometer electrodes: Glas

electrode (pHG 201), reference electrode (reference 201, Ag/AgCl). Titration curves were fitted by a weighted least squares method.

7.1.3.1 Methyl (*E*)-3-(4-fluorophenyl)-2-propenoate (8c**).** Concentrated H₂SO₄ (14 ml) was added to a solution of (*E*)-3-(4-fluorophenyl)-2-propenoic acid (20.0 g, 120 mmol) in CH₃OH (240 ml). The solution was heated under reflux for 2 h. H₂O was azeotroped off with CH₃OH (200 ml), CH₃OH (200 ml) was added, and the mixture was heated under reflux overnight. The mixture was evaporated to dryness, dissolved in ether (200 ml), and washed with iced H₂O (2×200 ml) and iced aqueous Na₂CO₃ (2×200 ml, 1 M). The organic phase was dried and evaporated to give **8c** (20.5 g, 95%); mp 39–40°C (lit. [42]: bp 114–116°C/0.6 Pa). ¹H NMR (60 MHz, CDCl₃) δ 3.81 (s, 3H), 6.55 (d, *J* = 15, 1H), 7.04–7.08 (m, 2H), 7.48–7.55 (m, 2H), 7.82 (d, *J* = 15, 1H).

7.1.3.2 Methyl (*E*)-3-(3-fluorophenyl)-2-propenoate (8b**).** Synthesized, as described for **8c**, by use of (*E*)-3-(3-fluorophenyl)-2-propenoic acid (5.0 g, 30.1 mmol), CH₃OH (60 ml) and concentrated H₂SO₄ (3.5 ml). Crude **8b** was obtained as a yellow oil (5.16 g, 95%) (lit. [42]: bp 68–70°C/6.0 Pa). ¹H NMR (60 MHz, CDCl₃) δ 3.70 (s, 3H), 6.20 (d, *J* = 15, 1H), 6.70–7.20 (m, 4H), 7.40 (d, *J* = 15, 1H).

7.1.3.3 Methyl (*E*)-3-(2-fluorophenyl)-2-propenoate (8a**).** Compound **8a** was synthesized, as described for **8c**, from (*E*)-3-(2-fluorophenyl)-2-propenoic acid (5.0 g, 30 mmol), CH₃OH (60 ml) and concentrated H₂SO₄ (3.5 ml). Crude **8a** was obtained as an oil (4.45 g, 82%) (lit. [42]: bp 122–123°C/1.2 Pa). ¹H NMR (60 MHz, CDCl₃) δ 3.82 (s, 3H), 6.55 (d, *J* = 16, 1H), 7.0–7.23 (m, 2H), 7.2–7.42 (m, 1H), 7.47–7.60 (m, 1H), 7.82 (d, *J* = 16, 1H).

7.1.3.4 Methyl 2,3-dibromo-3-(4-fluorophenyl)propanoate (9c**).** A solution of Br₂ (1.46 ml, 28.4 mmol) in CCl₄ (5 ml) was added to an ice-cooled suspension of **8c** (5.10 g, 28.3 mmol) in CCl₄ (5 ml). The reaction mixture was stirred at rt overnight and then evaporated. The resulting crystalline product was triturated with light petroleum with stirring for 2 h. The crystals were collected and dried in vacuo to give **9c** (8.11 g, 84%). A sample was recrystallized (light petroleum): mp 63–65°C. ¹H NMR (60 MHz, CDCl₃) δ 3.90 (s, 3H), 4.80 (d, *J* = 10, 1H), 5.40 (d, *J* = 10, 1H), 7.00–7.80 (m, 4H). Anal. (C₁₀H₉Br₂FO₂) C, H, Br.

7.1.3.5 Methyl 2,3-dibromo-3-(3-fluorophenyl)propanoate (9b**).** Synthesized, as described for **9c**, from **8b** (5.1 g, 28.3 mmol) and Br₂ (1.45 ml, 28.3 mmol). The crude **9b** was purified by flash chromatography and recrystallized (light petroleum) to give **9b** (6.51 g, 68%); mp 65–70°C. ¹H NMR (60 MHz, CDCl₃) δ 3.75 (s, 3H),

4.50 (d, $J = 10$, 1H), 5.15 (d, $J = 10$, 1H), 7.00–7.48 (m, 4H). Anal. ($C_{10}H_9Br_2FO_2$) C, H, Br.

7.1.3.6 Methyl 2,3-dibromo-3-(2-fluorophenyl)propanoate (9a). Compound **9a** was synthesized, as described for **9c**, from **8a** (4.90 g, 27.2 mmol) and Br_2 (1.41 ml, 27.5 mmol) to give crude **9a** (8.43 g, 91%). A sample was recrystallized (light petroleum): mp 55–60°C. 1H NMR (60 MHz, $CDCl_3$) δ 3.90 (s, 3H), 5.00 (d, $J = 12$, 1H), 5.60 (d, $J = 12$, 1H), 7.02–7.49 (m, 4H). Anal. ($C_{10}H_9Br_2FO_2$) C, H, Br.

7.1.3.7 5-(4-Fluorophenyl)-3-hydroxyisoxazole (10c). NaOH (9.88 g, 247 mmol) was added to an ice-cold solution of NH_2OH , HCl (6.13 g, 88.3 mmol) in CH_3OH (175 ml), and the mixture was stirred for 10 min. To this solution was portionwise added **9c** (12.0 g, 35.3 mmol) during 1 h, and stirring was continued at 0°C for an additional 1 h. The mixture was refluxed for 6 h and evaporated. The residue was dissolved in H_2O (70 ml), and the solution was acidified with HCl (4 M) at 0°C and stirred at 0°C for 30 min. The resulting crystals were collected, washed with H_2O and dried in vacuo to give **10c** (4.6 g, 73%). A sample was recrystallized (EtOAc–heptane): mp 207–208°C. 1H NMR ($CDCl_3$) δ 6.19 (s, 1H), 7.10–7.25 (m, 2H), 7.68–7.82 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 90.0, 114.6, 115.0, 123.1, 126.2, 126.4, 159.7, 167.3, 170.0. Anal. ($C_9H_7FNO_2$) C, H, N.

