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Synthesis and In Vitro Pharmacology at AMPA and Kainate Preferring Glutamate Receptors of 4-Heteroarylmethylidene Glutamate Analogues

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Abstract—2-Amino-3-[3-hydroxy-5-(2-thiazolyl)-4-isoxazolyl]propionic acid (**1**) is a potent AMPA receptor agonist with moderate affinity for native kainic acid (KA) receptors, whereas (*S*)-*E*-4-(2,2-dimethylpropylidene)glutamic acid (**3**) show high affinity for the GluR5 subtype of KA receptors and much lower affinity for the GluR2 subtype of AMPA receptors. As an attempt to develop new pharmacological tools for studies of GluR5 receptors, (*S*)-*E*-4-(2-thiazolylmethylene)glutamic acid (**4a**) was designed as a structural hybrid between **1** and **3**. **4a** was shown to be a potent GluR5 agonist and a high affinity ligand and to indiscriminately bind to the AMPA receptor subtypes GluR1–4 with lower affinities. Compounds **4b**–**h**, in which the 2-thiazolyl substituent of **4a** was replaced by other heterocyclic rings, which have previously been incorporated as 5-substituents in AMPA analogues, as exemplified by **1** were also synthesized. Compounds **4b**–**h** were either inactive (**4e**,**f**) or weaker than **4a** as affinity ligands for GluR1–4 and GluR5 with relative potencies comparable with those of the corresponding AMPA analogues as AMPA receptor agonists. Compounds **4a**–**h** may be useful tools for the progressing pharmacophore mapping of the GluR5 agonist binding site.

Introduction

Receptors for the major central excitatory amino acid neurotransmitter, (*S*)-glutamic acid (Glu) are divided into two heterogeneous classes, the ionotropic Glu receptors (iGluRs) and the metabotropic Glu receptors (mGluRs).[1–3] The iGluRs are subdivided into *N*-methyl-D-aspartic acid (NMDA), 2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid (AMPA), and kainic acid (KA) receptors. The eight mGluRs, so far identified, are subdivided into group I (mGluR1,5), group II (mGluR2,3), and group III (mGluR4,6,7,8).

AMPA receptors are homo- or heteromeric assembles of GluR1–4 subunits and KA receptors of GluR5–7, KA1 and KA2 subunits.[1,4,5] (*S*)-AMPA is the classical AMPA receptor agonist,[6] and a large number of heterocyclic Glu analogues, most of which are derived from AMPA, are potent and selective agonists at AMPA receptors.[3] Thus, (*S*)-2-amino-3-[3-hydroxy-5-(2-pyridyl)-4-isoxazolyl]propionic acid [(*S*)-2-Py-AMPA][7] and 2-amino-3-[3-hydroxy-5-(2-thiazolyl)-4-isoxazolyl]propionic acid (**1**)[8] (Fig. 1) are examples of very potent AMPA agonists, whereas (*R*)-2-Py-AMPA, 3-Py-AMPA (**2**), and 4-Py-AMPA are essentially inactive.[7,9] (*S*)-2-Amino-3-(5-*tert*-butyl-3-hydroxy-4-isoxazolyl)propionic acid [(*S*)-ATPA], but not (*R*)-ATPA,[10–12] on the other hand, is a very potent agonist at GluR5 receptors, but a relatively weak agonist at AMPA receptor subtypes.[10] Compound **3**, which is an acyclic analogue of (*S*)-ATPA, shows a very similar

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pharmacological profile.[13] In addition to powerful AMPA agonist effects, **1** shows the highest affinity for KA receptors within a large group of AMPA analogues containing a heterocyclic substituent in the 5-position of the 3-isoxazolol ring.[7–9]

These structure–activity relationships (SARs) prompted us to synthesize and pharmacologically characterize compound **4a** as a structural hybrid between **1** and **3** (Fig. 1). Furthermore, compounds **4b–h** were synthesized and profiled pharmacologically at AMPA (GluR1–4) and KA (GluR5,6) receptor subtypes. The SARs of **4a–h** were compared with those of the AMPA analogues containing the same heterocyclic rings as 5-substituents. The overall goal of this approach is to develop tools for the pharmacological characterization and ligand-based pharmacophore mapping of the GluR5 agonist binding site.[1,14]

Results

Chemistry

Compounds **4a–h** were synthesized in three steps from the activated pyroglutamate derivative **5** (Scheme 1), which was prepared as described by August et al.[15] Heterocyclic Grignard reagents were reacted with **5** in a 1,4-addition–elimination reaction to yield the intermediates **6a–h** in 24–86% yields. Only the *E* isomers of **6a–h** were obtained, which is in agreement with previous reports.[16,17] The 2- and 5-thiazole Grignard reagents were prepared from 2-bromothiazole and 2-trimethylsilanyl-thiazole, by bromine-lithium exchange and deprotonation, respectively, using *n*-BuLi and subsequent lithium–magnesium exchange using $MgBr_2$. The synthesis of 4-bromo-2-trimethylsilanyl-thiazole has been reported in the literature by the use of excess *n*-BuLi,[18] but this was not compatible with our subsequent reaction with **5**. This problem was solved using 2 equivalents of *t*-BuLi instead of *n*-BuLi. The pyridine and thiophene Grignard reagents were prepared by literature procedures.[19,20] Compounds **6a–h** were deprotected in two steps: The lactam rings were hydrolyzed by LiOH in H_2O/THF to yield *N*-Boc protected Glu esters, which were subsequently treated with HBr in glacial acetic acid to give the final products **4a–h** in 55–90% yields. Compounds **4a–b**, and **4f** were precipitated as zwitterions from H_2O/*i*-PrOH by propylene oxide because they were very hygroscopic as hydrobromides.

The stereochemical purity of compounds **4a–h** was investigated by chiral HPLC on a Sumichiral OA-5000 column. Compound **4a** was synthesized both in an optically pure form (see Experimental) and in a partially racemized form. This allowed the identification of two peaks in the chiral HPLC chromatogram with identical UV-profiles that correspond to the *S*- and *R*-form of **4a** ($t_R = 8$ and 11 min, respectively, ee = 66% for the *S*-form). The non-racemized sample of **4a** was found to have an ee = 99.5%. Compound **4c** was similarly observed to have two peaks with identical UV profiles ($t_R = 10$ and 12 min) corresponding to an ee = 91%. No enantiomeric impurity could be detected for the remaining compounds (**4b**,**4d–h**), indicating that the enantiomeric impurities were less than the detection limit, which was estimated to be $<0.5\%$ depending on the observed t_R. This corresponds to an ee $>99\%$ for these compounds. The alternative, that the *S*- and *R*-forms may co-elute, thus preventing determination of ee, cannot be ruled out for these compounds because no racemized or partially racemized samples were available (efforts to racemize these compounds using literature procedures[21] failed because the compounds ring closed into pyroglutamate derivatives faster than they racemized).

