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A stereochemical anomaly: the cyclised (*R*)-AMPA analogue (*R*)-3-hydroxy-4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridine-5-carboxylic acid [(*R*)-5-HPCA] resembles (*S*)-AMPA at glutamate receptors

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(*RS*)-3-Hydroxy-4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridine-5-carboxylic acid (5-HPCA) (**1**), which is a conformationally constrained cyclised analogue of AMPA has previously been described as causing glutamate receptor mediated excitations of spontaneously firing cat spinal interneurons in a similar fashion to AMPA. We have now prepared the enantiomers of **1** through chiral chromatographic resolution of (*RS*)-3-(carboxymethoxy)-4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridine-5-carboxylic acid (**2**) followed by a stereoconservative hydrolysis resulting in the enantiomers of **1** with high enantiomeric excess (% ee ≥ 99). The absolute configurations indicated by an X-ray analysis of (−)-**1** monohydrate were confirmed by comparing observed and *ab initio* calculated electronic circular dichroism spectra and by stereoconservative synthesis of (*S*)-**1** from (*S*)-AMPA, the pharmacologically active form of AMPA. The pharmacological effects at native and cloned (GluR1-4) AMPA receptors were shown to reside exclusively with (*R*)-(+)-**1**, in striking contrast to the usual stereoselectivity trend among AMPA receptor agonists. The reasons for this anomalous behaviour became clear upon docking both enantiomers of **1** to the agonist binding site of GluR2.

Introduction

(*S*)-Glutamic acid [(*S*)-Glu] (Fig. 1) is the predominant excitatory neurotransmitter in the central nervous system (CNS). (*S*)-Glu operates *via* activation of two major classes of receptor systems: ligand-gated ionotropic receptors (iGluRs), which mediate fast synaptic transmission, and metabotropic receptors (mGluRs), which are G-protein-coupled to second-messenger systems.[1–3] It is generally agreed that GluRs are vital to healthy CNS function and potential targets for therapeutic intervention in a number of CNS diseases.[1,2,4,5] The iGluRs are subdivided into three groups according to selective agonists, *N*-methyl-D-aspartic acid (NMDA), (*RS*)-2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid (AMPA), and kainic acid (KA) receptors. Functional iGluR ion channels assemble as tetramers from homo- or heteromeric combinations of various subunits.[1,6,7] NMDA receptors are constructed from subunits NR1, NR2A-D and NR3A,B, AMPA receptors from subunits GluR1-4, and KA receptors from subunits GluR5-7 and KA1,2.[1,8] In recent years, soluble constructs of the S1S2 ligand-binding domain[9] have provided detailed insights into the three-dimensional structure of the extracellular portion of AMPA receptors.[10,11] In addition to information on specific determinants for receptor–ligand interactions, successful co-crystallisation of the GluR2–S1S2 construct with a number of AMPA receptor ligands has also provided information on the processes of receptor activation, modulation and inhibition.[10–14]

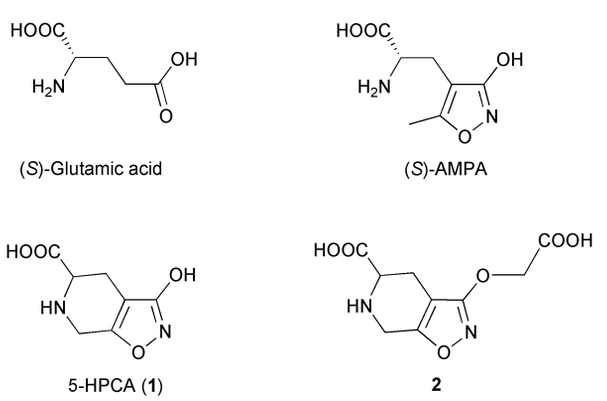

Fig. 1 Chemical structures of (*S*)-Glu, (*S*)-AMPA, 5-HPCA (**1**) and **2**.

With AMPA as a lead structure, a series of AMPA analogues has been synthesised and pharmacologically characterised.[1] At the early stage of these structure–activity studies, we synthesised the conformationally restricted AMPA analogue, (*RS*)-3-hydroxy-4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridine-5-carboxylic acid (5-HPCA) (**1**) and showed it to cause glutamate receptor mediated excitations of spontaneously firing cat spinal interneurons in a similar fashion to AMPA.[15]

By analogy with the stereoselective excitatory effects of (*S*)-Glu, one would expect AMPA receptors to be stereoselectively activated by analogues containing an *S*-α-amino acid moiety. In general, where AMPA analogues have been resolved, the agonistic effect can indeed be entirely attributed the *S*-enantiomer,[16–19] although in isolated cases the *R*-enantiomers also show some activity.[20,21]

However, conformational restriction and incorporation of the amino group into a ring structure set **1** apart from the majority of well-characterised AMPA receptor agonists, and we therefore sought to resolve and pharmacologically characterise its enantiomers using both native and cloned iGluRs. This study presents the complementary approaches by which we have definitively characterised (*R*)- and (*S*)-**1**: chiral

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Table 1 Receptor binding affinities at iGluRs in the rat cortical membranes[a]

	[^{3}H]AMPA IC_{50} (μM)	[^{3}H]KA IC_{50} (μM)	[^{3}H]CGP 39653 K_i (μM)
(*S*)-Glu	0.34[b]	0.38[c]	0.20 [0.16;0.26]
(*S*)-(+)-AMPA	0.021 [0.019;0.022]	24[b] [22;27]	>100
(*R*)-(−)-AMPA	76[d]	>100	>100
(*R*)-(+)-**1**	0.47 [0.43;0.51]	>100	>100[e]
(*S*)-(−)-**1**	>100	>100	>100[e]

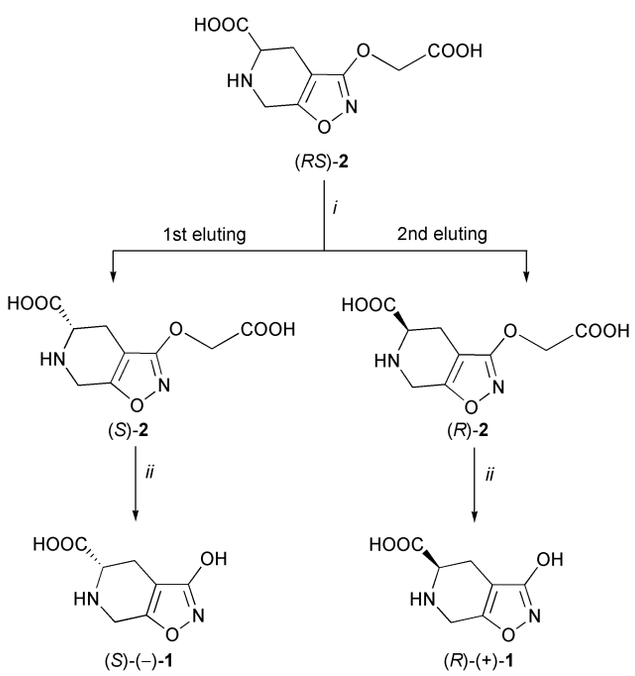
[a] Values are expressed as the antilog to the log mean of at least three individual experiments. The numbers in parentheses [min;max] indicate ± SEM according to a logarithmic distribution of IC_{50} or K_i. [b] Johansen *et al.*[21] [c] B_{max1} was 20% [d] Nielsen *et al.*[55] [e] [^{3}H]CPP.

chromatography and stereoconservative synthesis; crystallography and *ab initio* ECD spectral interpretation; and *in vitro* and *in silico* characterisation.