7.1.3.8 5-(3-Fluorophenyl)-3-hydroxyisoxazole (10b). Synthesized, as described for **10c**, from **9b** (1.70 g, 5.00 mmol), NH_2OH , HCl (0.87 g, 12.5 mmol) and NaOH (1.40 g, 35.0 mmol). Crude **10b** was extracted with hot EtOAc (2×15 ml), and the combined organic extracts were dried and evaporated to give **10b** (760 mg, 85%) as a yellowish solid. Two recrystallizations (EtOAc–light petroleum) gave **10b** (440 mg, 49%): mp 185–189°C. 1H NMR (60 MHz, $CDCl_3$) δ 6.20 (s, 1H), 7.05–7.21 (m, 1H), 7.36–7.59 (m, 3H). Anal. ($C_9H_6FNO_2$) H, N; C: calcd, 60.34; found, 59.98.

7.1.3.9 5-(2-Fluorophenyl)-3-hydroxyisoxazole (10a). Compound **10a** was synthesized, as described for **10c**, from **9a** (1.70 g, 5.00 mmol), NH_2OH , HCl (0.87 g, 12.5 mmol) and NaOH (1.40 g, 35.0 mmol). Crude **10a** was subjected to CC [eluent: tol containing AcOH (1%)] to give 0.67 g (75%) of **10a**. A sample was recrystallized (EtOAc): mp 170–174°C. 1H NMR ($CDCl_3$) δ 6.32 (d, $J = 4.5$, 1H), 7.08–7.51 (m, 3H), 7.82–8.00 (m, 1H). Anal. ($C_9H_6FNO_2$) C, H, N.

7.1.3.10 5-Cyclohexyl-3-hydroxyisoxazole (10d). N -Hydroxyurea (570 mg, 7.5 mmol) was added to a solution of NaOH (700 mg, 17.5 mmol) in CH_3OH (15 ml). A solution of **9d** [43] (1.64 g, 5.0 mmol) in CH_3OH (5 ml) was added dropwise, and the mixture was stirred at rt

for 6 h and then refluxed for 20 h. After evaporation, H_2O (15 ml) was added to the residue. The mixture was cooled in an ice-bath and acidified with concentrated HCl to precipitate crude **10d**. The product was extracted with boiling heptane, and the extracts were concentrated to give crystalline **10d** (506 mg, 61%): mp 118–119°C (lit. [44]: mp 123°C; lit. [45]: mp 125°C). 1H NMR ($CDCl_3$) δ 1.00–2.27 (m, 10H), 2.32–2.95 (m, 1H), 5.47 (s, 1H). Anal. ($C_9H_{13}NO_2$) C, H, N.

7.1.3.11 4-(Bromomethyl)-5-(4-fluorophenyl)-2-(methoxymethyl)isoxazolin-3-one (11c). A mixture of **10c** (200 mg, 1.10 mmol), 1,3,5-trioxane (149 mg, 1.66 mmol) and aqueous HBr (4 ml, 62%) in a sealed ampoule was placed in an oil bath at 60°C for 20 h. The reaction mixture was extracted with CH_2Cl_2 (3×25 ml) followed by addition of CH_3OH (50 ml) and then vigorous stirring for 2 h at rt. CH_2Cl_2 (50 ml) was added and the organic phase was washed with H_2O (3×75 ml), dried and evaporated to give **11c** (344 mg, 99%): mp 197–199°C. 1H NMR ($CDCl_3$) δ 3.47 (s, 3H), 4.40 (s, 2H), 5.30 (s, 2H), 7.20–7.32 (m, 2H), 7.80–7.92 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 20.3, 57.3, 75.6, 106.4, 116.4, 116.9, 122.4, 129.6, 129.8, 162.0, 166.0, 167.1. Anal. ($C_{12}H_{11}BrFNO_3$) C, H, N.

7.1.3.12 4-(Bromomethyl)-5-(3-fluorophenyl)-2-(methoxymethyl)isoxazolin-3-one (11b). Synthesized, as described for **11c**, from **10b** (500 mg, 2.79 mmol), 1,3,5-trioxane (373 mg, 4.14 mmol), and aqueous HBr (4 ml, 62%). Compound **11b** was obtained as a semicrystalline solid (845 mg, 96%). A sample was recrystallized (EtOAc): mp 190–192°C. 1H NMR ($CDCl_3$) δ 3.48 (s, 3H), 4.39 (s, 2H), 5.29 (s, 2H), 7.22–7.36 (m, 1H), 7.48–7.69 (m, 3H). Anal. ($C_{12}H_{11}BrFNO_3$) C, H, N.

7.1.3.13 4-(Bromomethyl)-5-(2-fluorophenyl)-2-(methoxymethyl)isoxazolin-3-one (11a). Synthesized, as described for **11c**, from **10a** (1.65 g, 9.20 mmol), 1,3,5-trioxane (1.20 g, 13.3 mmol) and aqueous HBr (13 ml, 62%). Compound **11a** was obtained as an oil (2.48 g, 85%) 1H NMR ($CDCl_3$) δ 3.49 (s, 3H), 4.31 (s, 2H), 5.30 (s, 2H), 7.20–7.42 (m, 2H), 7.53–7.69 (m, 2H). Anal. ($C_{12}H_{11}BrFNO_3$) C, H, N.

