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Use of the 4-Hydroxy-Triazole Moiety as a Bioisosteric Tool in the Development of Ionotropic Glutamate Receptor Ligands

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Abstract

We report a series of glutamate and aspartate analogues designed using the hydroxy-1,2,3-triazole moiety as a bioisostere for the distal carboxylic acid. Compound **6b** showed unprecedented selectivity among AMPA receptor subtypes, confirmed also by an unusual binding mode observed on the crystal structures in complex with the AMPA receptor GluA2 agonist binding domain. Here, a methionine (Met729) was highly disordered compared to previous agonist-bound structures. This observation provides a possible explanation for the pharmacological profile. In the structure with **7a**, an unusual organization of water molecules around the bioisostere arises compared to previous structures of ligands with other bioisosteres. Aspartate analogue **8** with the hydroxy-1,2,3-triazole moiety directly attached to glycine was unexpectedly able to activate both the glutamate and glycine agonist binding sites of the NMDA receptor. These observations demonstrate novel features that arise when employing a hydroxyl-triazole moiety as bioisostere for the distal carboxylic acid in glutamate receptor agonists.

Graphical Abstract.

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CONFLICTS OF INTEREST

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PDB CODES

PDB ID: 6Q54 (GluA2-ABD in complex with **6b**), PDB ID: 6Q60 (GluA2-ABD in complex with **7a**). Authors will release the atomic coordinates upon article publication.

ASSOCIATED CONTENT

Supporting info: a PDF containing ¹H-NMR and ¹³C-NMR spectra of all compounds. Molecular formula strings and biological data (CSV file).

Keywords

Bioisosterism; Glutamic Acid; AMPA; Hydroxy-1,2,3-Triazole; NMDA

1. INTRODUCTION

(*S*)-Glutamic acid (glutamate, Glu, **1**, Figure 1) is the major excitatory neurotransmitter in the central nervous system (CNS) and is crucial to normal brain functions. However, the neurotransmitter is also involved in a number of brain disorders.¹⁻⁵ The effects of Glu are mediated by a highly heterogeneous receptor population comprising G protein-coupled metabotropic Glu receptors and ionotropic Glu receptors (iGluRs).¹ The iGluRs are ligand-gated ion-channels that are divided into three major classes according to amino acid sequence identity and to their respective activation by the agonists *N*-methyl-*D*-aspartic acid (NMDA), (*S*)-2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propionic acid ((*S*)-AMPA, **2**) and kainic acid (kainate, KA).² The iGluRs are homo- or heterotetrameric assemblies of various subunits; NMDA receptors being made up of the GluN1, GluN2A-D, and GluN3A-B subunits, AMPA receptors of the GluA1-4 subunits, and KA receptors of the GluK1-5 subunits. The majority of functional NMDA receptors in the CNS are heteromeric GluN1/GluN2 receptors composed by two GluN1 subunits that recognize the endogenous co-agonist glycine (or D-serine) and two GluN2 subunits that recognize Glu, whereas KA and AMPA receptors exist as both homo- and heteromeric combinations of their respective cloned subunits. X-ray crystallographic studies of full-length AMPA, KA and NMDA receptors^{6, 7} have established that the iGluRs are tetrameric assemblies⁸⁻¹⁴ consisting of four structural subdomains: the intracellular C-terminal domain, the transmembrane domain forming the ion channel, the agonist binding domain (ABD), and the large extracellular *N*-terminal domain. A large number of structures of soluble iGluR ABDs constructs have revealed important information on the molecular basis for orthosteric ligand recognition, and the mechanisms underlying activation, desensitization, and allosteric modulation of the receptors.¹⁵⁻¹⁷