In vitro pharmacology

The affinity of reference compounds AMPA, KA, ATPA, **1** and **3** and of the new compounds **4a–h** for

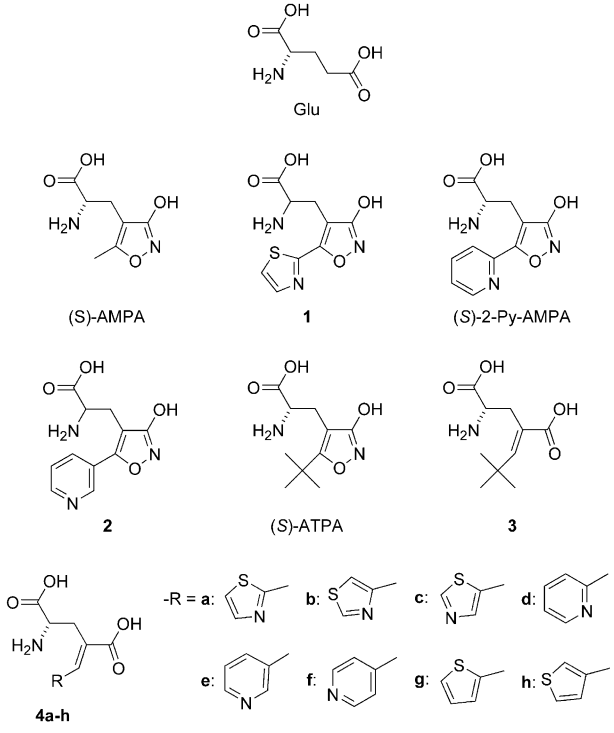
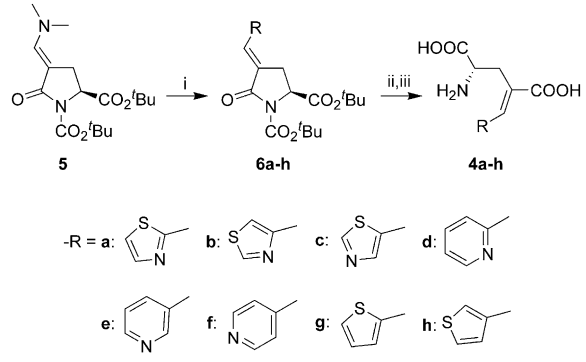


Figure 1. Structures of Glu, (*S*)-AMPA, 5-heteroaryl-AMPA analogues, (*S*)-ATPA, and the 4-methylidene Glu analogues **3** and **4a–h**.



Scheme 1. (i) RMgBr, THF or Et_2O, −78 °C; (ii) LiOH, H_2O, THF; (iii) HBr, AcOH.

native NMDA, AMPA and KA receptors was determined using the ligands [^{3}H]CGP39653, [^{3}H]AMPA, and [^{3}H]KA, respectively (Table 1). Weak affinity for NMDA receptors was observed for all of the new compounds except for **4a**, **4b** and **4d** and weak, but somewhat higher, affinity for AMPA receptors was observed for all new compounds except **4e** and **4f**. Only compounds **4a**, **4g**, and **4h** showed weak affinity for native KA receptors. These affinities generally corresponded well to the observed electrophysiological profiles determined on native receptors using a rat cortical slice model (Table 1). Compounds **4e**, **4f**, and **4h** were found to be NMDA receptor agonists, as their activity could be blocked by the NMDA receptor antagonist 3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP) (5 μM), and compound **4c** was a mixed NMDA/AMPA agonist; partially blocked by CPP (5 μM) or the AMPA receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(*f*)quinoxaline (NBQX) (5 μM). Compounds **4a**, **4b**, and **4d** were found to be AMPA receptor agonists (blocked by 5 μM NBQX), and compound **4g** was found to be a mixed AMPA/KA agonist (blocked partially by 5 μM NBQX and fully by 20 μM NBQX).

Table 1. Receptor binding and electrophysiological data

	[^{3}H]CGP39653 K_i (μM)	[^{3}H]AMPA IC_{50} (μM)	[^{3}H]KA K_i (μM)	Electrophysiology EC_{50} (μM)
AMPA[a]	>100[b]	0.04±0.02	>100	3.5±0.2[d]
KA[a]	>100[b]	4.0±1.2	0.007±0.002	nt
ATPA[a]	>100[b]	3.9±0.6	31±1	48[d]
3[a]	>100[b]	2.0±0.1	>100	nt
1[c]	>100	0.094	4.9	2.3[d]
4a	>100	4.0±0.5	28±10	49[d]
4b	>100	6.7±1.8	>100	175[d]
4c	75±10	8.5±0.8	>100	64[e]
4d	>100	23±3	>100	207[d]
4e	16±3	>100	>100	56[f]
4f	12±3	>100	>100	28[f]
4g	65±9	6.9±0.6	36±13	90[g]
4h	32±4	60±2	32±3	99[f]

Values represent mean±SEM, $n \geq 3$. nt: not tested.
[a]Ref 13.
[b][^{3}H]CPP.
[c]Ref 8.
[d]AMPA agonist (blocked by 5 μM NBQX).
[e]Mixed NMDA/AMPA agonist (blocked partially by 5 μM CPP or 5 μM NBQX).
[f]NMDA agonist (blocked by 5 μM CPP).
[g]Mixed AMPA/KA agonist (blocked partially by 5 μM NBQX and fully by 20 μM NBQX).

The affinity was also determined for cloned homomeric GluR1–4 expressed in recombinant baculovirus-infected Sf9 insect cells and GluR5–6 stably expressed in HEK293 cell lines (Table 2). The highest affinity was observed for compound **4a** on GluR5 ($K_i = 0.35$ μM), whereas the affinity of compound **4a** for GluR1–4 was 3–6 times lower. Similar profiles were observed for compounds **4b** and **4g**, though with somewhat weaker affinities. Compound **4c** showed equal affinities for GluR1–4 and GluR5, whereas compound **4d** showed some selectivity for GluR1 and GluR5 versus GluR2, 3 and 4 (approximately 6–16 times) Finally, compounds **4e** and **4f** were without affinity for GluR1–4 (K_i >100 μM) and GluR5 (K_i >30 μM), and all compounds under study were without affinity for GluR6 (K_i >30 μM).

The affinity of compound **4a** for GluR5 was further investigated by electrophysiological experiments performed on HEK293 cells expressing homomeric GluR5(*Q*). Compound **4a** was found to be a partial agonist (Fig. 2a and b). When **4a** was applied to the cells before and during application of Glu (3 mM) partial (0.3 μM **4a**) or full (10 μM **4a**) inhibition of the Glu response was observed (Fig. 2b). At higher concentrations (100 μM and above), **4a** showed partial agonist activity (Fig. 2a). As the aforementioned inhibition of the Glu response is achieved at **4a** concentrations that are 10–300 times lower than the concentration needed to elicit agonist activity, the inhibition cannot be attributed to partial antagonism, but is due to desensitization of the receptor. Concentration–response curves for **4a** and Glu are shown in Figure 2c and inhibition of the Glu response by **4a** is shown in Figure 2d. The desensitization of the Glu response by **4a** depicted in Figure 2d corresponds to an $IC_{50} = 0.7$ μM. None of the compounds **4a–h** showed detectable agonist or antagonist effects (at 1 mM) on mGluR1a, mGluR2, or mGluR4a representing group I, II, and III of the mGluRs, respectively (data not shown).