Results

Resolution

As a result of an initial evaluation of different approaches for preparing both enantiomers of **1**, a strategy was chosen based on chromatographic resolution of (*RS*)-3-(carboxymethoxy)-4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridine-5-carboxylate (**2**), a precursor of **1** (Scheme 1). Compound **2** was synthesised as previously described[22] and resolved using a Sumichiral OA-5000 chiral ligand-exchange column, which contains *N*,*S*-dioctyl-D-penicillamine as the chiral selector.[23] After removal of the mobile phase, both enantiomers of **2** were obtained in good yields (≥94%) and with enantiomeric excesses (ee) ≥99%, as determined on an analytical D-proline column.[24] The (+)- and (−)-enantiomers of **1** were obtained with 99.0% ee and 99.6% ee, respectively, by deprotection of the two enantiomers of **2** using 48% aqueous hydrobromic acid (Scheme 1). The conversion progressed without any significant racemisation.



Scheme 1 Chiral HPLC separation of compound **1**. (i) Sumichiral OA-5000; (ii) 48% aqueous HBr, reflux.

In vitro pharmacology

The affinities of the enantiomers of **1** and AMPA for the three classes of native iGluRs were tested using the radioligands [^{3}H]-(*RS*)-(*E*)-2-amino-4-phosphonomethyl-3-heptenoic acid ([^{3}H]CGP 39653)[25] or [^{3}H]-(*RS*)-3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid ([^{3}H]CPP),[26] [^{3}H]AMPA[27] and [^{3}H]KA[28] (Table 1). The (+)-enantiomer was found to be the eutomer and showed twenty times weaker affinity for [^{3}H]AMPA binding sites as compared to (*S*)-AMPA, and no detectable affinity for NMDA or KA receptors. The (−)-enantiomer of **1** was inactive in all three binding assays. (+)-**1** showed affinity for cloned homomeric AMPA receptors (GluR1-4) but not for cloned homomeric KA-receptors (GluR5 and GluR6) (Table 2). (−)-**1** did not show any detectable affinity for these cloned AMPA and KA receptors (Table 2). In addition, as with (*S*)-AMPA, (+)-**1** did not distinguish significantly between the individual AMPA receptor subtypes.

X-ray analysis

In order to obtain crystals with sufficient anomalous dispersion for determining the absolute configuration, several attempts were made to crystallise (−)-**1** as a hydrobromide salt, but only crystals containing the zwitterion (−)-**1**·H_2O were obtained (Fig. 2). The Flack absolute structure parameter[29,30] for the *S*-configuration was calculated to be $x = 0.02(14)$. The results strongly suggest that (−)-**1** possesses the *S*-configuration. However, an unequivocal statement is not possible due to the high standard deviation of the Flack parameter. This stereochemical assignment would place the AMPA receptor agonistic effects solely with the *R*-enantiomer of **1**, without precedent among other AMPA analogues and the natural ligand (*S*)-Glu where the *S*-enantiomer is the eutomer. Total confidence in the correct assignment of the absolute configurations of the enantiomers of **1** is therefore crucial for correct interpretation of the pharmacological data. We therefore turned to alternative methods of determining the absolute stereochemistry.

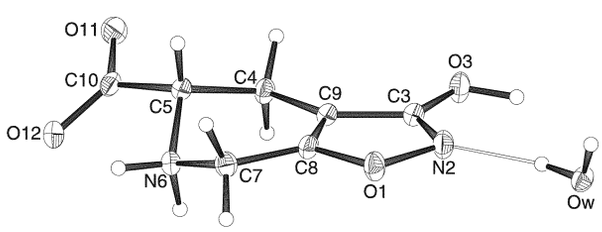


Fig. 2 Perspective drawing[56] of the molecular structure of (*S*)-(−)-**1**·H_2O with the crystallographic atomic labelling. Displacement ellipsoids enclose 50% probability. Hydrogen atoms are represented by spheres of arbitrary size. The isoxazole ··· H_2O hydrogen bond is indicated by a hollow line. The isoxazole ring is planar and the six-membered ring is in a half-chair conformation with the carboxylate group in an equatorial position. The N6–H ··· O12 *intra*molecular interaction stabilises this preferred conformation. The packing of the molecules in the crystal structure is stabilised by hydrogen bonds. All five hydrogen atoms bonded to nitrogen and oxygen atoms are utilised in the formation of hydrogen bonds.

Electronic circular dichroism

Empirical correlation of electronic circular dichroism (ECD) within series of compounds is sometimes relied upon to determine configuration.[31] However, in recent years, the necessary theory and computing power have been developed for calculating the optical properties of chiral molecules directly from the response of an *ab initio* electronic wavefunction, which can

Table 2 Receptor binding affinity at cloned AMPA and KA receptors expressed in Sf9 cells[a]

	(*RS*)-AMPA K_i (μM)	(*R*)-(+)-**1** K_i (μM)	(*S*)-(−)-**1** K_i (μM)
$GluR1_o$	0.0219 ± 0.0044	0.36 ± 0.03	>100
$GluR2_o$	0.0166 ± 0.0029	0.60 ± 0.17	>100
$GluR3_o$	0.0206 ± 0.0026	0.84 ± 0.11	>100
$GluR4_o$	0.04 ± 0.02	0.64 ± 0.02	>100
GluR5	1.15 ± 0.11	10.1 ± 2.1	>100
GluR6	>1000	>100	>100

[a] Mean ± S.D. from at least three experiments.

be computed from the nuclear positions.[32–34] It is therefore now technically feasible to non-empirically determine the absolute configuration of molecules of biological interest, independently of crystallography, by comparing observed and calculated spectra elicited by plane or circularly polarised light (ORD and ECD spectroscopy, respectively). The former is more demanding in terms of the basis set requirements, and therefore more difficult to calculate or less reliable given limited computer resources.[35]