A large number of ligands showing selectivity among the three classes of iGluRs have been disclosed, but few ligands have been developed with specificity for the individual receptor subtypes within the classes, presumably due to high sequence identity among the subunits within each class. In particular, selectivity towards individual AMPA receptor subunits has been difficult to achieve. Nevertheless, some compounds (Figure 1) such as (*RS*)-2-amino-3-(4-chloro-3-hydroxyisoxazol-5-yl)propionic acid (Cl-HIBO, **3**) and (*RS*)-3-hydroxy-4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridine-7-carboxylic acid (7-HPCA, **4**) are able to selectively

activate GluA1/2,^{18, 19} exploiting the only difference between GluA1/2 and Glu3/4 among the amino acids in direct contact with Glu. Some preference towards GluA1 and GluA3 has been achieved by the continued development of MeTetAMPA (**5a**) through BnTetAMPA (**5b**) to aminomethyl and chloro substituted BnTetAMPA analogues **5c** and **5d**, respectively.²⁰⁻²² The protrusion of the benzyl group in BnTetAMPA into a less conserved area is enabled by the movement of a conserved methionine (Met729 in GluA2) in the binding pocket, leading to the observed variation in the selectivity profiles of these analogues.

In recent years, we have been focusing on improving the strategy of scaffold hopping to replace the acidic moieties by acidic hydroxylated azoles in different lead compounds.²³ Using this strategy, we successfully designed a new class of DHODH (dihydroorotate dehydrogenase) inhibitors,²⁴⁻³¹ AKR1C3 (Aldo-Keto Reductase 1C3) inhibitors,³²⁻³⁴ inhibitors of the canonical NF- κ B cascade³⁵⁻³⁶ GABA (gamma-aminobutyric acid) analogues³⁷⁻³⁹ as well as Glu analogues.⁴⁰ In light of the promising results obtained in this previous work, and in an attempt to explore the selectivity towards iGluRs, the hydroxy-1,2,3-triazole scaffold was used to bioisosterically replace the distal carboxylic group of Glu. For the AMPA analogues **6a** and **7a**⁴¹ we previously reported that the hydroxy-1,2,3-triazole scaffold provides the opportunity to place a substituent oriented in two different directions as opposed to the isoxazolol moiety in AMPA. Here, we describe the synthesis and extensive pharmacological characterization at native and recombinant iGluRs of the Glu analogues **6a-c**, **7a-c** and the aspartate analogue **8** along with X-ray crystallographic studies of compounds **6b** and **7a** bound in the GluA2 ABD.

2. RESULTS

2.1 Chemistry: synthesis of target compounds **6a-c**, **7a-c** and **8**.

The synthesis of racemic hydroxy-1,2,3-triazole Glu analogues **6a-c** and **7a-c** (Scheme 1) follows our previously published procedure, starting from the isomeric triazole building blocks **9a-c** and **10a-c**,⁴¹ which are obtained in a robust methodology for preparation of regio-substituted hydroxy-1,2,3-triazoles. Compounds **9a-c** and **10a-c** were first reduced to their corresponding alcohols **11a-c** and **12a-c** with NaBH₄ in EtOH, subsequently reacted with N-bromosuccinimide (NBS) to yield **13a-c** and **14a-c**. Reaction with diethyl-2-acetamidomalonate in the presence of NaH followed by catalytic hydrogenation at atmospheric pressure afforded compounds **17a-c** and **18a-c**. Final deprotection of **17a-c** and **18a-c** in 6N HCl at reflux gave, after ion exchange chromatography, the zwitterion racemic mixture of compounds **6a-c** and **7a-c** in quantitative yields.

The synthesis of the zwitterion racemic mixture of triazole NMDA analogue **8** (Scheme 2), started from **14a** treated with NaCN in ethanol to yield the nitrile **19**. The latter subsequently reacted with trimethylchlorosilane (TMSCl) and ethanol to afford the ester **20**. The NMDA analogues **8** was obtained by nitrosation of **20** and subsequent reduction of oxime **21** by catalytic hydrogenation at atmospheric pressure, followed by immediate hydrochloric acid hydrolysis of the intermediate ester **22**.

2.2 Pharmacology.

Initially, compounds **6b-c**, **7b-c**, and **8** were characterized at the major iGluR subgroups in a radioligand binding assay using rat cortical synaptosomes (data for **6a** and **7a** were previously obtained and are reported in ref. 37 Table 1). Compounds like **6a-6c** and **7a** showed selective but moderate (μM) activity at AMPA receptors. **6a** is a close analogue of AMPA and displayed an IC_{50} value of $1.4 \mu\text{M}$ in the $[^3\text{H}]\text{AMPA}$ binding assay, which is 100-fold higher than that displayed by AMPA itself. The ethyl and propyl substituted analogues **6b** and **6c** displayed similar potencies of 0.98 and $5.20 \mu\text{M}$, respectively. In **7a**, the movement of the methyl group to the neighboring nitrogen gives **7a** similar potency as **6a** at AMPA receptors ($\text{IC}_{50} = 3.1 \mu\text{M}$), although in this case, ethyl and propyl substitutions (**7b** and **7c**) were not tolerated. Compound **8** displayed slightly weaker affinity to NMDA receptors ($K_i = 34 \mu\text{M}$) than NMDA itself ($K_i = 6.2 \mu\text{M}$).