Table 2. Affinity for cloned homomeric AMPA (GluR1–4) and KA (GluR5,6) receptors

	$GluR1_o$	$GluR2_o(R)$	$GluR3_o$ K_i (μM)	$GluR4_o$	GluR5(*Q*)	GluR6(*V*,*C*,*R*)
AMPA	0.022±0.004[a]	0.017±0.003[a]	0.021±0.003[a]	0.040±0.020	1.9±0.2[b]	>100[b]
ATPA	2.8±0.6	1.7±0.3	2.0±0.1	2.1±0.5	0.0076±0.0021[b]	>100[b]
3[b]	nt	3.0±0.5	nt	nt	0.024±0.005	>100
4a	1.1±0.2	1.3±0.3	2.3±0.7	1.4±0.2	0.35±0.13	>30
4b	5.8±0.9	5.9±1.1	5.2±0.9	4.7±0.5	2.0±0.7	>30
4c	6.9±3.7	6.1±0.7	4.1±2.3	2.9±0.1	3.7±1.6	>30
4d	3.6±1.1	14±3	39±15	14±2	2.5±0.7	>30
4e	>100	>100	>100	>100	>30	>30
4f	>100	>100	>100	>100	>30	>30
4g	7.5±1.1	7.5±0.8	14±7	6.6±0.5	2.7±0.4	>30
4h	39±10	33±12	32±17	23±1	15±2	>30

Values represent mean±SEM, $n \geq 3$. nt, not tested.
[a]Ref 36.
[b]Ref 13.

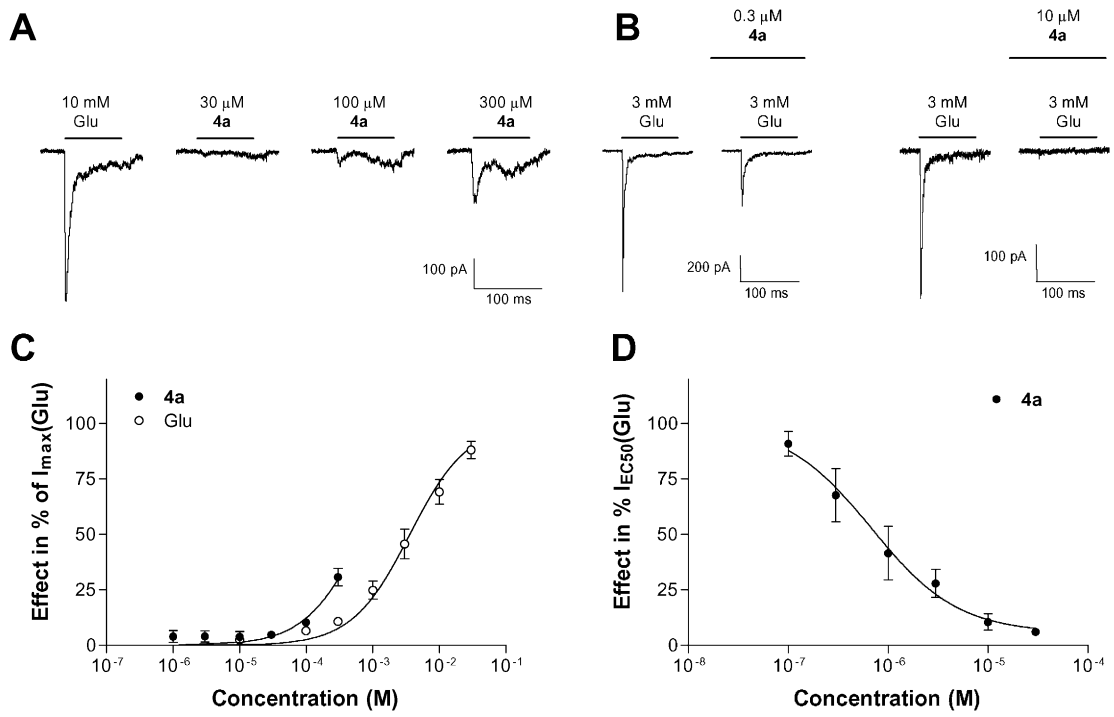


Figure 2. Recordings from patch clamp experiments on HEK293 cells expressing homomeric GluR5; currents obtained by application of: (A) Glu or compound **4a**. (B) Glu alone or Glu applied to cells that have been pre-incubated with **4a**. (C) Concentration–response curves obtained with Glu or compound **4a** and (D) inhibition of the Glu induced current by increasing concentrations of **4a**.

Discussion

Accumulating evidence supports the view that iGluRs as well as mGluRs play important roles in the healthy as well as the diseased central nervous system and that all subtypes of these receptors are potential therapeutic targets in a number of diseases and disease conditions.[1–3,22] Both classes of GluRs are heterogeneous, and it is of interest to develop subtype-selective GluR ligands as tools and leads for the design of receptor specific therapeutic agents. A prerequisite for the design of such compounds on a rational basis is the availability of information about the pharmacophore characteristics of the recognition sites of the individual GluRs.

A large number of heterocyclic Glu analogues,[23,24] including (*S*)-AMPA,[3] (*S*)-2-Py-AMPA,[7] and **1**[8] are very potent agonists at native AMPA receptors, and SAR analyses of this comprehensive series of compounds have shed indirect light on the pharmacophore characteristics of AMPA receptor recognition sites. More recently, X-ray crystallographic analyses of complexes between the ligand-binding domain of the AMPA receptor subtype, GluR2, and a number of AMPA agonists, including (*S*)-AMPA, have provided direct insight into the structural basis for these receptor–ligand interactions.[25,26] It has been shown that the fundamental interactions between the GluR2 recognition site and the amino acid parts of the agonists are conserved, whereas the anionic heterocyclic units of the agonist molecules show binding modes remarkably different from that of the distal carboxylate group of Glu.[27] The binding of these heterocyclic groups are stabilized by hydrogen bonds directly to the receptor protein and/or through water-mediated hydrogen bonds. Structural information of this nature is not yet available for other iGluR subtypes, including the KA-preferring GluR subtype, GluR5. Comparative mutation studies on GluR1 and GluR5 have, however, provided some insight into the structural basis for ligand-recognition site interactions at these receptor subtypes.[14] Thus, Ser741 in GluR5 and Met722 in GluR1 appear to play a key role in the potent and relatively weak binding of (*S*)-ATPA to GluR5 and GluR1, respectively, although other structural factors also play a role in these interactions.