Compound **1** is an ideal case for these methods, because it displays asymmetry in the vicinity of a chromophore, a necessary condition for ECD activity. Moreover, because of conformational restriction, the Boltzmann conformational distribution can be satisfactorily represented by the global energy minimum, which was calculated to be the same in gas phase and solution as observed crystallographically (Fig. 2) and determined by NMR.[36] We have therefore measured and calculated the ECD spectra of the enantiomers of **1**. These spectra of (+)- and (−)-**1** were recorded in aqueous hydrochloric acid (Fig. 3A). The suspected eutomer (*R*)-**1** gave a positive Cotton effect at 219 nm, $\Delta\varepsilon = +0.2$ m² mol^{-1}. Again, this behaviour would be the opposite of that normally seen for AMPA analogues, where the pharmacologically inactive *R*-isomer typically shows a negative Cotton effect of around the same magnitude (0.2 m² mol^{-1}). The predicted spectrum calculated on the cationic form of (*R*)-**1** is shown in Fig. 3B. The magnitude, shape and sign of the calculated and experimental ECD spectra of (*R*)-**1** and (+)-**1** are in excellent agreement, in accordance with the crystallographic indication. However, the calculated spectrum lies approximately 25 nm higher in energy, which is consistent with systematic overestimation of the energy of low-lying electronic transitions in conjugated systems by methods based on the response of the Hartree–Fock wavefunction. *Ab initio* prediction of the optical rotation of (*R*)-**1** gave $[a]_D = +119.3$ (*cf.* $[a]_D^{25} = +91$ measured). While solvation is known to affect $[a]_D$, the unusually strong optical rotation of **1** gives confidence that we have correctly and independently identified (+)-**1** based on the gas phase electronic structure of (*R*)-**1**. Similar calculations on the known chiral compound (*R*)-(+)-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid gave similar agreement with experiment ($[a]_D^{20} = +138$ (exp),[37,38] $[a]_D = +156$ (cal)) confirming the ability of *ab initio* optical spectral interpretation to establish the absolute configuration for these conformationally restricted compounds.

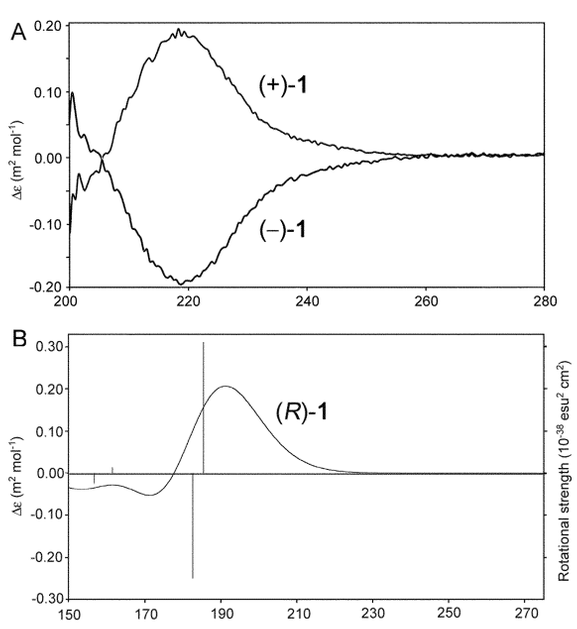
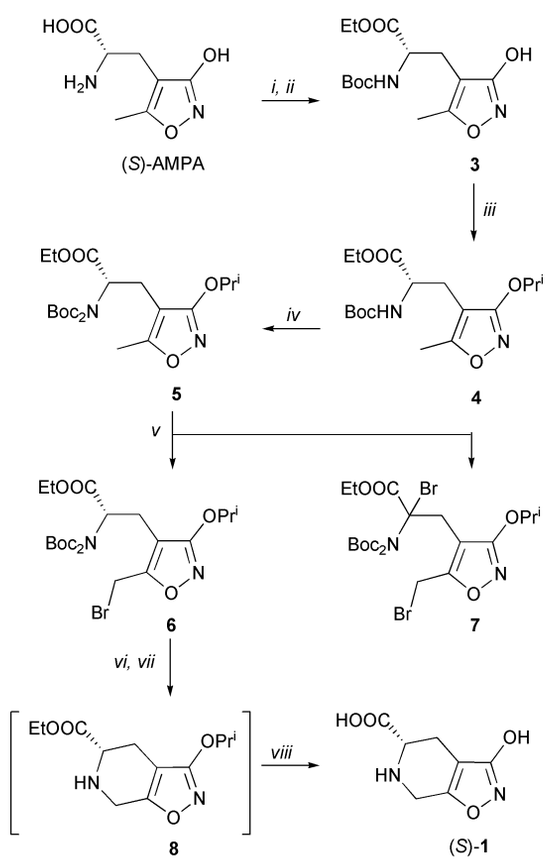


Fig. 3 **A**: Electronic circular dichroism spectra of (+)-**1** and (−)-**1**. **B**: Rotational strengths (10^{-38} esu² cm², vertical bars) and $\Delta\varepsilon$ (m² mol^{-1}, curve) with Gaussian line broadening of 0.25 eV according to HF/cc-pVDZ for cationic (*R*)-**1**.

Stereoconservative synthesis

To finally verify the configurational assignment made above, we undertook a synthesis of (*S*)-**1** starting from (*S*)-AMPA (Scheme 2). (*S*)-AMPA was protected stepwise to give compound **5**. An NBS bromination gave rise to a 6 : 1 mixture of compound **6** and the dibrominated product **7**. Competing bromination takes place at the chiral center, apparently due to



Scheme 2 Stereoconservative synthesis of (*S*)-**1** from (*S*)-AMPA. (i) AcCl, EtOH; (ii) Boc_2O, Et_3N, THF; (iii) $BrCH(CH_3)_2$, K_2CO_3, DMF; (iv) Boc_2O, DMAP, CH_3CN; (v) NBS, CCl_4, dibenzoyl peroxide; (vi) TFA, CH_2Cl_2; (vii) K_2CO_3, DMF; (viii) 6 M HCl/AcOH.

electronic effects elicited by the two Boc groups on the amino group, as mono-Boc protection of this group gave mainly bromination at the methylene group. Selective deprotection of the amino group with trifluoroacetic acid, followed by *intra*-molecular ring closure in the presence of potassium carbonate in *N*,*N*-dimethylformamide gave the protected form of (*S*)-**1** (**8**). The volatility of **8** made it difficult to isolate, and **8** was therefore directly extracted into aqueous hydrochloric acid. Deprotection and subsequent purification on reverse phase HPLC gave (*S*)-**1**, together with dimethylamine hydrochloride arising from hydrolysis of *N*,*N*-dimethylformamide.