The AMPA receptor selectivity among compounds **6a-c**, and **7a-c** prompted an initial functional screening in a $\text{Ca}^{2+}/\text{Fluo-4}$ assay using HEK293 cell lines expressing GluA1-, GluA2(Q)-, GluA3- and GluA4 (Table 2) to probe whether the compounds are agonists at the AMPA receptors. This demonstrated that all the compounds having affinity towards AMPA receptors displayed significant agonist activity ($>5\%$ of the maximal response induced by (*S*)-Glu) with an order of magnitude lower potency than Glu at GluA4. Full curves could not be generated at the other subtypes due to weaker agonist potencies in general using this assay, and therefore it was not possible to determine any subtype selectivity of the compounds.

Following these results, we decided to investigate whether subtype selectivity could be observed among the AMPA receptor subtypes with these new bioisosteric analogues. We determined the binding affinities of selected compounds at cloned rat AMPA receptor subtypes expressed in *Sf9* insect cells (Table 3). In general, the binding affinities were of the same magnitude (low micromolar) as in the synaptosome assay, but showing variation among subtypes. Some compounds (**6a-c** and **7a**) showed an order of magnitude weaker affinity towards GluA1 than GluA2. The most potent compound **6b**, with an affinity of $0.71 \mu\text{M}$ at GluA2 showed an order of magnitude weaker binding at both GluA1 and GluA4. This compound was further characterized functionally using two-electrode voltage-clamp electrophysiology with *Xenopus* oocytes expressing recombinant homomeric rat GluA2(Q)_i. This assay demonstrated that the compound **6b** was a partial agonist with EC_{50} of $65 \pm 6 \mu\text{M}$ and I_{max} of 0.863 ± 0.015 , where $\text{Glu I}_{\text{max}} = 1.000$ (Figure 2).

We investigated the activity of compound **8** at recombinant NMDA receptors using two-electrode voltage-clamp electrophysiology. Initially, the compound was tested at $100 \mu\text{M}$ for the ability to activate GluN1/2A-D receptors in the absence of both Glu and Gly. Unexpectedly, the compound ($100 \mu\text{M}$) was able to activate several recombinant NMDA receptors in absence of both Glu and Gly, with the exception of the GluN1/2A subtype (Figure 3B).

We determined the activity of **8** in the presence of either Glu or Gly to probe the activity on GluN2 and GluN1 agonist binding sites, respectively (Figure 3C-D, Table 4). In the presence of $300 \mu\text{M}$ Glu (i.e. for activity at GluN1), compound **8** was a full agonist at GluN1 when

expressed with GluN2C and GluN2D with potencies of 90 μ M and 35 μ M, respectively. The compound was a weaker agonist at GluN1/2A and GluN1/2B receptors (full curves could not be generated). We then tested the activity of compound **8** at GluN1/2A-D in the presence of 100 μ M Gly (i.e. for activity at GluN2). Compound **8** was a partial agonist at GluN1/2B and GluN1/2D with agonist efficacies of 22% and 58% and potencies of 108 μ M and 58 μ M, respectively. No response was seen at GluN1/2A and a weak response at GluN1/2C was observed at 300 μ M (full curve could not be generated).

2.3 Structure.

We crystallized **6b** and **7a** with the agonist binding domain of GluA2 (GluA2-ABD). Compound **6b** displays the greatest affinity at GluA2 within this new series of compounds, whereas **7a** allowed us to explore a novel substitution pattern on the five-membered ring. Both structures crystallized as a GluA2-ABD dimer (Figure 4), and X-ray diffraction data were collected to a resolution of 1.4 Å (**6b**) and 1.55 Å (**7a**) (Table 5). Unambiguous electron density was seen for both ligands (Figures 5A and 5D). Full GluA2-ABD domain closure around **6b** (22° for chain A and 20° for chain B) and **7a** (21° for chain A and B) were observed. The domain closure is similar to that induced by AMPA⁴⁴ and supports that **6b** and **7a** function as agonists.