Both **3** and (*S*)-ATPA are very potent at the GluR5 and less active at the other GluR subunits tested (GluR2,6 for **3**, and GluR1–4,6 for (*S*)-ATPA, see Table 2),[13] whereas compound **1** exhibits the converse affinity profile, being a very potent inhibitor of [^{3}H]AMPA binding and a relatively weak inhibitor of the binding of [^{3}H]KA (Table 1).[8] Compound **1** does, however, bind more tightly to KA-preferring iGluRs than a number of other AMPA analogues containing heterocyclic 5-substituents.[8] These SARs prompted us to synthesize **4a** as a structural hybrid between **1** and **3** as a potentially selective ligand for GluR5, and, furthermore, **4a** was used as a lead for the synthesis of analogues **4b–h**.

Compound **4a** was shown to be much weaker than the corresponding AMPA analogue **1** as an AMPA agonist and as an inhibitor of the binding of [^{3}H]AMPA to native AMPA receptors (Table 1). The GluR5 agonist effect of **4a** was demonstrated electrophysiologically using GluR5(*Q*) receptors expressed homomerically in HEK293 cells. In agreement with previous observations for Glu at GluR5,[28] the GluR5 receptors were shown to desensitize very rapidly after application of **4a** alone or

co-administrated with Glu (Fig. 2a and b). Only at high concentrations (>100 μM) could the agonist effect of **4a** be recorded, indicating that **4a** is a GluR5 agonist that induces receptor desensitization but not an antagonist at this iGluR subtype.

In binding studies using homomeric recombinant iGluRs, **4a** was almost an order of magnitude weaker than **3** as an affinity ligand for GluR5, but still with markedly higher affinity than the analogues **4b–h**. Compound **4a** shows higher affinity for GluR5 than **4b** and **4c**, which contain isomeric thiazolyl substituents (Fig. 1), and in general, the SAR of **4a–h** discloses that the binding affinity of the compounds is strongly dependent on the heterocyclic substituent. Thus, the 2-pyridyl analogue **4d** is a moderately potent inhibitor, whereas the 3- and 4-pyridyl isomers, **4e** and **4f**, are inactive. Similarly, the 2-thienyl analogue **4g** is substantially more potent than the 3-thienyl isomer **4h** (Table 2). In the 'horizontal' SAR based on receptor binding data listed in Tables 1 and 2, it is striking that the relative affinities of the compounds for GluR5 are similar to their relative order of affinities for the cloned AMPA receptor subtypes, GluR1–4 (Table 2) and also for native AMPA receptors (Table 1). Whereas the AMPA analogues corresponding to **4b**,**c** have not been reported, the AMPA analogues containing the heteroaromatic groups of **4a**,**d–h** as 5-substituents have been synthesized and pharmacologically characterized, as exemplified by compounds **1**,[8] **2**,[9] and (*S*)-2-Py-AMPA[7] (Fig. 1). Interestingly, the relative affinities of **4a**,**d–h** for GluR1-4 and GluR5 are very similar to the relative affinities of the corresponding AMPA analogues as AMPA receptor agonists.[7–9,29]

To summarize the SARs, it is evident from previous observations and from the data listed in Tables 1 and 2 that the presence of a *tert*-butyl in (*S*)-ATPA and in **3** greatly facilitates the interaction of these compounds with the GluR5 recognition site. Steric effects and a not fully understood influence of Ser741 at the recognition site of GluR5 are primarily responsible for the GluR5 selectivity of (*S*)-ATPA and **3**. On the other hand, the remarkably similar relative affinities of **4a–h** for GluR5 and for the AMPA receptor subtypes GluR1–4 strongly suggest that the recognition sites for these receptors share some structural characteristics of as yet unknown nature. Future X-ray crystallographic analyses of the binding domain of GluR5, co-crystallized with some of the compounds shown in Figure 1 and compared with the structural data obtained for GluR2 will shed light on receptor topographical characteristics of major importance for the design of GluR5 ligands on a rational basis.

Experimental

General methods

All reagents are commercially available unless stated otherwise. All reactions were carried out under a nitrogen atmosphere. Melting points were measured on a capillary melting point apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on a Varian 300 MHz spectrometer. Residual solvent peaks were used as an internal reference in the NMR spectra except for experiments run in D_2O where 1,4-dioxane was used. Optical rotations were measured on a Perkin-Elmer 241 polarimeter.

HPLC analysis

Analytical HPLC for determination of ee was performed using a Sumichiral OA-5000 column (4.6 × 150 mm) equipped with a Sumichiral guard column (4 × 10 mm) (Sumika Chemical Analysis Service, Japan). The column was kept at 60 °C and eluted at 1.0 mL/min with a mobile phase consisting of 85–95% of an aqueous solution [10 or 50 mM NH_4OAc and 0.1 mM $Cu(OAc)_2$, pH adjusted to 4.7 using AcOH], and 15–5% 2-PrOH. The column was connected to a TSP HPLC system (Bie & Berntsen A/S, Copenhagen, Denmark) consisting of a P2000 pump, an AS3000 autoinjector and an SM5000 PDA detector.

Preparation of Grignard reagents

All the Grignard reagents were prepared in situ and reacted immediately with the appropriate electrophile as described in the general procedure below. 2-, 3-, and 4-pyridylmagnesium bromides were prepared from bromopyridines in THF as described by Trecourt et al.[19] 3-Thienylmagnesium bromide was prepared from 3-bromothiophene in THF as described by Gronowitz and Pettersson.[20] 2-Thienylmagnesium bromide was prepared by reacting 2-bromothiophene with magnesium in THF. 2-Thiazolylmagnesium bromide was prepared in Et_2O from 2-bromothiazole as described by Dondoni et al.[30]

4-Thiazolylmagnesium bromide. To *tert*-BuLi (10 mL, 1.7 M in hexane; 17 mmol) in dry Et_2O (25 mL) at −78 °C was added, dropwise over 30 min, a solution of 4-bromo-2-trimethylsilanyl-thiazole[18] (2.01 g, 8.5 mmol) in Et_2O (20 mL). The mixture was stirred for additional 30 min and then $MgBr_2$ (3.4 mL, 2.5 M in Et_2O; 8.5 mmol) was added dropwise and the mixture stirred for 30 min.

5-Thiazolylmagnesium bromide. To *n*-BuLi (17.7 mL, 1.6 M in hexane; 28.4 mmol) in dry Et_2O (200 mL) at −78 °C was added, dropwise over 30 min, a solution of 2-trimethylsilanyl-thiazole[18] (4.38 g, 27.9 mmol) in Et_2O (20 mL). The mixture was stirred for additional 15 min and then $MgBr_2$ (11.4 mL, 2.5 M in Et_2O; 28.4 mmol) was added dropwise and the mixture stirred for 30 min.