7.1.3.14 4-(Bromomethyl)-5-cyclohexyl-2-(methoxymethyl)isoxazolin-3-one (11d). Synthesized, as described for **11c**, from **10d** (1.45 g, 8.67 mmol), 1,3,5-trioxane (1.17 g, 1.30 mmol) and aqueous HBr (12 ml, 62%). Compound **11d** was obtained as a semicrystalline solid (2.14 g, 81%). A sample was recrystallized (EtOAc): mp 199–201°C. 1H NMR ($CDCl_3$) δ 1.20–2.04 (m, 10H), 2.81 (tt, $J = 3.3$, $J = 11.7$, 1H), 3.38 (s, 3H), 4.22 (s, 2H), 5.16 (s, 2H); ^{13}C NMR ($CDCl_3$) δ 18.8, 25.2, 25.4 (2C), 29.1 (2C), 36.9, 57.0, 75.3, 105.4, 166.1, 175.1. Anal. ($C_{12}H_{18}BrNO_3$) C, H, N.

7.1.4 Ethyl 2-acetamido-2-(ethoxycarbonyl)-3-[5-(4-fluorophenyl)-2-(methoxymethyl)-3-oxoisoxazolin-4-yl]propanoate (12c)

A suspension of NaH in mineral oil (241 mg, 60%, 6.02 mmol) was added during 30 min at 0°C to a solution of diethyl acetamidomalonate (1.13 g, 6.02 mmol) in dry DMF (25 ml). After stirring for further 30 min, a solution of **11c** (1.73 g, 5.47 mmol) in dry DMF (10 ml) was added during 15 min. After stirring at rt for 20 h, the mixture was evaporated, dissolved in CH₂Cl₂ (75 ml) and washed with ice-cold NaOH (80 ml, 1 M) and with ice-cold H₂O (2×100 ml). The organic phase was dried and evaporated. CC of the residue [eluent: tol:EtOAc (1:1)] gave **12c** (1.71 g, 70%) as an oil. ¹H NMR (CDCl₃) δ 1.24 (t, *J* = 7, 6H), 1.54 (s, 3H), 3.42 (s, 3H), 3.66 (s, 2H), 4.05–4.35 (m, 4H), 5.23 (s, 2H), 6.82 (br s, 1H), 7.10–7.25 (m, 2H), 7.65–7.75 (m, 2H); ¹³C NMR (CDCl₃) δ 13.6 (2C), 20.6 (2C), 21.9, 57.1, 62.7, 75.1, 101.2, 114.2, 114.8, 124.4, 128.4, 130.0, 130.5, 159.8, 164.6, 167.2, 169.3, 170.8, 174.0. Anal. (C₂₁H₂₅FN₂O₈) C, H, N.

7.1.5 Methyl 2-acetamido-3-[5-(3-fluorophenyl)-2-(methoxymethyl)-3-oxoisoxazolin-4-yl]-2-(methoxycarbonyl)propanoate (12b)

Synthesized, as described for **12c**, from **11b** (5.0 g, 15.8 mmol), a suspension of NaH in mineral oil (530 mg, 80%, 17.7 mmol) and dimethyl acetamidomalonate (3.30 g, 17.4 mmol). After stirring at rt for 20 h the reaction mixture was neutralized with AcOH and evaporated. The residue was dissolved in EtOAc (50 ml) and washed with ice-cold H₂O (2×100 ml). The organic solution was dried and evaporated. CC of the residue [eluent: tol:EtOAc (2:1), AcOH (1%)] produced **12b** as an oil (3.20 g, 47.5%). ¹H NMR (CDCl₃) δ 1.52 (s, 3H), 3.42 (s, 3H), 3.65 (s, 2H), 3.73 (s, 6H) 5.23 (s, 2H), 6.80 (br s, 1H), 7.18–7.30 (m, 1H), 7.34–7.56 (m, 3H). Anal. (C₁₉H₂₁FN₂O₈) C, H, N.

7.1.5.1 Methyl 2-acetamido-3-[5-(2-fluorophenyl)-2-(methoxymethyl)-3-oxoisoxazolin-4-yl]-2-(methoxycarbonyl)propanoate (12a). Synthesized, as described for **12c**, from **11a** (2.40 g, 7.59 mmol), a suspension of NaH in mineral oil (332 mg, 60%, 8.30 mmol) and dimethyl acetamidomalonate (1.60 g, 8.30 mmol). CC of the residue [eluent: tol:EtOAc (1:1)] gave **12a** (2.39 g, 74%) as a semicrystalline solid. A sample was recrystallized (EtOAc): mp 203.5–204.5°C. ¹H NMR (CDCl₃) δ 1.55 (s, 3H), 3.46 (s, 3H), 3.55 (s, 2H), 3.70 (s, 6H), 5.23 (s, 2H), 6.80 (br s, 1H), 7.12–7.36 (m, 3H), 7.45–7.61 (m, 1H). Anal. (C₁₉H₂₁FN₂O₈) C, H, N.

7.1.5.2 Methyl 2-acetamido-3-[5-cyclohexyl-2-(methoxymethyl)-3-oxoisoxazolin-4-yl]-2-(methoxycarbonyl)propanoate (12d). Synthesized, as described for **12c**, from **11d** (7.02 g, 23.1 mmol), a suspension of NaH in

mineral oil (1.0 g, 60%, 25 mmol), and dimethyl acetamidomalonate (4.8 g, 25 mmol). CC of the residue [eluent: tol:EtOAc (2:1)] gave **12d** (9.49 g, 99%) as a semicrystalline solid. A sample was recrystallized (tol-light petroleum): mp 115.5–116.5°C. ¹H NMR (CDCl₃) δ 1.1–1.9 (m, 10H), 2.02 (s, 3H), 2.6–2.7 (m, 1H), 3.30 (s, 2H), 3.36 (s, 3H), 3.82 (s, 6H), 5.10 (s, 2H), 7.24 (s, 1H). Anal. (C₁₉H₂₈N₂O₈) C, H, N.