We used chiral chromatography performed on a D-proline column to confirm the stereochemical identity of the chromatographically resolved (−)-**1**. By comparing the chromatograms of racemic **1**[15] (Fig. 4A) and synthesised (*S*)-**1** (96.5% ee) (Fig. 4B) we can see that no appreciable racemisation has taken place during the 6-step synthesis from (*S*)-AMPA (97.5% ee). Furthermore, the presence of any residual (*S*)-AMPA would not interfere with the analysis as (*S*)-AMPA elutes after 2.8 min. When the synthesised (*S*)-**1** (Fig. 4B) was mixed with the chromatographically resolved (−)-enantiomer of **1** (Fig. 4C) in a 1 : 1 ratio (Fig. 4D), the two compounds co-eluted. It was therefore concluded that the (−)-enantiomer of **1** has the *S*-configuration.

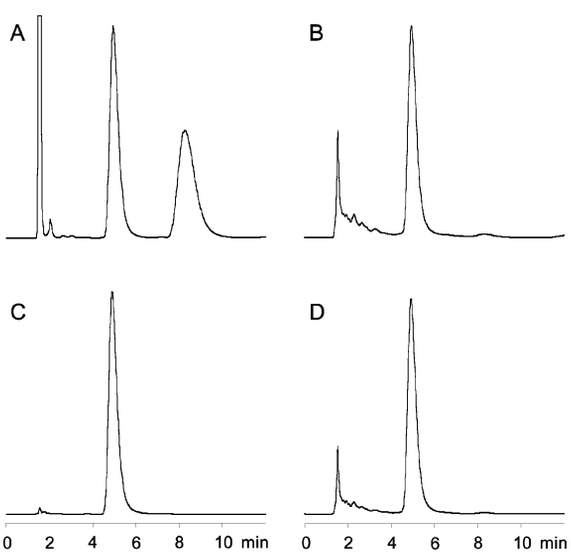


Fig. 4 HPLC traces (UV, 215 nm) showing the comparison of synthesised (*S*)-**1** with resolved (−)-**1**. **A**. compound **1**,[15] **B**. (*S*)-**1** (synthesised), **C**. (−)-**1** (resolved), **D**. (−)-**1** (resolved) and (*S*)-**1** (synthesised) in a ratio of 1 : 1.

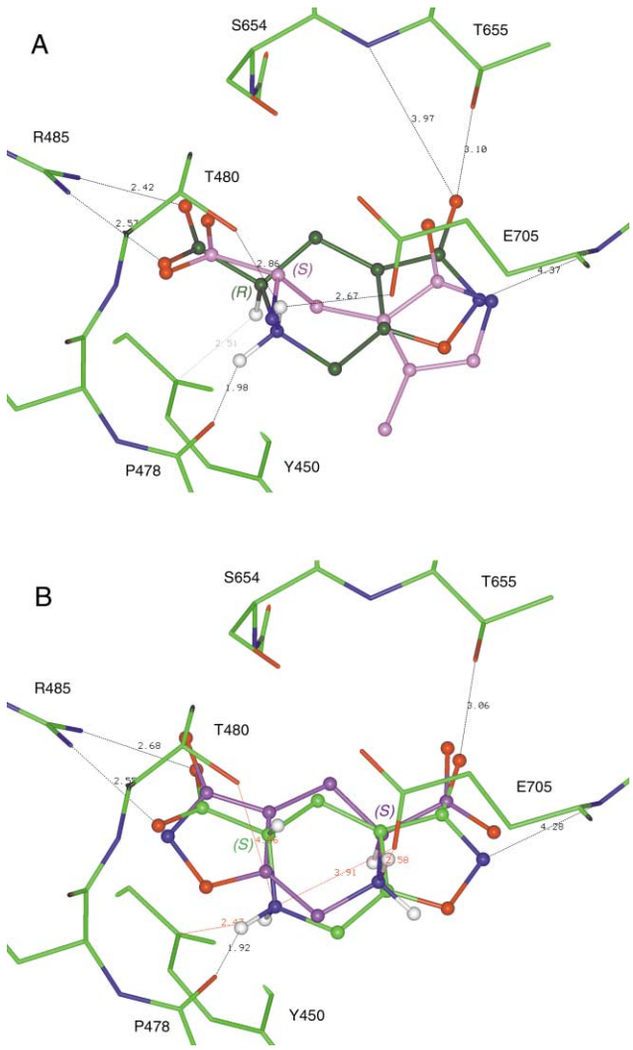


Fig. 5 Docking of tri-ionised (*R*)- and (*S*)-**1** to the GluR2 agonist binding site. Hydrogens are omitted except for those of the α-carbon and ammonium centres. Atoms colour coded by type except for ligand carbon atoms. Important hydrogen bonds shown as dashed black lines and poor contacts by dashed red lines. **A**: lowest energy pose of (*R*)-**1** (dark green carbons). Experimental (*S*)-AMPA position[11] (pink carbons) shown for comparison. Limiting approach of (*R*)-α-proton to Y450 shown in grey. **B**: (*S*)-**1** docked to GluR2. Lowest energy pose (purple carbons) shows an inverted binding mode (isoxazole on the left) while the more (*S*)-AMPA-like pose (light green carbons) is higher in energy. Neither pose of (*S*)-**1** is able to fulfil α-amino acid recognition by hydrogen bond donation to T480 and E705 (missing hydrogen bonds and poor interactions in red).

Docking

To examine the structural basis of the observed enantiopharmacology, we docked the global minima of both enantiomers of tri-ionised **1** to a crystal structure of GluR2[39] in an agonised state using Glide 1.8.[40] The results are summarised in Fig. 5. Glide ranks posed on the basis of a model energy score, Emodel. For (*R*)-**1** (Fig. 5A), the best ranked pose (Emodel −123.8) allows the *R*-α-amino acid moiety to reproduce the hydrogen bonding interactions with R485, P478, T480 in domain 1 and E705 in domain 2, known to be important experimentally.[11,41]

The three-centred hydrogen bond of the axial ammonium proton is more reminiscent of the binding mode of KA bound at GluR2 than that of (*S*)-AMPA.[11] In this binding mode, the anionic isoxazole ring of (*R*)-**1** comes to occupy a similar region of the receptor as the isoxazole ring of (*S*)-AMPA; we do not require exact overlap of these moieties since co-crystallisation of (*S*)-Glu analogues has revealed some variability in the positioning of the distal anion, depending on the ligand structure, induced fit and degree of domain closure.[12] The relatively close approach of the proton on the α-carbon of (*R*)-**1** to Y450 is a reminder that domain 1 has evolved to recognise *S*-α-amino acids, and that (*R*)-**1** is a barely tolerated special case due to conformational restriction.