General procedure for the preparation of *tert*-butyl (*S*)-*E*-1-(*tert*-butoxycarbonyl)-4-(heteroarylmethylene)pyroglutamates (6a–h). To a solution of the appropriate Grignard reagent (18.6 mmol in 150 mL dry Et_2O for **6a–c**, and in 50 mL dry THF for **6d–h**) at −78 °C was added dropwise a solution of *tert*-butyl (*S*)-(*tert*-butoxycarbonyl)-4-(dimethylamino-methylene)pyroglutamate[15] **5** (3.15 g, 9.3 mmol) in dry THF (25 mL) and the

mixture stirred for one h at −78 °C. The mixture was allowed to reach rt slowly and stirred for additional 4 h. The mixture was quenched with saturated NH_4Cl and the aqueous layer extracted with EtOAc (3 × 150 mL). The combined organic layers were washed with brine, dried with Na_2SO_4, and evaporated under vacuum to give the crude products **6a–h**.

***tert*-Butyl (*S*)-*E*-1-(*tert*-butoxycarbonyl)-4-(thiazol-2-yl-methylene)pyroglutamate (6a).** Prepared according to the general procedure. The crude product was purified by flash chromatography (EtOAc/heptane, 1:2) and recrystallized (EtOAc/heptane) to yield **6a** (1.1 g, 24%) as colorless crystals. $[\alpha]_D^{25}$ +36.3° (*c* 1.0, $CHCl_3$). Mp 144–145 °C. Elemental analysis ($C_{18}H_{24}N_2O_5S$): calcd 56.82% C, 6.36% H, 7.36% N, found 56.81% C, 6.44% H, 7.35% N. ^{1}H NMR ($CDCl_3$) δ 7.99 (1H, d, *J* = 3.2 Hz), 7.64 (1H, t, *J* = 3.0 Hz), 7.51 (1H, d, *J* = 3.2 Hz), 4.61 (1H, dd, *J* = 10.0, 3.2 Hz), 3.42 (1H, ddd, *J* = 19.9, 10.0, 3.2 Hz), 3.14 (1H, dt, 19.9, 3.0 Hz), 1.54 (9H, s), 1.46 (9H, s). ^{13}C NMR ($CDCl_3$) δ 170.1, 166.4, 162.9, 149.7, 145.1, 132.3, 124.5, 121.8, 83.7, 82.4, 56.9, 28.8, 27.8, 27.7.

***tert*-Butyl (*S*)-*E*-1-(*tert*-butoxycarbonyl)-4-(thiazol-4-yl-methylene)pyroglutamate (6b).** Prepared according to the general procedure. The crude product was dissolved in 30 mL THF and treated with TBAF (1.5 equivalents) and stirred for 30 min. The solution was diluted with 100 mL EtOAc and washed with 30 mL H_2O and then 30 mL brine. The organic phase was dried with Na_2SO_4 and evaporated under vacuum. The crude product was purified by flash chromatography (EtOAc/heptane, 1:4) and recrystallized (EtOAc/heptane) to yield **6b** (600 mg, 37%) as colorless crystals. $[\alpha]_D^{25}$ +33.8° (*c* 1.0, $CHCl_3$). Mp 160–161 °C. Elemental analysis ($C_{18}H_{24}N_2O_5S$): calcd 56.82% C, 6.36% H, 7.36% N, found 56.82% C, 6.39% H, 7.17% N. ^{1}H NMR ($CDCl_3$) δ 8.85 (1H, s), 7.54 (1H, s), 7.49 (1H, d, *J* = 3.2 Hz), 4.55 (1H, dd, *J* = 10.0, 3.2 Hz), 3.45 (1H, ddd, *J* = 19.5, 10.0, 3.2 Hz), 3.21 (1H, dt, 19.5, 3.2 Hz), 1.52 (9H, s), 1.44 (9H, s). ^{13}C NMR ($CDCl_3$) δ 171.1, 166.1, 149.7, 154.0, 152.3, 134.6, 121.4, 121.7, 83.5, 82.6, 56.9, 28.6, 28.2, 27.9.

***tert*-Butyl (*S*)-*E*-1-(*tert*-butoxycarbonyl)-4-(thiazol-5-yl-methylene)pyroglutamate (6c).** Prepared according to the general procedure. The crude product was purified by flash chromatography (EtOAc/heptane, 4:1) and recrystallized (EtOAc/heptane) to yield **6c** (2.2 g, 62%) as colorless crystals: $[\alpha]_D^{25}$ +26.9° (*c* 1.0, $CHCl_3$). Mp 175–177 °C (decomp). Elemental analysis ($C_{18}H_{24}N_2O_5S$): calcd 56.82% C, 6.36% H, 7.36% N, found 56.73% C, 6.44% H, 7.33% N. ^{1}H NMR ($CDCl_3$) δ 8.93 (1H, s), 8.08 (1H, s), 7.77 (1H, t, *J* = 3.0 Hz), 4.60 (1H, dd, *J* = 10.0, 3.0 Hz), 3.18 (1H, ddd, *J* = 18.0, 10.0, 3.0 Hz), 2.76 (1H, dt, *J* = 18.0, 3.0 Hz), 1.53 (9H, s), 1.46 (9H, s). ^{13}C NMR ($CDCl_3$) δ 169.9, 165.9, 155.6, 149.6, 146.9, 133.6, 128.6, 124.1, 83.9, 82.8, 56.7, 28.2, 28.1, 28.0.

***tert*-Butyl (*S*)-*E*-1-(*tert*-butoxycarbonyl)-4-(pyridin-2-yl-methylene)pyroglutamate (6d).** Prepared according to the general procedure. The crude product was purified by flash chromatography (EtOAc/heptane, 1:1) and recrystallized (EtOAc/heptane) to yield **6d** (0.99 g, 45%) as colorless crystals. $[\alpha]_D^{25}$ +49.6° (*c* 1.0, $CHCl_3$). Mp 130–131 °C. Elemental analysis ($C_{20}H_{26}N_2O_5$): calcd 64.15% C, 7.00% H, 7.48% N, found 64.11% C, 7.05% H, 7.39% N. ^{1}H NMR ($CDCl_3$) δ 8.68 (1H, br d, *J* = 4.7 Hz), 7.70 (1H, dt, *J* = 7.7, 1.8 Hz), 7.45 (1H, t, *J* = 2.9 Hz), 7.4 (1H, br d, *J* = 7.7 Hz), 7.19 (1H, ddd, ?*J* = 7.7, 4.8, 1.0 Hz), 4.57 (1H, dd, *J* = 9.9, 3.4 Hz), 3.55 (1H, ddd, *J* = 20.0, 9.9, 3.2 Hz), 3.32 (1H, dt, *J* = 20.0, 3.1 Hz), 1.54 (9H, s), 1.45 (9H, s). ^{13}C NMR ($CDCl_3$) δ 170.6, 167.5, 154.3, 150.0, 149.9, 136.5, 132.7, 132.1, 127.1, 122.9, 82.4, 82.2, 57.3, 28.9, 27.9, 27.8.