7.1.5.3 Methyl 2-acetamido-3-[2-(acetoxymethyl)-5-(3-fluorophenyl)-3-oxoisoxazolin-4-yl]-2-(methoxycarbonyl)propanoate (13b). BF₃·Et₂O (800 μL, 6.36 mmol) was added to a mixture of **12b** (2.0 g, 4.71 mmol), CHCl₃ (30 ml) and (Ac)₂O (30 ml). After stirring overnight at rt, water (75 ml) was carefully added at 0°C, and stirring was continued for 1 h at rt. The phases were separated and the aqueous phase was extracted with CHCl₃ (3×70 ml). The combined organic phases were dried and evaporated. The crude reaction product was subjected to CC [eluent: tol:EtOAc (1:1)] to give TLC pure **13b** (1.54 g, 72%). ¹H NMR (CDCl₃) δ 1.52 (s, 3H), 2.10 (s, 3H), 3.65 (s, 2H), 3.72 (s, 6H), 5.86 (s, 2H), 6.72 (br s, 1H), 7.15–7.33 (m, 3H), 7.44–7.62 (m, 1H).

7.1.5.4 Methyl 2-acetamido-3-[2-(acetoxymethyl)-5-(2-fluorophenyl)-3-oxoisoxazolin-4-yl]-2-(methoxycarbonyl)propanoate (13a). Synthesized, as described for **13b**, from **12a** (1.29 g, 3.04 mmol), BF₃·Et₂O (520 μL, 4.14 mmol), CHCl₃ (20 ml), and (Ac)₂O (20 ml). The crude reaction product was subjected to CC [eluent: tol:EtOAc (1:2)] to give TLC pure **13a** (784 mg, 57%). ¹H NMR (CDCl₃) δ 1.52 (s, 3H), 2.12 (s, 3H), 3.51 (s, 2H), 3.70 (s, 6H), 5.86 (s, 2H), 6.72 (br s, 1H), 7.13–7.37 (m, 3H), 7.44–7.62 (m, 1H).

7.1.5.5 Methyl 2-acetamido-3-[2-(acetoxymethyl)-5-cyclohexyl-3-oxoisoxazolin-4-yl]-2-(methoxycarbonyl)propanoate (13d). Synthesized, as described for **13b**, from **12d** (4.0 g, 9.70 mmol), BF₃·Et₂O (1.34 ml, 10.7 mmol), CH₂Cl₂ (50 ml), and (Ac)₂O (2.0 ml). CC of the residue [eluent: tol:EtOAc (2:1)] gave **13d** (3.0 g, 70%) as a semicrystalline solid. A sample was recrystallized (EtOAc): mp 153.5–154.0°C. ¹H NMR (CDCl₃) δ 1.1–1.9 (m, 10H), 2.02 (s, 3H), 2.08 (s, 3H), 2.5–2.8 (m, 1H), 3.31 (s, 2H), 3.82 (s, 6H), 5.73 (s, 2H), 7.08 (s, 1H). Anal. (C₂₀H₂₈N₂O₉) C, H, N.

7.1.5.6 Methyl 2-acetamido-3-[5-(3-fluorophenyl)-3-hydroxyisoxazol-4-yl]-2-(methoxycarbonyl)propanoate (14b). A mixture of Na (114 mg, 4.56 mg-atom) in CH₃OH (80 ml) and **13b** (1.54 g, 3.31 mmol) was refluxed for 3 h. The reaction mixture was cooled, acidified with AcOH, evaporated and re-evaporated with tol. H₂O (75 ml) was added, and the water phase was extracted with CH₂Cl₂ (3×75 ml). The combined

organic phases were dried and evaporated. The crude product was subjected to CC [eluent: tol:EtOAc (1:1), AcOH (1%)] to give **14b** (1.03 g, 82%). A sample was recrystallized [CH₃OH:ether:light petroleum (3:1:1)]: mp 206–208°C. ¹H NMR (CDCl₃) δ 1.62 (s, 3H), 3.62 (s, 6H), 3.72 (s, 2H), 6.58 (br s, 1H), 7.13–7.35 (m, 3H), 7.44–7.61 (m, 1H). Anal. (C₁₇H₁₇FN₂O₇) C, H, N.

7.1.5.7 Methyl 2-acetamido-3-[5-(2-fluorophenyl)-3-hydroxyisoxazol-4-yl]-2-(methoxycarbonyl)propanoate (14a). Synthesized, as described for **14b**, from Na (97 mg, 4.22 mg-atom), CH₃OH (70 ml) and **13a** (1.27 g, 2.81 mmol) to give **14a** (640 mg, 60%). A sample was recrystallized (2-propanol): mp 203.5–204.5°C. ¹H NMR (CDCl₃) δ 1.58 (s, 3H), 3.64 (s, 6H), 3.72 (s, 2H), 6.60 (s, 1H), 7.13–7.35 (m, 3H), 7.44–7.61 (m, 1H). Anal. (C₁₇H₁₇FN₂O₇) C, H, N.

7.1.5.8 Methyl 2-acetamido-3-(5-cyclohexyl-3-hydroxyisoxazol-4-yl)-2-(methoxycarbonyl)propanoate (14d). Synthesized, as described for **14b**, from Na (230 mg, 10 mg-atom), CH₃OH (100 ml) and **13d** (3.3 g, 7.5 mmol). The crude product, which contained about 20% of a compound proposed to be the 2-hydroxy-methyl derivative of **14d** [¹H NMR (CDCl₃) δ 5.26 (s, CH₂OH)], was refluxed in CH₃OH (100 ml) for 3 h, evaporated and dried to give ¹H NMR pure **14d** (2.21 g, 79%). A sample was recrystallized (EtOAc): mp 205.0–205.5°C. ¹H NMR (CDCl₃) δ 1.1–1.9 (m, 10H), 2.03 (s, 3H), 2.4–2.7 (m, 1H), 3.39 (s, 2H), 3.82 (s, 6H), 6.77 (s, 1H). Anal. (C₁₇H₂₄N₂O₇) C, H, N.