(*S*)-**1** presents a markedly different picture (Fig. 5B) and is clearly less well accommodated by the receptor. The best scored pose (Emodel −113.8) presents an unlikely inverted binding mode, with the anionic isoxazole ring bound to R485, and the NH_2^+ interacting with E705 at a poor angle (Fig. 5B, purple carbons). While it is possible to dock (*S*)-**1** in a more (*S*)-AMPA-like binding mode (Fig. 5B, green carbons), the score is poorer (Emodel −94.0) and the axial ammonium proton is directed towards the face of Y450. Most critically, conformational restriction of the *S*-α-amino acid in this way has abolished the ability of the ammonium group to donate a hydrogen bond to E705, which according to all structural evidence to date is a prerequisite for agonism of iGluRs.[11,12,42] These results support the conclusion that only (*R*)-**1** is able to activate AMPA receptors.

Discussion

The conformationally restricted analogue of AMPA, compound **1** (5-HPCA), was originally synthesised in order to study the receptor-active conformation of AMPA. We have now had the opportunity to resolve and characterise the enantiomers of **1**, as well as evaluate them in receptor binding experiments. We found that (+)-**1** possesses the *R*-configuration, by examining X-ray analysis of the zwitterion monohydrate, by comparing the measured optical rotation and ECD spectra with *ab initio* calculations, and by chemical correlation with (*S*)-AMPA. (*R*)-(+)-**1** turned out to be the active enantiomer, showing similar affinity for all four homomeric AMPA receptors (Table 2).

Conformational analysis by molecular mechanics places the carboxylate group of tri-ionised **1** in an equatorial orientation as the lowest energy conformation in solution, in keeping with the similar half-chair conformation identified in the solid state using X-ray crystallography (Fig. 2) and in solution by NMR.[36] (*R*)-**1** but not (*S*)-**1** can be docked in a reasonable binding mode to the ligand binding site of GluR2, and is predicted to display this same conformation at the receptor (Fig. 5A). The conformational restriction of (*R*)-**1** forces its major pharmacophore elements into a similar spatial orientation to those of more flexible (*S*)-AMPA analogues when bound to AMPA receptors. At the same time, it is the spatial relationship between the aromatic chromophore and the elements around the stereogenic centre, which indirectly gives rise to the sign and strength of the optical properties. That (*R*)-**1** has such a large optical rotation compared to conformationally less restricted AMPA analogues[16,18,21] may be understood as being related to the conformational restriction of the asymmetric centre to a position near but not coplanar with the chromophore, whereas free amino acid moieties yield an ensemble of conformers that partially cancel each other's optical effects. We note that the enantioselectivity is associated here not with the absolute configuration of the amino acid, but with the sign of the optical rotation as well as the sign of the Cotton effect. We tentatively suggest that this may be because the same spatial features recognised by the receptor are those that have most influence on the ECD spectrum, and that biological activity can in some cases follow chiroptical properties rather than absolute stereochemistry—that is, that the sign of the Cotton effect may in some cases be a better predictor of biological activity than the absolute configuration.

Finally, supported by the results of this study, we would sound a note of caution, that one cannot assume that a new analogue, presenting similar optical, and apparently similar chiral chromatographic and biological enantioselectivity once resolved, in fact has the same absolute configuration as its known cousins—the unequivocal determination of absolute configuration remains an important and challenging enterprise in understanding molecular recognition.

Conclusion

We conclude that the (*R*)-(+)-isomer of **1** is the pharmacologically active enantiomer of **1**, binding selectively to AMPA receptors, but less potently than (*S*)-AMPA. Similarly to (*S*)-AMPA, (*R*)-(+)-**1** does not distinguish between the individual AMPA receptor subtypes. This is the first reported AMPA analogue where the *R*-enantiomer is the eutomer but the *S*-enantiomer is inactive, the opposite of the general trend for similar AMPA analogues.

Experimental

^{1}H and ^{13}C NMR spectra were recorded on a Varian Gemini (300 MHz) spectrometer and chemical shifts are reported in ppm, with coupling constants (*J*) in Hz. Flash column chromatography (FC) was performed on Merck silica gel 60 (70–230 mesh). Most reactions were followed on TLC (silica gel 60 F_{254} plates; Merck) using UV and $KMnO_4$, or ninhydrin spraying reagents. Melting points were determined in open capillaries and are uncorrected. Dry *N*,*N*-dimethylformamide and acetonitrile were obtained by storing over 4 Å molecular sieves. Dry ethanol was obtained by distillation over magnesium. Dry potassium carbonate was obtained by heating *in vacuo*. Elemental analyses were performed at Microanalytical Laboratory at the Department of Physical Chemistry, University of Vienna, Austria. FAB MS data were obtained at Mass Spectrometry Research Unit, Department of Chemistry, University of Copenhagen, Denmark. Optical rotations were measured in thermostated cuvettes on a Perkin-Elmer 241 polarimeter. $[a]_D$ values are given in 10^{-1} deg cm^2 g^{-1}. UV spectra were recorded in 1.0 cm cuvettes on a Perkin-Elmer Lambda 2 spectrophotometer. Electronic circular dichroism (ECD) was recorded in 1.0 cm cuvettes at room temperature on a Jasco J-720 spectropolarimeter.

Liquid chromatography

Preparative chromatography was performed with an HPLC system consisting of a Jasco 880 HPLC pump, a Rheodyne 7125 injector equipped with a 5 cm^3 sample loop, and a Waters 481 UV detector, set at 210 nm, attached to a Hitachi D-2000 Chromato-Integrator. Preparative resolution was performed on a Sumichiral OA-5000 chiral ligand exchange column (10 × 250 mm) connected to a Sumichiral OA-5000 guard column (10 × 4.0 mm) (Sumika Chemical Analysis Service, Japan). The column was eluted at 4 cm^3 min^{-1} with an aqueous solution of ammonium acetate (10 mM), adjusted to pH 4.7 using acetic acid and containing copper(II) acetate (0.1 mM) and 2-propanol (10% v/v). The temperature was kept at 50 °C by an LKB 2155 HPLC column oven. Removal of copper ions was performed on an XK16/20 column, packed with Chelating Sepharose Fast Flow (capacity: 0.7–1.0 mmol copper ions). The column was connected to a Waters 510 pump, a Waters U6K injector equipped with a 2 cm^3 sample loop and a Waters 991 photodiode array detector, set at 254 nm. The Chelating Sepharose column was eluted at 1 cm^3 min^{-1} with milli-Q water as the eluent.