***tert*-Butyl (*S*)-*E*-1-(*tert*-butoxycarbonyl)-4-(pyridin-3-yl-methylene)pyroglutamate (6e).** Prepared according to the general procedure. The crude product was purified by flash chromatography (EtOAc/CH_2Cl_2, 1:1) and recrystallized (EtOAc/heptane) to yield **6e** (1.89 g, 86%) as colorless crystals. $[\alpha]_D^{25}$ +13.4° (*c* 1.0, $CHCl_3$). Mp 182–184 °C. Elemental analysis ($C_{20}H_{26}N_2O_5$): calcd 64.15% C, 7.00% H, 7.48% N, found 63.92% C, 7.01% H, 7.37% N. ^{1}H NMR ($CDCl_3$) δ 8.75 (1H, d, *J* = 2.1 Hz), 8.61 (1H, dd, *J* = 4.9, 1.6 Hz), 7.76 (1H, dt, *J* = 8.1, 1.6 Hz), 7.53 (1H, t, *J* = 2.8 Hz), 7.39 (1H, dd, *J* = 4.9, 8.1 Hz), 4.62 (1H, dd, *J* = 10.0, 3.2 Hz), 3.35 (1H, ddd, *J* = 18.2, 10.0, 3.2 Hz), 2.91 (1H, dt, *J* = 18.2, 3.2 Hz), 1.55 (9H, s), 1.47 (9H, s). ^{13}C NMR ($CDCl_3$) δ 170.0, 166.5, 150.9, 150.1, 149.8, 136.7, 131.3, 130.7, 129.9, 123.8, 83.7, 82.6, 56.5, 27.9, 27.8, 27.8.

***tert*-Butyl (*S*)-*E*-1-(*tert*-butoxycarbonyl)-4-(pyridin-4-yl-methylene)pyroglutamate (6f).** Prepared according to the general procedure. The crude product was purified by flash chromatography (EtOAc/CH_2Cl_2, 3:2) and recrystallized (EtOAc/heptane) to yield **6f** (1.68 g, 81%) as colorless crystals. $[\alpha]_D^{25}$ +8.4° (*c* 1.0, $CHCl_3$). Mp 175–176 °C. Elemental analysis ($C_{20}H_{26}N_2O_5$): calcd 64.15% C, 7.00% H, 7.48% N, found 64.01% C, 7.01% H, 7.40% N. ^{1}H NMR ($CDCl_3$) δ 8.68 (2H, br d, *J* = 6.0 Hz), 7.44 (1H, t, *J* = 2.8 Hz), 7.30 (2H, br d, *J* = 6.0 Hz), 4.61 (1H, dd, *J* = 10.0, 3.2 Hz), 3.34 (1H, ddd, 18.2, 10.0, 3.2 Hz), 1.54 (9H, s), 1.45 (9H, s). ^{13}C NMR ($CDCl_3$) δ 169.9, 166.2, 150.5, 149.7, 141.7, 132.7, 131.9, 123.6, 83.9, 82.7, 56.5, 27.9, 27.8, 27.7.

***tert*-Butyl (*S*)-*E*-1-(*tert*-butoxycarbonyl)-4-(thien-2-ylmethylene)pyroglutamate (6g).** Prepared according to the general procedure. The crude product was purified by flash chromatography (heptane/CH_2Cl_2/EtOAc, 6:2:1) and recrystallized (EtOAc/heptane) to yield **6g** (1.96 g, 78%) as colorless crystals. $[\alpha]_D^{25}$ +26.3° (*c* 1.0, $CHCl_3$). Mp 158–159 °C. Elemental analysis ($C_{19}H_{25}NO_5S$): calcd 60.14% C, 6.64% H, 3.69% N, found 60.07% C, 6.65% H, 3.61% N. ^{1}H NMR ($CDCl_3$) δ 7.76 (1H, t, *J* = 3.0 Hz), 7.56 (1H, d, *J* = 5.2 Hz), 7.32 (1H, D, *J* = 3.6 Hz), 7.15 (1H, dd, *J* = 5.2, 3.6 Hz), 4.61 (1H, dd, *J* = 10.1, 3.2 Hz), 3.22 (1H, ddd, *J* = 18.2, 10.1, 3.2 Hz), 2.83 (1H, dt, *J* = 18.2, 3.2 Hz), 1.54 (9H, s), 1.47 (9H, s). ^{13}C NMR ($CDCl_3$) δ 170.2, 166.9, 149.9, 138.9, 132.2, 130.0, 128.1, 127.6, 124.9, 83.3, 82.3, 56.5, 27.8, 27.7, 27.6.

***tert*-Butyl (*S*)-*E*-1-(*tert*-butoxycarbonyl)-4-(thien-3-ylmethylene)pyroglutamate (6h).** Prepared according to the general procedure. The crude product was purified by flash chromatography (heptane/CH_2Cl_2/EtOAc, 6:2:1) and recrystallized (EtOAc/heptane) to yield **6h** (1.53 g, 81%) as colorless crystals. $[\alpha]_D^{25}$ +8.0° (*c* 1.0, $CHCl_3$). Mp 176–177 °C. Elemental analysis ($C_{19}H_{25}NO_5S$): calcd 60.14% C, 6.64% H, 3.69% N, found 60.17% C, 6.65% H, 3.60% N. ^{1}H NMR ($CDCl_3$) δ 7.56 (1H, t, *J* = 2.7 Hz), 7.51 (1H, br d, *J* = 3.3 Hz), 7.39 (1H, dd, *J* = 5.1, 2.7 Hz), 7.24 (1H, dd, *J* = 5.1, 1.0 Hz), 4.59 (1H, dd, *J* = 10.2, 3.2 Hz), 3.26 (1H, ddd, *J* = 18.0, 10.2, 3.2 Hz), 2.84 (1H, dt, *J* = 18.0, 3.1 Hz), 1.54 (9H, s), 1.46 (9H, s). ^{13}C NMR ($CDCl_3$) δ 170.3, 167.2, 149.9, 136.9, 128.7, 128.5, 128.0, 126.7, 125.9, 83.3, 82.3, 56.5, 27.8, 27.7.

General procedure for the preparation of (*S*)-*E*-4-(heteroarylmethylene)glutamic acids (4a–h). To the appropriate *tert*-butyl (*S*)-*E*-1-(*tert*-butoxycarbonyl)-4-(heteroarylmethylene)pyroglutamate **6a–h** (2.63 mmol), dissolved in 25 mL THF, was added LiOH (18.4 mL, 2.5 M; 46.0 mmol) and the solution was stirred at rt for 3 h before the pH was set to 3.5 with 1 M HCl. The solution was extracted with EtOAc (3 × 50 mL) and the organic phases dried with Na_2SO_4 and evaporated under reduced pressure. The crude product was purified by flash chromatography (CH_2Cl_2/MeOH/AcOH, 100:5:2) to yield the *N*-Boc monoester. This ester was dissolved in AcOH (40 mL) and HBr (7 mL, 33% in AcOH) was added dropwise. The mixture was stirred for 1 h at rt and then evaporated under reduced pressure to yield the crude product.