7.1.5.9 Methyl 2-acetamido-3-[3-(benzoyloxy)-5-cyclohexylisoxazol-4-yl]-2-(methoxycarbonyl)propanoate (15). To a solution of **14d** (2.08 g, 5.65 mmol) in CH₂Cl₂ (50 ml) was added Et₃N (787 μl, 5.65 mmol) and benzoyl chloride (656 μl, 5.65 mmol) and the resulting solution was stirred at rt for 1 h. Evaporation, followed by CC [tol:EtOAc (3:2)] gave **15** (2.34 g, 88%). A sample was recrystallized (tol–light petroleum): mp 120.5–121.0°C. ¹H NMR (CDCl₃) δ 1.22–1.38 (m, 6H), 1.4–2.0 (m, 5H), 1.84 (s, 3H), 3.50 (s, 2H), 3.68 (s, 6H), 6.68 (s, 1H), 7.52 (m, 2H), 7.67 (m, 1H), 8.16 (m, 2H); ¹³C NMR (CDCl₃) δ 22.6, 25.1 (2C), 25.2 (2C), 25.7, 30.6 (2C), 35.8, 53.4, 65.6, 100.7, 127.6, 128.5 (2C), 130.3 (2C), 134.2, 162.6, 165.8, 167.6 (2C), 169.5, 177.4 Anal. (C₂₄H₂₈N₂O₈) C, H, N.

7.1.5.10 Methyl 2-acetamido-3-[3-(benzoyloxy)-5-(1-bromocyclohexyl)isoxazol-4-yl]-2-(methoxycarbonyl)propanoate (16). A solution of **15** (3.15 g, 6.67 mmol) in CCl₄ (125 ml) was treated under reflux with NBS (a total of 1.88 g, 10.53 mmol) and benzoylperoxide (a total of 173 mg, 0.71 mmol) for 48 h. Filtration and evaporation to dryness followed by CC [CH₂Cl₂:ether (19:1)] gave **16** as crystals (1.29 g, 35.1%). ¹H NMR (CDCl₃) δ 1.51–

1.85 (m, 6H), 1.88 (s, 3H), 2.29–2.53 (m, 4H), 3.67 (s, 8H), 6.86 (s, 1H), 7.48–7.71 (m, 3H), 8.06–8.21 (m, 2H); ¹³C NMR (CDCl₃) δ 23.0, 23.1 (2C), 24.5 (2C), 26.3, 29.4, 38.4, 53.7, 60.9, 65.3, 103.1, 127.4, 128.7 (2C), 130.5 (2C), 134.5, 162.6, 166.2, 167.5 (2C), 169.9, 172.0.

7.1.5.11 (RS)-2-Amino-3-(5-cyclohexenyl-3-hydroxyisoxazol-4-yl)propanoic acid (5e). A mixture of **16** (1.16 g, 2.10 mmol) and aqueous HBr (100 ml, 0.20 mol, 2 M) was refluxed for 7 h. The reaction mixture was filtered and evaporated twice from H₂O (2×10 ml). The residue was dissolved in H₂O (40 ml). The solution was washed with EtOAc (3×40 ml) followed by evaporation to dryness. The resulting crude product was dissolved in H₂O (2 ml), and pH of the solution was adjusted to 3 using NaOH (1 M). The resulting crystals were collected and dried and recrystallised twice (H₂O) to give **5e** (205 mg, 39%): mp 223–224°C (decomp.). ¹H NMR (DMSO-*d*₆) δ 1.69–1.90 (m, 4H), 2.28–2.51 (m, 4H), 2.68 (m, 2H), 3.70–3.75 (m, 1H), 6.31–6.37 (m, 1H); ¹³C NMR (DMSO) δ 23.0, 23.5, 25.0, 26.7, 26.8, 55.8, 101.9, 127.8, 135.9, 167.5, 174.8, 182.3. Anal. (C₁₂H₁₆N₂O₄) C, H, N.

7.1.5.12 (RS)-2-Amino-3-[5-(4-fluorophenyl)-3-hydroxyisoxazol-4-yl]propanoic Acid (4-F-APPA) (5c). A mixture of **12c** (812 mg, 1.91 mmol) and aqueous CF₃COOH (10 ml, 10 mmol, 1 M) was refluxed for 12 h. The reaction mixture was evaporated and re-evaporated twice from H₂O and subsequently twice from tol. The dried residue was subjected to preparative TLC [eluent: CH₃CN:H₂O:AcOH (8:1:1); Rf 0.31]. The crude product was recrystallized twice from H₂O to give **5c** (331 mg, 65%): mp 245–247°C (decomp). pK_a-values [10.1±0.3 (3σ), 5.0±0.3 (3σ), 1.5±0.3 (3σ)]. ¹H NMR (D₂O) δ 3.20 (d, *J* = 6.55, 2H), 4.27 (t, *J* = 6.55, 1H), 7.15–7.28 (m, 2H), 7.60–7.68 (m, 2H); ¹³C NMR (D₂O) δ 23.4, 52.7, 102.4, 113.9, 116.9, 117.3, 119.6, 130.3, 130.4, 169.2, 172.8, 173.1. Anal. (C₁₂H₁₁FN₂O₄·1/4H₂O) C, H, N.

7.1.5.13 (RS)-2-Amino-3-[5-(3-fluorophenyl)-3-hydroxyisoxazol-4-yl]propanoic acid (3-F-APPA) (5b). Compound **14b** (840 mg, 2.21 mmol) was refluxed in aqueous CF₃COOH (50 ml, 100 mmol, 2 M) for 20 h. The reaction mixture was evaporated and re-evaporated from H₂O and twice from tol. The residue was dissolved in H₂O (3 ml) and an aqueous solution of Et₃N in H₂O (0.45 M) was added to pH 3. The resulting crude product was dissolved in H₂O (100 ml), and the solution washed with EtOAc (50 ml), concentrated to 80 ml and left at 5°C. The resulting crystals were collected and recrystallized (H₂O) to give **5b**·H₂O (204 mg, 32%): mp 226–227°C (decomp.). pK_a-values [9.8±0.2 (3σ), 4.6±0.2 (3σ), 1.6±0.2 (3σ)]. ¹H NMR (D₂O) δ 2.57 (dd, *J* = 9.3, *J* = 14.7, 1H), 2.82 (dd, *J* = 4.9, *J* = 14.7, 1H),

3.39 (dd, $J = 4.9$, $J = 9.3$, 1H), 7.09–7.26 (m, 1H), 7.32–7.55 (m, 3H). Anal. (C₁₂H₁₁FN₂O₄·H₂O) C, H, N.