The analytical determination of the enantiomeric excess (ee) of the isolated enantiomers of **1** and **2**, and the correlation with stereoconservatively synthesised (*S*)-**1** were performed on a D-proline column (4.6 × 120 mm) prepared as previously described.[24] The column was thermostated at 50 °C and connected to a Jasco 880 HPLC pump or a TSP pump and a TSP HPLC system consisting of an AC 3000 autoinjector, and a SM 5000 PDA detector. For HPLC control, data collection, and data handling the TSP PC1000 software was used. The D-proline column was eluted at 1 cm^3 min^{-1} with an aqueous solution of potassium dihydrogen phosphate (50 mM) and 2-propanol (10% v/v). Purification of synthesised (*S*)-**1** was performed on a Polarity reverse phase column (2.1 × 150 mm) eluting with 0.1% aqueous trifluoroacetic acid at 0.6 cm^3 min^{-1}. The column was connected to a Jasco 880 HPLC pump, a Rheodyne injector and a Shimadzu SPD-6A UV spectrophotometric detector, set at 215 nm, attached to a Hitachi D2000 Chromato-Integrator.

Chromatographic resolution of (*RS*)-3-(carboxymethoxy)-4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridine-5-carboxylic acid (2)

A total of 490 mg (2.02 mmol) of **2** dissolved in water (5.7 mg cm^{-3}) was resolved using 0.7 cm^3 (4 mg) injections. Fractions containing the first eluting enantiomer were pooled, as were those containing the second eluting enantiomer, and the solvent removed *in vacuo* to give blue residues. The individual residues were dissolved in water and purified on a Chelating Sepharose column to give the first eluting enantiomer of **2** (231.2 mg, 94%,

99.9% ee) and the second eluting enantiomer of **2** (240.0 mg, 98%, 99.0% ee), respectively, as pale yellow oils.

Synthesis

(*S*)-(−)-3-Hydroxy-4,5,6,7-tetrahydroisoxazolo[5,4-*c*]-pyridine-5-carboxylic acid [(−)-1]. The first eluting enantiomer of **2** (231.2 mg, 0.95 mmol) was dissolved in 48% aqueous hydrobromic acid and refluxed for 30 min in a preheated oil bath. The solvent was removed *in vacuo* to give brown crystals that were re-evaporated with water (3 × 5 cm^3). The colour was partly removed by coal filtration. Re-crystallisation (water) gave (−)-**1** (73.3 mg, 42%, 99.6% ee) as brownish crystals; mp > 220 °C (decomp.); $[\alpha]_D^{25} = -94$ (*c* 0.31 in 0.1 M HCl); $\Delta\varepsilon$ (219 nm) = −0.2 m^2 mol^{-1}; λ_{max} (log ε): 205.0 nm (3.8). Found: C, 41.0; H, 4.7; N, 13.0. $C_7H_8N_2O_4{\cdot}H_2O$ requires C, 41.6; H, 5.0; N, 13.9%; FAB MS found: 185.0576 requires 185.0562 (+7.2 ppm); δ_H(300 MHz; D_2O; acetonitrile δ 2.06) 2.64 (1 H, ddd, J_{AB} 16.5, J_{AX} 10.2 and ^5J 2.6, 1 × CHCH_2), 2.97 (1 H, ddd, J_{AB} 16.5, J_{BX} 5.4 and ^5J 1.2, 1 × CHCH_2), 3.95 (1 H, dd, J_{AX} 10.2 and J_{BX} 5.4, CHCH$_2$), 4.22 (1 H, dd, J_{AB} 16.3 and ^5J 2.6, 1 × NCH_2), 4.32 (1 H, dd, J_{AB} 16.3 and ^5J 1.2, 1 × NCH_2).

(*R*)-(+)-3-Hydroxy-4,5,6,7-tetrahydroisoxazolo[5,4-*c*]-pyridine-5-carboxylic acid [(+)-1]. The second eluting enantiomer of **2** (240.0 mg, 0.99 mmol) was dissolved in 48% aqueous hydrobromic acid (5 cm^3) and refluxed for 28 min in a preheated oil bath. The solvent was removed *in vacuo* and re-evaporated with water (3 × 5 cm^3). Re-crystallisation (water) gave (+)-**1** (91.4 mg, 48%, 99% ee); as brownish crystals; mp > 220 °C (decomp.); $[\alpha]_D^{25} = +91$ (*c* 0.31 in 0.1 M HCl); $\Delta\varepsilon$ (219 nm) = +0.2 m^2 mol^{-1}; λ_{max} (log ε): 205.0 nm (3.8). Found: C, 41.0; H, 4.7; N, 13.4. $C_7H_8N_2O_4{\cdot}H_2O$ requires C, 41.6; H, 5.0; N, 13.9%; FAB MS found: 185.0582 requires 185.0562 (+10.5 ppm). The ^{1}H NMR spectrum was identical with that of the *S*-enantiomer.

Ethyl (*S*)-2-(*tert*-butoxycarbonylamino)-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionate (3). Dry ethanol (12.4 cm^3) was cooled to 0 °C and distilled acetyl chloride (1.24 cm^3) was slowly added. (*S*)-AMPA (250 mg, 1.34 mmol) was added and the solution refluxed for 24 h. The solvent was removed *in vacuo* and triethylamine (0.9 cm^3) and water (9 cm^3) were added. Di-*tert*-butyl dicarbonate (411 mg, 1.88 mmol) dissolved in tetrahydrofuran (9 cm^3) was added and the solution was stirred for 16 h. The solvent was removed *in vacuo* and water (30 cm^3) was added. The aqueous phase was washed with ether (3 × 15 cm^3), pH was adjusted to 2 with $KHSO_4$ (1 M) and then extracted with ethyl acetate (3 × 30 cm^3). The combined ethyl acetate phases were dried ($MgSO_4$) and the solvent removed *in vacuo*. Re-crystallisation (acetone) gave **3** (261 mg, 62%) as white crystals; mp 128–131 °C; δ_H(300 MHz; $CDCl_3$; TMS) 1.27 (3 H, t, *J* 7.5, CH$_2$CH_3), 1.43 (9 H, s, C(CH_3)$_3$), 2.26 (3 H, s, CCH_3), 2.75 (2 H, m, CHCH_2), 4.12–4.25 (2 H, m, CH_2CH$_3$), 4.56 (1 H, m, CHCH$_2$), 5.49 (1 H, d, *J* 7.8, NH); δ_C(75 MHz; $CDCl_3$; TMS) 11.4, 14.0, 24.1, 28.2, 53.0, 61.6, 79.7, 100.8, 154.9, 167.4, 169.7, 171.1.