(*S*)-*E*-4-(Thiazol-2-ylmethylene)glutamic acid (4a). Prepared according to the general procedure. The crude product was purified by recrystallization (EtOH/Et_2O) to yield the dihydrobromide salt of **4a** (510 mg, 78%). A portion of the salt (202 mg, 0.5 mmol) was dissolved (2-PrOH/H_2O, 2:1) and treated with propylene oxide (600 mg, 10 mmol) at rt for 1 h. This gave zwitterionic **4a** (78 mg, 64%) as colorless crystals. $[\alpha]_D^{25}$ +22.2° (*c* 0.6, 0.1 M HCl). Mp 183–186 °C (decomp). Elemental analysis ($C_9H_{10}N_2O_4S{\cdot}1/3H_2O$): calcd 43.54% C, 4.33% H, 11.28% N, found 43.49% C, 4.34% H, 11.16% N. ^{1}H NMR (D_2O) δ 8.06 (1H, d, *J* = 3.3 Hz), 7.69 (1H, s), 7.89 (1H, d, *J* = 3.3 Hz), 4.39 (1H, t, *J* = 7.1 Hz), 3.54 (1H, dd, *J* = 14.0, 6.9 Hz), 3.46 (1H, dd, *J* = 14.0, 7.4 Hz). ^{13}C NMR (D_2O) δ 171.3, 169.7, 162.4, 143.2, 132.2, 129.5, 124.4, 51.7, 28.3.

(*S*)-*E*-4-(Thiazol-4-ylmethylene)glutamic acid (4b). Prepared according to the general procedure. The crude product was purified by recrystallization (EtOH/Et_2O) to yield **4b** (338 mg, 81%) as the dihydrobromide. The salt was dissolved (2-PrOH/H_2O, 2:1) and treated with propylene oxide (600 mg, 10 mmol) at rt for 1 h. This gave zwitterionic **4b** (130 mg, 68%) as colorless crystals. $[\alpha]_D^{25}$ +16.6° (*c* 0.5, 0.1 M HCl). Mp 179–180 °C. Elemental analysis ($C_9H_{10}N_2O_4S$): calcd 44.62% C, 11.56% H, 6.93% N, found 44.43% C, 11.47% H, 6.82% N. ^{1}H NMR (D_2O) δ 9.14 (1H, d, *J* = 2.0 Hz), 7.96 (2H, br m), 4.35 (1H, t, *J* = 7.0 Hz), 3.53 (1H, dd, *J* = 14.0, 6.7 Hz), 3.47 (1H, dd, *J* = 14.0, 7.4 Hz). ^{13}C NMR (D_2O) δ 171.3, 170.4, 155.6, 149.5, 134.7, 125.6, 125.2, 52.2, 27.9.

(*S*)-*E*-4-(Thiazol-5-ylmethylene)glutamic acid (4c) dihydrobromide. Prepared according to the general procedure. The crude product was purified by recrystallization (EtOH/Et_2O) to yield **4c** dihydrobromide (730 mg, 86%) as colorless crystals. $[\alpha]_D^{25}$ −20.1° (*c* 0.5, 0.1 M HCl). Mp 207–208 °C (decomp). Elemental analysis ($C_9H_{12}Br_2N_2O_4S$): calcd 26.75% C, 2.99% H, 6.93% N, found 26.80% C, 3.13% H, 6.93% N. ^{1}H NMR (D_2O) δ 9.57 (1H, s), 8.35 (1H, s), 8.10 (1H, s), 4.31 (1H, t, *J* = 7.4 Hz), 3.32 (1H, dd, *J* = 14.8, 7.4 Hz), 3.23 (1H, dd, *J* = 14.8, 7.4 Hz). ^{13}C NMR (D_2O) δ 171.6, 170.5, 159.3, 143.2, 134.0, 132.1, 128.5, 52.0, 29.2.

(*S*)-*E*-4-(Pyridin-2-ylmethylene)glutamic acid (4d) dihydrobromide. Prepared according to the general procedure. The crude product was purified by recrystallization (EtOH/Et_2O) to yield **4d** dihydrobromide (380 mg, 76%) as colorless crystals. $[\alpha]_D^{25}$ +4.5° (*c* 0.4, 0.1 M HCl). Mp 213–215 °C (decomp). Elemental analysis ($C_{11}H_{14}Br_2N_2O_4{\cdot}0.5H_2O$): calcd 32.46% C, 3.71% H, 6.88% N, found 32.37% C, 3.80% H, 6.86% N. ^{1}H NMR (D_2O) δ 8.84 (1H, dd, *J* = 6.0, 1.6 Hz), 8.62 (1H, dt, *J* = 8.0, 1.6 Hz), 8.10 (1H, br d, *J* = 7.8 Hz), 8.04 (1H, ddd, *J* = 8.0, 6.0, 1.6 Hz), 7.96 (1H, s), 4.29 (1H, t, *J* = 6.8 Hz), 3.16 (2H, d, *J* = 6.8 Hz). ^{13}C NMR (D_2O) δ 171.5, 168.8, 147.0, 146.8, 142.3, 136.8, 132.4, 127.8, 126.7, 51.7, 28.0.

(*S*)-*E*-4-(Pyridin-3-ylmethylene)glutamic acid (4e) dihydrobromide. Prepared according to the general procedure. The crude product was purified by recrystallization (EtOH/Et_2O) to yield **4e** (620 mg, 90%) as colorless crystals. $[\alpha]_D^{25}$ −8.8° (*c* 0.9, 0.1 M HCl). Mp 210 °C (decomp). Elemental analysis ($C_{11}H_{14}Br_2N_2O_4{\cdot}0.5H_2O$): calcd 32.46% C, 3.71% H, 6.88% N, found 32.22% C, 4.04% H, 6.68% N. ^{1}H NMR (D_2O) δ 8.86 (1H, m), 8.80 (1H, dd, *J* = 5.8, 1.0 Hz), 8.61 (1H, dd, *J* = 8.2, 1.0 Hz), 8.15 (1H, dd, *J* = 8.2, 5.8 Hz), 7.95 (1H, s), 4.10 (1H, t, *J* = 7.0 Hz), 3.04 (2H, br d, *J* = 7.1 Hz). ^{13}C NMR (D_2O) δ 172.0, 169.7, 146.7, 140.8, 140.7, 135.9, 134.7, 133.4, 127.4, 52.4, 27.8.