7.1.5.14 (RS)-2-Amino-3-[5-(2-fluorophenyl)-3-hydroxyisoxazol-4-yl]propanoic acid (2-F-APPA) (5a). Synthesized, as described for **5b**, from **14a** (580 mg, 1.52 mmol) and aqueous CF₃COOH (50 ml, 100 mmol, 2 M). The residue was dissolved in H₂O (8 ml) and an aqueous solution of Et₃N in H₂O (1.4 M) was added to pH 3. The resulting crystals were washed with EtOAc and recrystallized twice from H₂O to give **5a** (77 mg, 19%): mp 212–214°C (decomp.). p*K*_a-values [9.9 ± 0.1 (3σ), 4.6 ± 0.1 (3σ), 2.1 ± 0.1 (3σ)]. ¹H NMR (D₂O) δ 2.41 (dd, $J = 9.4$, $J = 14.9$, 1H), 2.74 (dd, $J = 4.6$, $J = 14.9$, 1H), 3.31 (dd, $J = 4.6$, $J = 9.4$, 1H), 7.15–7.36 (m, 2H), 7.40–7.60 (m, 2H). Anal. (C₁₂H₁₁FN₂O₄) C, H, N.

7.1.5.15 (RS)-2-Amino-3-(5-cyclohexyl-3-hydroxyisoxazol-4-yl)propanoic acid (5d). Synthesized, as described for **5c**, from **12d** (514 mg, 1.17 mmol) and aqueous CF₃COOH (10 ml, 10 mmol, 1 M). The crude product was purified by preparative TLC [eluent: CH₃CN:H₂O:AcOH (8:1:1), *R*_f 0.35]. Recrystallization

(H₂O) gave **5d** (176 mg, 59%): mp 220–222°C (decomp.). p*K*_a-values [10.0 ± 0.2 (3σ), 5.1 ± 0.2 (3σ), 2.2 ± 0.2 (3σ)]. ¹H NMR (D₂O) δ 1.20–1.90 (m, 10H), 2.67 (tt, $J = 3.3$, $J = 11.3$, 1H), 3.10 (d, $J = 6.9$, 2H), 4.28 (t, $J = 6.9$, 1H). Anal. (C₁₂H₁₈N₂O₄) C, H, N.

7.1.5.16 (R)-(-)-2-Amino-3-[5-(4-fluorophenyl)-3-hydroxyisoxazol-4-yl]propanoic acid [(R)-(-)-4-F-APPA] (7). To a suspension of 4-F-APPA (**5c**) (620 mg, 2.33 mmol) in C₂H₅OH (35 ml) was added (*S*)-(-)-PEA (590 μL, 4.66 mmol). The mixture was heated to reflux, and the resulting solution was filtered and cooled. Ether (30 ml) was added, and after standing for 24 h at 5°C the jelly-like precipitate was collected, washed with ether, and dried in vacuo. The salt of (*R*)-(-)-4-F-APPA and (*S*)-(-)-PEA was recrystallized three times from C₂H₅OH:ether containing ca 100% excess of (*S*)-(-)-PEA. The salt [141 mg, de 99.9%, Crownpak CR(-)] was dissolved in H₂O (10 ml) and the solution was acidified with AcOH to pH 3. Recrystallization from H₂O gave 67 mg (22%) of **7**: mp 247°C (decomp.); [α]_D²⁵ -38.5° (*c* 0.54, HCl 1 M); *ee* 99.6%. ¹H NMR (D₂O/NaOD) δ 2.56 (dd, $J = 9.5$, $J = 14.9$, 1H), 2.82