In the GluA2-ABD structure with **6b**, the amino-acid moiety of **6b** forms the same hydrogen bonds to protein residues as usually seen for agonists (Figure 5B)⁴⁴ The 4-hydroxy-1,2,3-triazole moiety of **6b** makes two direct hydrogen bonds to the receptor: one from the (a)-nitrogen atom to the backbone nitrogen atom of Glu726 and one from the 4-hydroxyl group to the side-chain hydroxyl group of Thr676 (Figure 5B). In addition, the 4-hydroxyl group of **6b** forms two water-mediated contacts: one through W1 to Thr676 (backbone N) and the carboxylate group of **6b** as well as one through W2 to Leu671 (backbone N) and Leu724 (backbone O). The 1-ethyl substituent is within van der Waals distance (4 Å cut-off) of Glu423, Tyr471, Pro499, Glu726, Met729 and Tyr753 (Figure 5B); a region in GluA2-ABD that has previously been denoted the hydrophobic pocket.⁴⁴ The side chain of Met729 is poorly defined in the electron density, indicating a high degree of flexibility (Figure 5A). This is in contrast to other binding site residues that are well-defined (see Arg506 in Figure 5A). Met729 has previously been shown to undergo an induced fit depending of the ligand bound.⁴⁴ In the structure with **6b**, the side chain of Met729 bends away from the 1-ethyl substituent.

Interestingly, **6b** binds in a mode similar to AMPA (Figure 5C), whereas other AMPA-related agonists containing a larger substituent than a methyl group in the 5-position (corresponding to the N(c)-position in **6b**), such as t-butyl group (ATPA)⁴⁶, 2-methyl-tetrazole (MeTetAMPA)⁴⁷ or benzyl-tetrazole (BnTetAMPA),²² have been shown to switch to a binding mode similar to Glu.⁴⁴ Therefore, compound **6a** (N(c)-methyl) would most likely also bind in the AMPA binding mode, whereas **6c** (N(c)-propyl) probably would switch into the glutamate binding mode due to otherwise steric clash with the residues forming the hydrophobic pocket.

Compound **7a** also binds in the AMPA binding mode, and the amino acid part and 1,2,3-triazole moiety of **7a** form the same hydrogen bonds to protein residues as seen for **6b** (Figure 5E). The introduction of a methyl group in the N(b)-position in **7a** and absence of a substituent in the N(c)-position sequesters an additional water molecule (W3), forming contacts to the nitrogen atom in the N(c)-position of **7a**, the hydroxyl group of Tyr471 and one water molecule (W4) (Figures 5E and 5F). Both W3 and W4 are absent in the structure of GluA2-ABD with AMPA (PDB code 1FTM⁴⁸), whereas only W3 is absent in the structure with **6b**. The major difference in the GluA2-ABD between the AMPA and **6b** / **7a** bound structures is the conformation of Met729 that moves further away from the ligand in the structures with **6b** and **7a**, also affecting the nearby surroundings (Figure 5F). These structural changes might lead to the sequestering of W4, forming contacts to the side chain hydroxyl groups of Tyr426, Thr728 and Tyr753, and also to Met729 (backbone N) in the structure with **6b**. In the AMPA bound structure where W4 is not present the side chains of Thr728 and Tyr753 form a direct contact with each other as also seen in the structure with **7a**.

Based on the binding affinities of **7b** and **7c** containing an ethyl and n-propyl group, respectively, in the N(b)-position it was possible to rule out that larger substituents than a methyl group at this position can be accommodated in the receptor (Table 3). This is in agreement with the crystal structure of GluA2-ABD with **7a**, where the distance between the N(b)-methyl group and the C β atom of Met729 is only 3.4 Å. Therefore, introduction of a larger substituent in the 2-position would lead to steric clash with Met729 or require major structural changes in the receptor.