(*S*)-*E*-4-(Pyridin-4-ylmethylene)glutamic acid (4f). Prepared according to the general procedure. The crude product was purified by recrystallization (EtOH/Et_2O) to yield **4f** (540 mg, 55%) as the dihydrobromide. A portion of the salt (188 mg, 0.47 mmol) was dissolved (2-PrOH/H_2O, 2:1) and treated with propylene oxide (600 mg, 10 mmol) at rt for 1 h. This gave zwitterionic **4f** (90 mg, 77%) as colorless crystals. $[\alpha]_D^{25}$ −20.1° (*c* 0.9, 0.1 M HCl). Mp 204–207 °C (decomp). Elemental analysis ($C_{11}H_{12}N_2O_4$): calcd 55.93% C, 5.12% H, 11.86% N, found 55.57% C, 5.21% H, 11.69% N. ^{1}H NMR (D_2O) δ 8.77 (2H, m), 7.97 (3H, m), 4.16 (1H, t, *J* = 7.2 Hz), 3.02 (2H, d, *J* = 7.2 Hz) ^{13}C NMR (D_2O) δ 171.1, 169.1, 153.5, 141.3, 138.1, 133.7, 126.9, 51.6, 27.7.

(*S*)-*E*-4-(Thien-2-ylmethylene)glutamic acid (4g). Prepared according to the general procedure. The crude product was triturated with Et_2O and recrystallized (H_2O) to yield zwitterionic **4g** (260 mg, 57%) as colorless crystals. $[\alpha]_D^{25}$ −31.6° (*c* 0.5, 0.1 M HCl). Mp 205–206 °C (decomp). Elemental analysis ($C_{10}H_{11}NO_4S$): calcd 49.78% C, 4.60% H, 5.81% N, found 50.08% C, 4.61% H, 5.58% N. ^{1}H NMR (D_2O) δ 8.11 (1H, s), 7.75 (1H, dt, J = 5.2, 1.0 Hz), 7.50 (1H, d, J = 3.5 Hz), 7.22 (1H, dd, J = 5.2, 3.5 Hz), 4.28 (1H, d, J = 7.7 Hz), 3.25 (1H, d, J = 8.0 Hz), 3.19 (1H, dd, J = 7.8 Hz). ^{13}C NMR (DMSO-d_6) δ 174.1, 170.0, 139.2, 131.0, 129.5, 128.2, 128.1, 122.0, 52.2, 30.1.

(*S*)-*E*-4-(Thien-3-ylmethylene)glutamic acid (4h). Prepared according to the general procedure. The crude product was triturated with Et_2O and recrystallized (H_2O) to yield zwitterionic **4h** (260 mg, 64%) as colorless crystals. $[\alpha]_D^{25}$ −51.8° (*c* 0.5, 0.1 M HCl). Mp 220–222 °C (decomp). Elemental analysis ($C_{10}H_{11}NO_4S$): calcd 49.78% C, 4.60% H, 5.81% N, found 49.69% C, 4.58% H, 5.62% N. ^{1}H NMR (D_2O) δ 7.80 (1H, br s), 7.64 (1H, br s), 7.40 (1H, m), 7.21 (1H, m), 4.07 (1H, t, J = 7.1 Hz), 3.10 (1H, dd, J = 14.8, 7.5 Hz), 3.03 (1H, dd, J = 14.8, 7.5 Hz). ^{13}C NMR (DMSO-d_6) δ 174.6, 170.9, 137.6, 129.3, 128.7, 127.8, 127.4, 123.2, 52.5, 30.4.

Pharmacology

Native receptor binding assays. Affinities for the native NMDA, AMPA, and KA receptors were determined using 2 nM [^{3}H]CGP39653,[31] 5 nM [^{3}H]AMPA,[32] and 5 nM [^{3}H]KA,[33] respectively, with modifications previously described.[12] Rat brain membrane preparations used in the receptor binding experiments were prepared according to the method described by Ransom and Stec.[34]

Recombinant receptor binding assays. Sf9 cells were infected with recombinant GluR1–4 baculovirus and membranes prepared and used for binding. The affinities of compounds at GluR1–4 were determined from competition experiments with [^{3}H]AMPA as previously described.[35–37] HEK293 cells with stable expression of GluR5(*Q*) or GluR6(*V,C,R*) were prepared and used as previously described.[13] Binding to GluR5 receptors was performed using 3 nM [^{3}H]ATPA (previously described as a GluR5 radioligand[38]). GluR6 receptors were labelled with [^{3}H]KA (5 nM).

Cortical slice electrophysiology. A rat cortical slice preparation[39] in a modified version[40] was used for determination of depolarizing effects of the analogues under study at native receptors. Agonists were applied for 90 s. Receptor selectivity was determined by blockade of the responses obtained at agonist concentrations corresponding to their EC_{50} values. AMPA receptor responses could be blocked with 5 μM NBQX, KA receptor responses with 20 μM NBQX, and NMDA receptor responses with 5 μM CPP. Antagonists were pre-applied for 90 s followed by a co-application of agonist and antagonist.

Cell culture and patch clamp electrophysiology

HEK293 cells stably transfected with GluR5 were maintained according to standard procedures.[42] All electrophysiological measurements were obtained in voltage-clamp using conventional whole-cell patch clamp techniques as described in Nielsen et al.[41] The cells were clamped at −60 mV and the measurements were performed at room temperature (20–22 °C). The internal solution was composed of (in mM): 120 KCl, 31 KOH, 10 EGTA, 10 HEPES and 1.8 $MgCl_2$ (pH adjusted to 7.2 with KOH). The external bath solution contained (in mM): 140 NaCl, 10 HEPES, 10 $CaCl_2$, 5 KCl and 1 $MgCl_2$ (pH adjusted to 7.4 with NaOH). Rapid exchange of control and test solutions was obtained using double-barreled application tubes mounted at a piezoelectric device (PZS-100HS, Burleigh Instruments) connected to a piezo-driver (PZ-150M, Burleigh Instruments) and driven by TTL pulses from the amplifier. Agonistic effect was calculated as test compound induced current amplitude divided with the maximal inducible current amplitude (induced by 10 mM Glu). EC_{50} values were determined from data points fitted to the equation $y = 100/(1 + D/IC_{50})$, where D is the concentration of the test compound. The antagonistic desensitizing effect was evaluated by preincubating the cells with test compound for 1 min before Glu application (3 mM) and measuring the Glu response. IC_{50} values were determined from data points fitted to the equation $y = 100/(1 + IC_{50}/D)$.

Metabotropic receptor assays. The mGluR subtypes mGluR1a, mGluR2, and mGluR4a were expressed in Chinese hamster ovary cell lines, which were maintained as previously described.[43] Pharmacological assays were performed by measurement of intracellular inositol phosphates by ion-exchange chromotography (mGluR1a) or cAMP using a scintillation proximity assay (SPA) (mGluR2/4a) as previously described. All compounds were tested for agonist and antagonist activity up to 1 mM concentrations.[43]

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