Elemental analysis

		Calcd(%)				Found(%)			
		C	H	N	Br	C	H	N	Br
9c	C ₁₀ H ₉ Br ₂ FO ₂	35.32	2.67		47.00	35.55	2.76		46.87
9b	C ₁₀ H ₉ Br ₂ FO ₂	35.32	2.67		47.00	35.49	2.74		47.34
9a	C ₁₀ H ₉ Br ₂ FO ₂	35.32	2.67		47.00	35.52	2.62		46.72
10c	C ₉ H ₆ FNO ₂	60.34	3.38	7.82		59.97	3.35	7.88	
10b	C ₉ H ₆ FNO ₂	60.34	3.38	7.82		59.77	3.45	7.81	
10a	C ₉ H ₆ FNO ₂	60.34	3.38	7.82		60.23	3.37	7.83	
10d	C ₉ H ₁₃ NO ₂	65.65	7.84	8.38		64.47	7.81	8.44	
11c	C ₁₂ H ₁₁ BrFNO ₃	45.59	3.51	4.43		45.19	3.17	4.15	
11b	C ₁₂ H ₁₁ BrFNO ₃	45.59	3.51	4.43		45.84	3.54	4.44	
11a	C ₁₂ H ₁₁ BrFNO ₃	45.59	3.51	4.43		45.30	3.40	4.47	
11d	C ₁₂ H ₁₈ BrNO ₃	47.38	5.96	4.60		47.51	5.73	4.78	
12c	C ₂₁ H ₂₅ FN ₂ O ₈	55.75	5.57	6.19		56.03	5.51	6.23	
12b	C ₁₉ H ₂₁ FN ₂ O ₈	53.77	4.99	6.60		53.94	4.78	6.63	
12a	C ₁₉ H ₂₁ FN ₂ O ₈	53.77	4.99	6.60		54.04	4.94	6.71	
12d	C ₁₉ H ₂₈ N ₂ O ₈	55.33	6.84	6.79		55.55	6.94	6.69	
13b	C ₂₀ H ₂₁ FN ₂ O ₉	53.16	4.68	6.19		53.35	4.52	6.19	
13a	C ₂₀ H ₂₁ FN ₂ O ₉	53.16	4.68	6.19		53.46	4.62	6.27	
13d	C ₂₀ H ₂₈ N ₂ O ₉	54.54	6.41	6.36		54.40	6.54	6.45	
14b	C ₁₇ H ₁₇ FN ₂ O ₇	53.69	4.51	7.37		53.73	4.50	7.17	
14a	C ₁₇ H ₁₇ FN ₂ O ₇	53.69	4.51	7.37		53.65	4.56	7.39	
14d	C ₁₇ H ₂₄ N ₂ O ₇	55.43	6.57	7.60		55.88	6.54	7.54	
15	C ₂₄ H ₂₈ N ₂ O ₈	61.01	5.97	5.93		60.73	5.98	6.13	
5e	C ₁₂ H ₁₆ N ₂ O ₄	57.13	6.39	11.10		57.45	6.37	11.19	
5c	C ₁₂ H ₁₁ FN ₂ O ₄ ·1/4H ₂ O	52.35	4.39	10.22		52.09	4.11	9.84	
5b	C ₁₂ H ₁₁ FN ₂ O ₄ ·H ₂ O	50.69	4.61	9.89		50.85	4.36	9.84	
5a	C ₁₂ H ₁₁ FN ₂ O ₄	54.14	4.16	10.52		53.78	4.29	10.34	
5d	C ₁₂ H ₁₈ N ₂ O ₄	56.68	7.13	11.01		56.73	7.03	10.89	
6	C ₁₂ H ₁₁ FN ₂ O ₄	54.14	4.16	10.52		54.16	4.12	10.48	
7	C ₁₂ H ₁₁ FN ₂ O ₄	54.14	4.16	10.52		54.12	4.06	10.45	

(dd, $J = 4.9$, $J = 14.9$, 1H), 3.40 (dd, $J = 4.9$, $J = 9.5$, 1H), 7.22 (dd, $J = 5.6$, $J = 8.7$, 2H), 7.67 (dd, $J = 5.6$, $J = 8.7$, 2H). Anal (C₁₂H₁₁FN₂O₄) C, H, N.

7.1.5.17 (S)-(+)-2-Amino-3-[5-(4-fluorophenyl)-3-hydroxyisoxazol-4-yl]propanoic acid [(S)-(+)-4-F-APPA] (6). The mother liquors from the two first crystallizations of the salt of (R)-(-)-4-F-APPA and (S)-(-)-PEA were evaporated. The residue consisted of a mixture of the diastereomeric salts with a diastereomeric excess of the salt of (S)-(+)-4-F-APPA and (S)-(-)-PEA (de 65%, Crownpak CR(-)). The mixture of the diastereomeric salts was dissolved in H₂O (10 ml) and the solution was acidified with AcOH to pH 3. The resulting crystals were collected and dried in vacuo. The partly resolved **6** (225 mg, 0.85 mmol) was processed, as described above for **7**, using (R)-(+)-PEA (215 mg, 1.69 mmol). The salt of (S)-(+)-4-F-APPA and (R)-(+)-PEA was recrystallized three times. The salt [125 mg, de 99.8%, Crownpak CR(-)] was dissolved in H₂O (10 ml) and the solution was acidified with AcOH to pH 3. Recrystallization from water gave 32 mg (14%) of **6**: mp 246°C decomp.; $[\alpha]_D^{25} + 37.9^\circ$ (c 0.25, HCl 1 M); ee 99.8%. The ¹H NMR spectrum of **6** was identical with that of **7**. Anal (C₁₂H₁₁FN₂O₄) C, H, N.

7.1.6 Radioligand binding assays

The membrane preparation used in the [³H]AMPA, [³H]kainic acid, [³H]CPP, [³H]MK-801, [³H]glycine and [³H]CNQX binding assays was prepared as described [21]. [³H]AMPA [23], [³H]kainic acid [22], [³H]CPP [20] and [³H]CNQX [24,27] binding were performed following published procedures. [³H]MK-801 binding to fully stimulated membranes was performed essentially as described earlier [46]. [³H]Glycine binding was carried out by a modified version of the method described [47], using filtration through Whatman GF/B filters instead of centrifugation to isolate bound ligand.

7.1.7 In vitro electrophysiology

A rat cortical wedge preparation for testing the depolarizing activity of EAAs described by Harrison and Simmonds [48] was used in a modified version. Wedges (500 μm thick) of rat brain containing cerebral cortex and corpus callosum were placed with the cortex part between two layers of absorbent fiber and the corpus callosum part between two other layers of absorbent fiber. The two halves were electrically insulated from each other with a grease gap. The cortical part was constantly perfused with a Mg²⁺-free, oxygenated Krebs buffer to which the compounds tested were added, whereas the corpus callosum part was perfused with a Mg²⁺- and Ca²⁺-free Krebs buffer. The two parts were each in contact with an Ag/AgCl electrode through which DC potentials were measured and plotted on a chart recorder.

7.1.8 Computational methods

Semiempirical calculations were performed by the use of SPARTAN 4.1.1. [49].