A 40-fold and 250-fold lower binding affinity of **6b** and **7a** at GluA2, respectively, compared to AMPA was observed (Table 3). Due to the very similar binding mode of **6b**, **7a** and AMPA, we find that the most likely explanation for this difference in binding affinity is the difference in pK_a values for the 4-hydroxy-1,2,3-triazole moiety (**6b**: 6.42 and **7a**: 6.11)⁴¹ compared to that of AMPA (4.8).⁴⁹ Furthermore, the structural changes of Met729 in the GluA2-ABD accompanied by the difference in binding site water network might also contribute to the observed differences in binding affinities.

3. DISCUSSION.

We have reported the synthesis and pharmacological characterization of a series of glutamate and aspartate analogues with the hydroxy-1,2,3-triazole as bioisostere for the distal carboxylic acid. Four analogues showed selectivity among AMPA receptors, most pronounced with compound **6b** that shows moderate binding affinity towards GluA2, but an order of magnitude lower affinity towards GluA1 and GluA4. This unprecedented selectivity profile is not mediated by differences in amino acid residues in direct contact with the ligand. The only sequence difference in the orthosteric site that mediates the selectivity of Cl-HIBO (**3**) is a tyrosine in GluA1/2 that is a phenylalanine in GluA3/4.⁵⁰ However, **6b** shows a different pharmacological profile. Previously, we have demonstrated that selectivity in BnTetAMPA analogues arise from movement of a methionine corresponding to Met729 in GluA2. This movement likely has varying energy costs in different AMPA subunits due to a subunit specific environment in this area, where the sequence differs among the subunits. It

is therefore notable that the X-ray structure of **6b** in GluA2-ABD shows an unprecedented weak electron density for Met729 in GluA2, indicating that **6b** binding results in increased flexibility of this residue in GluA2-ABD compared to other ligands for which crystal structures exist. This could reduce the entropic cost of binding to GluA2-ABD and thereby explain the higher affinity towards GluA2-ABD compared to other subtypes. The structure of **7a** shows that substituents larger than a methyl group cannot be accommodated which is in line with the observed lack of activity of compounds **7b** and **7c**.

The use of the hydroxy-1,2,3-triazole in aspartate analogues lead to compound **8** that shows activity among NMDA receptor subtypes. Most notable is the ability of compound **8** to activate both the GluN1 and GluN2 subunits that harbours the Gly and Glu binding sites, respectively, of the NMDA receptor. This is surprising, since the size requirement for agonists binding the Gly site is normally quite strict leaving little space for substituents on Gly. However, recently we have demonstrated that larger substituents can be accommodated.⁵¹ Since compound **8** also activates the Glu site, the hydroxy-1,2,3-triazole moiety is the first bioisostere capable of enabling the simultaneous activation of the Glu and Gly sites. It seems probable that the bioisostere of compound **8** is recognized in two different states: negatively charged in the Glu binding pocket and neutral in the Gly binding pocket. This would be enabled by the pK_a of the bioisostere being close to 7 (6.4 for **6a**).⁴¹

4. CONCLUSIONS.

This study provides structural insight into factors determining selectivity among iGluRs and identifies the hydroxy-1,2,3-triazole moiety as a novel bioisostere for a carboxylic acid, leading to important changes in the interaction patterns of the carboxylic acid binding pocket in iGluRs. This hydroxyazole system, beside providing the opportunity to place a substituent oriented in two different directions as opposed to the isoxazolol moiety in AMPA, because a relatively weak pK_a (close to 7) was able to act in differently states depending from the different binding pockets requirements. The synthesis and the study of the properties investigation of enantiomerically pure hydroxy-1,2,3-triazole Glu analogues are in progress; the results will be presented in a following publication.