Ethyl (*S*)-2-(*tert*-butoxycarbonylamino)-3-(3-isopropoxy-5-methyl-4-isoxazolyl)propionate (4). Dry potassium carbonate (229 mg, 1.66 mmol) was added to a solution of **3** (261 mg, 0.83 mmol) in dry *N*,*N*-dimethylformamide (3 cm^3) and the suspension was stirred at 60 °C for 20 min. 2-Bromopropane (233 mm^3, 2.48 mmol) was added and the suspension was stirred for 15 h. The suspension was cooled to room temperature and water (40 cm^3) was added. The aqueous phase was extracted with pentane (6 × 20 cm^3). The combined organic phases were washed with water (2 × 10 cm^3) and brine (1 × 10 cm^3), dried ($MgSO_4$) and the solvent was removed *in vacuo*. FC (9 : 1, toluene/ethyl acetate) gave **4** (198 mg, 67%) as a colourless oil. δ_H(300 MHz; $CDCl_3$; TMS) 1.23 (3 H, t, *J* 6.9, CH$_2$CH_3), 1.40 (6 H, d, *J* 6.3, CH(CH_3)$_2$), 1.43 (9 H, s, C(CH_3)$_3$), 2.24 (3 H, s, CCH_3), 2.75 (2 H, d, *J* 5.7, CHCH_2), 4.06–4.25 (2 H, m, CH_2CH$_3$), 4.38 (1 H, dt, *J* 7.5 and 5.7, CHCH$_2$), 4.94 (1 H, hep, *J* 6.3, CH(CH$_3$)$_2$), 5.49 (1 H, d, *J* 7.5, NH); δ_C(75 MHz; $CDCl_3$; TMS) 11.4, 14.0, 21.8, 21.9, 24.1, 28.2, 53.3, 61.2, 73.2, 79.6, 100.4, 154.9, 167.0, 169.4, 171.2.

Ethyl (*S*)-2-[*N*,*N*-(di-*tert*-butoxycarbonyl)amino]-3-(3-isopropoxy-5-methyl-4-isoxazolyl)propionate (5). Compound **4** (198 mg, 0.56 mmol) was dissolved in dry acetonitrile (4 cm^3) and 4-dimethylaminopyridine (3.4 mg, 0.03 mmol) and di-*tert*-butyl dicarbonate (243 mg, 1.12 mmol) were added. The solution was stirred for 48 h at room temperature and additional 4-dimethylaminopyridine (4.2 mg, 0.04 mmol) and di-*tert*-butyl dicarbonate (200 mg, 0.92 mmol) were added and the solution stirred for another 44 h. The solvent was removed *in vacuo* and water (20 cm^3) was added. The aqueous phase was extracted with ethyl acetate (4 × 20 cm^3). The combined organic phases were dried ($MgSO_4$) and the solvent removed *in vacuo*. FC (8 : 1, pentane/ethyl acetate) afforded **5** (247 mg, 0.54 mmol, 97%) as a colourless oil. δ_H(300 MHz; $CDCl_3$; TMS) 1.27 (3 H, t, *J* 7.2, CH$_2$CH_3), 1.38 (6 H, d, *J* 6.0, CH(CH_3)$_2$), 1.44 (18 H, s, 2 × C(CH_3)$_3$), 2.21 (3 H, s, CCH_3), 2.99 (2 H, d, *J* 7.8, CHCH_2), 4.21 (2 H, q, *J* 7.2, CH_2CH$_3$), 4.87 (1 H, hep, *J* 6.0, CH(CH$_3$)$_2$), 4.99 (1 H, t, *J* 7.5, CHCH$_2$); δ_C(75 MHz; $CDCl_3$; TMS) 11.2, 14.0, 21.8, 21.86, 21.88, 27.7, 57.3, 61.2, 72.7, 82.7, 101.1, 151.6, 166.5, 169.6, 169.7.

Ethyl (*S*)-2-[*N*,*N*-(di-*tert*-butoxycarbonyl)amino]-3-(5-bromomethyl-3-isopropoxy-4-isoxazolyl)propionate (6) and ethyl (*RS*)-2-bromo-2-[*N*,*N*-(di-*tert*-butoxycarbonyl)amino]-3-(5-bromomethyl-3-isopropoxy-4-isoxazolyl)propionate (7). Compound **5** (150 mg, 0.33 mmol) was dissolved in carbon tetrachloride (3 cm^3) and *N*-bromosuccinimide (70.2 mg, 0.39 mmol) and dibenzoyl peroxide (catalytic) were added in four portions during 3 h. The solution was refluxed for 17 h. The solvent was removed *in vacuo* and FC (8 : 2, pentane/ether) gave a mixture of **6** and **7** in a 6 : 1 ratio (51 mg). The mixture was used without further purification. Compound **6**: δ_H(300 MHz; $CDCl_3$; TMS) 1.28 (3 H, t, *J* 7.2, CH$_2$CH_3), 1.39 (3 H, d, *J* 6.0, CHCH_3), 1.39 (3 H, d, *J* 6.0, CHCH_3), 1.44 (18 H, s, 2 × C(CH_3)$_3$), 3.05 (1 H, dd, J_{AB} 14.7 and J_{AX} 4.3, 1 × CHCH_2), 3.10 (1 H, dd, J_{AB} 14.7 and J_{BX} 10.7, 1 × CHCH_2), 4.20 (2 H, q, *J* 7.2, CH_2CH$_3$), 4.29 (2 H, s, CH_2Br), 4.88 (1 H, hep, *J* 6, CH(CH$_3$)$_2$), 5.09 (1 H, dd, J_{AX} 4.3 and J_{BX} 10.7, CHCH$_2$). Compound **7**: δ_H(300 MHz; $CDCl_3$; TMS) 1.23 (3 H, t, *J* 7.2, CH$_2$CH_3), 1.42 (3 H, d, *J* 6, CHCH_3), 1.43 (3 H, d, *J* 6, CHCH_3), 1.44 (18 H, s, 2 × C(CH_3)$_3$), 2.99 (2 H, s, CCH_2), 4.10 (2 H, q, *J* 7.2, CH_2CH$_3$), 4.42 (1 H, d, *J* 12.0, 1 × CH_2Br), 4.57 (1 H, d, *J* 12.0, 1 × CH_2Br), 4.95 (1 H, hep, *J* 6, CH(CH$_3$)$_2$).

(*S*)-(−)-3-Hydroxy-4,5,6,7-tetrahydroisoxazolo[5,4-*c*]-pyridine-5-carboxylic acid. [(*S*)-1]. Compound **6** (47 mg, 0.088 mmol) was dissolved in CH_2Cl_2 (500 mm^3) and trifluoroacetic acid (200 mm^3) was added. The solution was stirred for 50 min. Water (20 cm^3) was added and the aqueous phase washed with ether (3 × 10 cm^3). The pH was adjusted to 10 with saturated sodium carbonate, and the aqueous phase was extracted with ether (3 × 20 cm^3). The combined ether phases were dried ($MgSO_4$) and the solvent removed *in vacuo*. The residue was dissolved in *N*,*N*-dimethylformamide (1 cm^3) and potassium carbonate was added (18 mg, 0.13 mmol) and the suspension was stirred for 15 h. Water (50 cm^3) was added and the aqueous phase extracted with ether (6 × 10 cm^3). The combined ether phases were extracted with 12 M hydrochloric acid (2 × 5 cm^3). To the combined aqueous phases was added acetic acid (10 cm^3) and the solution was refluxed for 12 h. The solvent was removed *in vacuo* and the residue re-evaporated with water (3 × 5 cm^3). The residue was purified on a Polarity reverse phase HPLC column to give (*S*)-**1** (3.2 mg, 20% based on ^{1}H NMR). δ_H (300

MHz; D_2O; dioxane δ 3.75) 2.84 (1 H, dd, J_{AB} 16.3 and J_{AX} 9.9, CHCH_2), 3.12 (1 H, dd, J_{AB} 16.3 and J_{BX} 5.4, CHCH_2), 4.21 (1 H, dd, J_{AX} 9.9 and J_{BX} 5.4, CH), 4.36 (1 H, d, J_{AB} 16.2, 1 × NCH_2), 4.50 (1 H, d, J_{AB} 16.2, 1 × NCH_2).