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References

- [1] Krogsgaard-Larsen P, Hansen JJ, editors. Excitatory Amino Acid Receptors: Design of Agonists and Antagonists. Chichester: Ellis Horwood, 1992.
- [2] Collingridge GL, Watkins JC, editors. The NMDA Receptor. Oxford: Oxford University, 1994.
- [3] Wheal HV, Thomson AM, editors. Excitatory Amino Acids and Synaptic Transmission. London: Academic, 1995.
- [4] Monaghan DT, Wenthold RJ, editors. The Ionotropic Glutamate Receptors. New Jersey: Humana, 1997.
- [5] Conn PJ, Patel J, editors. The Metabotropic Glutamate Receptors. New Jersey: Humana, 1994.
- [6] Danysz W, Parsons CG, Bresink I, Quack G. Drug News Perspect 1995;8:261.
- [7] Knöpfel T, Kuhn R, Allgeier H. J Med Chem 1995;38:1417.
- [8] Deutsch SI, Morihisa JM. Clin Neuropharmacol 1988;11:18.
- [9] Greenamyre JT, Young AB. Neurobiol Ag 1989;10:593.
- [10] Bowen DM. Br J Psychiat 1990;157:327.
- [11] Madsen U, Ebert B, Krogsgaard-Larsen P. Biomed Pharmacother 1994;48:305.
- [12] Carlsson M, Carlsson A. Trends Neurosci 1990;13:272.
- [13] Ulas J, Cotman CW. Schizophrenia Bull 1993;19:105.
- [14] Deutsch SI, Mastropaolo J, Schwartz BL, Rosse RB, Morihisa JM. Clin Neuropharmacol 1989;12:1.
- [15] Sams-Dodd F. Behav Pharmacol 1996;7:3.
- [16] Hansen JJ, Krogsgaard-Larsen P. Med Res Rev 1990;10:55.
- [17] Krogsgaard-Larsen P, Ebert B, Lund TM, Bräuner-Osborne H, Sløk FA, Johansen TN, Brehm L, Madsen U. Eur J Med Chem 1996;31:515.
- [18] Foster AC, Fagg GE. Eur J Pharmacol 1987;133:291.
- [19] Thomson AM. Prog Neurobiol 1990;35:53.
- [20] Murphy DE, Schneider J, Boehm C, Lehmann J, Williams K. J Pharmacol Exp Ther 1987;240:778.
- [21] Ransom RW, Stec NL. J Neurochem 1988;51:830.
- [22] Braitman DJ, Coyle JT. Neuropharmacology 1987;26:1247.
- [23] Honoré T, Nielsen M. Neurosci Lett 1985;54:27.
- [24] Honoré T, Drejer J, Nielsen EØ, Nielsen M. Biochem Pharmacol 1989;38:3207.
- [25] Christensen IT, Reinhardt A, Nielsen B, Ebert B, Madsen U, Nielsen EØ, Brehm L, Krogsgaard-Larsen P. Drug Des Del 1989;5:57.
- [26] Ebert B, Lenz SM, Brehm L, Bregndal P, Hansen JJ, Frederiksen K, Bøgesø KP, Krogsgaard-Larsen P. J Med Chem 1994;37:878.

- [27] Ebert B, Madsen U, Lund TM, Lenz SM, Krogsgaard-Larsen P. *Neurochem Int* 1994;24:507.
- [28] Ebert B, Madsen U, Søby KK, Krogsgaard-Larsen P. *Neurochem Int* 1996;29:309.
- [29] Ebert B, Søby KK, Madsen U, Krogsgaard-Larsen P. In: Fog R, Gerlach J, Hemmingsen R, editors. *Schizophrenia—An Integrated View*. Copenhagen: Munksgaard, 1995:379–398.
- [30] Johansen TN, Ebert B, Falch E, Krogsgaard-Larsen P. *Chirality* 1997;9:274.
- [31] Tomita K. *Ann Sankyo Res Lab* 1973;25:1.
- [32] Frølund B, Kristiansen U, Brehm L, Hansen AB, Krogsgaard-Larsen P, Falch E. *J Med Chem* 1995;38:3287.
- [33] Bennouna C, Petrus F, Verducci J. *Bull Soc Chim Fr II* 1980:478.
- [34] Begtrup M, Sløk FA. *Synthesis* 1993:861.
- [35] Gübitz G, Jellenz W, Santi W. *J Liq Chromatogr* 1981;4:701.
- [36] Gübitz G, Jellenz W, Santi W. *J Chromatogr* 1981;203:377.
- [37] Shinbo T, Yamagushi T, Nishimura K, Sugiura M. *J Chromatogr* 1987;405:145.
- [38] Lazareno S, Birdsall NJM. *Trends Pharmacol Sci* 1993;14:237.
- [39] Bang-Andersen B, Lenz SM, Skjærbaek N, Søby KK, Hansen HO, Ebert B, Bøgesø KP, Krogsgaard-Larsen P. *J Med Chem* 1997;40:2831.
- [40] Burfield DR, Smithers RH. *J Org Chem* 1978;43:3966.
- [41] Still WR, Kahn M, Mitra A. *J Org Chem* 1978;43:2923.
- [42] Beck M, Jaunich S, Frahm AW. *Arch Pharm* 1986;319:29.
- [43] Quaglia W, Pignini M, Tayebati SK, Piergentili A, Giannela M, Marrucci G, Melchiorre C. *J Med Chem* 1993;36:1520.
- [44] Jacquier R, Petrus C, Petrus F, Verducci J. *Bull Soc Chim Fr* 1970;5:1978.
- [45] Jacquier R, Petrus C, Petrus F, Verducci J. *Bull Soc Chim Fr* 1970;7:2685.
- [46] Foster AC, Wong EHF. *Br J Pharmacol* 1987;91:403.
- [47] Kemp JA, Foster AC, Leeson PD, Priestly T, Tridgett R, Iversen LL, Woodruff GN. *Proc Natl Acad Sci USA* 1988;85:6547.
- [48] Harrison NL, Simmonds MA. *Br J Pharmacol* 1985;84:381.
- [49] SPARTAN SGI version 4.1.1 GL Wavefunction, Inc., 18401 von Karman Ave., suite no. 370, Irvine, CA 92715.