5. EXPERIMENTAL SECTION.

5.1 Chemistry.

5.1.1 General methods.—All chemical reagents were obtained from commercial sources (Sigma Aldrich, Alfa Aesar and FluoroChem) and used without further purification. Analytical grade solvents (acetonitrile, diisopropyl ether, diethyl ether, dichloromethane [DCM], dimethylformamide [DMF], ethanol 99.8% v/v [EtOH], ethyl acetate [EtOAc], methanol [MeOH], petroleum ether b.p. 40-60 °C [petroleum ether]) were used without further purification. When needed, solvents were dried on 4 Å molecular sieves. Tetrahydrofuran (THF) was distilled immediately prior to use from Na and benzophenone under N₂. Thin layer chromatography (TLC) on silica gel was carried out on 5 × 20 cm plates with 0.25 mm layer thickness to monitor the process of reactions. Anhydrous Na₂SO₄ was used as a drying agent for the organic phases. Purification of compounds was achieved with flash column chromatography on silica gel (Merck Kieselgel 60, 230-400 mesh ASTM)

using the eluents indicated or by CombiFlash Rf 200 (Teledyne Isco) with 5-200 mL/min, 200 psi (with automatic injection valve) using RediSep Rf Silica columns (Teledyne Isco) with the eluents indicated. Compounds synthesized in our laboratory generally varied between 90-99% purity. The biological experiments were only employed on compounds with a purity of at least 95%. Purity was checked using three analytical HPLC methods. HPLC analyses were performed on an UHPLC chromatographic system (Perkin Elmer, Flexar). The analytical columns were an HPLC Ascentis® Express RP-Amide (4.9 × 100 mm, 2.7 μm), HPLC Thermo Aquasil C18 (4.6 × 200 mm, 5 μm) and UHPLC Acquity CSH Fluoro-Phenyl (2.1 × 100 mm, 1.7 μm) (Waters). Compounds were dissolved in water with 0.1% trifluoroacetic acid and injected through a 20 μL loop. The mobile phase for the first method consisted of water / acetonitrile (ratio between 90/20 and 40/60, depending on the compound's retention factor). UHPLC retention times were obtained at flow rates of 0.5 mL/min, and the column effluent was monitored at 262, 254 and 242 nm, referenced against a 360 nm wavelength. The mobile phase for the second method consisted of water / acetonitrile (ratio between 100/0 and 40/60, depending on the compound's retention factor). UHPLC retention times were obtained at flow rates of 1.0 mL/min, and the column effluent was monitored at 262, 254 and 242 nm, referenced against a 360 nm wavelength. The mobile phase for the third method consisted of water / acetonitrile with 0.1% trifluoroacetic acid (ratio between 90/20 and 40/60, depending on the compound's retention factor). UHPLC retention times were obtained at flow rates of 0.5 mL/min, and the column effluent was monitored at 266, 262, 254 and 242 nm, referenced against a 360 nm wavelength. Melting points (m.p.) were measured on a capillary apparatus (Büchi 540). The final m.p. determination was achieved by placing the sample at a temperature 10 °C below the m.p. and applying a heating rate of 1 °C/min. All compounds were routinely checked by ¹H- and ¹³C-NMR and mass spectrometry. MS spectra were performed on Finnigan-Mat TSQ-700 (70 eV, direct inlet for chemical ionization [CI]) or Waters Micromass ZQ equipped with ESCi source for electrospray ionization mass spectra. ¹H- and ¹³C-NMR spectra were performed on a Bruker Avance 300 instrument. For coupling patterns, the following abbreviations are used: *br*= broad, *s*= singlet, *d*= doublet, *dd*= doublet of doublets, *t*= triplet, *q*= quartet, *h*= sextet, *m*= multiplet. Chemical shifts (δ) are given in parts per million (ppm). Values marked with an asterisk are interchangeable. HRMS spectra were recorded on an LTQ Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany), equipped with an atmospheric pressure interface and an ESI ion source instrument. Compounds **9a-c** and **10a-c** were prepared following already described procedures.^{41, 52}

General procedure for synthesis of compounds 6a-c and 7a-c.: A suspension of appropriate protected glutamate triazole derivatives (17a-c or 18a-c, 150 mg) in 6N HCl (25 mL) in a microwave vial, was stirring in a monomodal MW reactor at 100 °C for 4 h and then concentrated *in vacuo*. The crude solid material was then triturated with EtOAc, dissolved in water (10 mL) and the resulting solution concentrated *in vacuo* to afford the hydrochloric salt of the desired compound as a yellow solid. This latter was then converted to zwitterion using a Dowex 50W-X8 (200-400 mesh, capacity 1.7 meq/mL wet bed volume) ion-exchange resin affording the title compound. The resin activation was performed according to the following method. The resin was washed with water (three volumes of resin), 10 % w/w HCl (up to acidic pH), water (up to neutral pH), 10 % w/w NH₃ (up to

basic pH), water (up to neutral pH) and then 10% w/w HCl until acid pH was reached. The resin was then washed with water until neutrality of the eluate, then a solution of the hydrochloride salt, dissolved in slightly acid water to help solubility, was loaded on the top the column. The column was eluted with water until neutral pH, then with 10% w/w NH₃ solution to recover the desired compound in zwitterionic form.