In vitro pharmacology

Receptor binding assays. Affinities for the native AMPA, KA and NMDA receptors were determined using 5 nM [^{3}H]AMPA (55.5 Ci mmol^{-1}),[27] 5 nM [^{3}H]KA (58.0 Ci mmol^{-1})[28] and 2 nM [^{3}H]CGP 39653 (K_d = 6 nM, 50.0 Ci mmol^{-1})[25] or 5 nM [^{3}H]CPP (36.0 Ci mmol^{-1}),[26] respectively, with minor modifications as previously described.[43] Rat brain membrane preparations used in the receptor binding experiments were prepared according to the method described by Ransom and Stec.[44]

Recombinant receptor binding assays. Sf9 cells were infected with recombinant baculovirus of rat AMPA receptors (GluR1-4) or rat KA receptors (GluR5 and 6) and membranes prepared and used for binding as previously detailed.[45] The affinities of compounds at GluR1, 2(*R*), 3, and 4 (all as *flop* isoforms) were determined from competition experiments with 2–5 nM [^{3}H]AMPA (55.5 Ci mmol^{-1}); at GluR5(*Q*) with 1–2 nM [^{3}H]SYM 2081[46] (K_d = 0.66 ± 0.08 nM, data not shown) (50.6 Ci mmol^{-1}) and using 5 nM [^{3}H]KA (K_d = 11.9 ± 3.2 nM; 58.0 Ci mmol^{-1}) at GluR6 (*V*,*C*,*R*). Italic letters in parentheses indicate the RNA-edited isoforms of the subunits used.

Crystallographic analysis

Colourless plate shaped crystals of (*S*)-(−)-**1** monohydrate were grown by slowly cooling a heated aqueous solution of the compound. Crystal data: $C_7H_8O_4N_2 \cdot H_2O$, M = 202.17 g mol^{-1}, monoclinic, a = 6.9444(11) Å, b = 6.4862(10) Å, c = 9.160(2) Å, β = 99.437(14)°, V = 407.01(13) Å^3, space group $P2_1$ (No. 4), Z = 2, D_c = 1.650 Mg m^{-3}, $F(000)$ = 212, μ(Cu K$_\alpha$) = 1.23 mm^{-1}, T = 110 × 0.5 K. Crystal dimensions: 0.05 × 0.15 × 0.35 mm.

Diffraction data were collected on an Enraf-Nonius CAD-4-diffractometer,[47] using graphite monochromated Cu K$_\alpha$ radiation (λ = 1.5418 Å). Unit cell dimensions were determined by least-squares refinement of 20 reflections (θ range: 34.21–45.44°). Intensities were measured in the $\omega/2\theta$ scan mode (θ: 4.89–74.88°, h: −8⟶8, k: −8⟶8, l: −11⟶11). Data were reduced by using the Enraf-Nonius Structure Determination Package (SDP).[48] Three standard reflections were monitored every 10^4 s (decay 2%, corrected). The data were corrected for absorption effects.[49] A total of 3340 reflections were averaged according to the point group symmetry 2 resulting in 1675 unique reflections (R_{int} = 0.024 on intensities), 1625 with $F_o > 3.0\ \delta F_o$. The structure was solved by direct methods using the program SHELXS86[50] and refined using the program CRYSTALS.[29] Fu1l-matrix least-squares refinement on F was performed with anisotropic displacement parameters for the non-hydrogen atoms. The positions of all the hydrogen atoms were located on intermediate difference electron density maps and refined with U_{iso}(CH and NH) equal to 1.2 × U_{eq} or U_{iso}(OH) equal to 1.5 × U_{eq}(OH) of the parent atoms. An auto-statistical weighting scheme was applied.[29,51] The refinement (1625 reflections, 158 parameters) with the molecule having the *S*-configuration converged at R = 0.0207, wR = 0.0287. The Flack parameter x[49,51] is 0.02(14). The large standard deviation on x is due to the atom types present in the compound (C, H, N and O) which produce minor anomalous scattering. Complex atomic scattering factors for neutral atoms were as incorporated in CRYSTALS.[29] A final difference Fourier synthesis showed minimum and maximum residual electron densities of −0.26 and 0.32 e Å^{-3}. †

† CCDC reference number 218716. See http://www.rsc.org/suppdata/ob/b3/b310450h/ for crystallographic data in.cif or other electronic format.

Molecular modelling

The conformational distributions of both cationic and tri-ionised (*R*)-**1** and (*S*)-**1** were examined using the MMFFs force field, including GB-SA treatment of aqueous solvation, in Macromodel 8.0.[40] Selected minima were re-optimised with density functional theory (DFT), at B3LYP/6-311+G(d.p) in Gaussian 98,[52] all confirming the conformation in the crystal structure (carboxylate group equatorial) to be preferred in gas phase and solution. The DFT geometry of cationic (*R*)-**1** was used to perform calculations of the ECD rotational strengths (HF/cc-pVTZ) and the optical rotation (HF/cc-pVTZ) at the sodium line (589.298126 nm) in Dalton 1.2.1.[53] Both calculations employed London orbitals. For comparison with experiment, the rotational strengths in 10^{-40} esu^2 cm^2 were converted to $\Delta\varepsilon$ (m^2 mol^{-1}) with Gaussian line broadening of 0.25 eV, according to the method of Diedrich and Grimme.[54]

The extracellular construct of the AMPA receptor binding domain, GluR2-S1S2J, developed by Gouaux *et al.*[11] in complex with the AMPA analogue thio-ATPA[39] was prepared using Impact 1.8,[40] according to the protein preparation method recommended in First Discovery.[40] The experimentally observed ligand and water molecules were removed, and the tri-ionised enantiomers of **1** were docked using Glide 1.8.[40] Van der Waals radii of weakly charged atoms of the ligand and receptor were scaled by 0.9 Å. The 30 best of 1000 initial poses were ranked by Glidescore,[40] saved and inspected.

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