(RS) 2-Amino-3-(4-hydroxy-1-methyl-1H-1,2,3-triazol-5-yl)propanoic acid (6a).: White solid (m.p. = 220.2-221.4 °C; from trituration with diethyl ether). Yield 92% ¹H-NMR (300 MHz, D₂O): δ 3.31 (*dd*, *J* = 6.5, 1.3 Hz, 2H, -CH₂CH-), 3.93 (*s*, 3H, -NCH₃), 4.36 (*t*, *J* = 6.5 Hz, 1H -CH₂CH-); ¹³C-NMR (75 MHz, D₂O): δ 23.3 (-CH₂CH-), 37.3 (-NCH₃), 51.2 (-CH₂CH-), 115.9 (*C-d*), 155.8 (*C-e*), 170.7 (COOH). MS (ES⁺): 187 (M+1), (ES⁻): 185 (M-1). ESI-HRMS (m/z) [M + H]⁺ calcd. for C₆H₁₁N₄O₃ 187.0826, obsd. 187.0825.

(RS) 2-Amino-3-(1-ethyl-4-hydroxy-1H-1,2,3-triazol-5-yl)propanoic acid (6b).: White solid (m.p. = 233.0-239.7 °C (dec.), from trituration with diethyl ether). Yield 72% ¹H-NMR (300 MHz, D₂O + MeOH): δ 1.25 (*t*, *J* = 7.1 Hz, 3H, -NCH₂CH₃), 2.60 (*dd*, *J* = 15.0, 8.7 Hz, 1H, -CH₂CH-), 2.81 (*dd*, *J* = 15.0, 5.1 Hz, 1H, -CH₂CH-), 3.26-3.39 (*m*, 1H, -CH₂CH-), 4.04 (*q*, *J* = 7.1 Hz, 2H, -NCH₂CH₃); ¹³C-NMR (75 MHz, D₂O + MeOH): δ 15.3 (-NCH₂CH₃), 28.6 (-CH₂CH-), 44.6 (-NCH₂CH₃), 56.2 (-CH₂CH-) 115.9 (*C-d*), 165.0 (*C-e*), 182.5 (-COOH). MS (ES⁺): 201 (M+1), (ES⁻): 199 (M-1). ESI-HRMS (m/z) [M + H]⁺ calcd. for C₇H₁₃N₄O₃ 201.0982, obsd. 201.0981.

(RS) 2-Amino-3-(4-hydroxy-1-propyl-1H-1,2,3-triazol-5-yl)propanoic acid (6c).: White solid, (m.p. = 233.0-241.7 °C (dec.), from trituration with diethyl ether). Yield 88%. ¹H-NMR (300 MHz, D₂O + MeOH): δ 0.85 (*t*, *J* = 7.4 Hz, 3H, -NCH₂CH₂CH₃), 1.83 (*h*, *J* = 7.3 Hz, 2H, -NCH₂CH₂CH₃), 3.23 (*dd*, *J* = 6.1, 1.4 Hz, 2H, -CH₂CH-), 4.05 (*dd*, *J* = 6.4, 5.6 Hz, 1H, -CH₂CH-), 4.21 (*t*, *J* = 7.2 Hz, 2H, -NCH₂CH₂CH₃); ¹³C-NMR (75 MHz, D₂O + MeOH): δ 10.7 (-NCH₂CH₂CH₃), 23.0 (-NCH₂CH₂CH₃) 24.3 (-CH₂CH-), 52.2 (-NCH₂CH₂CH₃), 53.8 (-CH₂CH-), 116.1 (*C-d*), 157.2 (*C-e*), 173.3 (-COOH). MS (ES⁺): 215 (M+1), (ES⁻) 213 (M-1). ESI-HRMS (m/z) [M + H]⁺ calcd. for C₈H₁₅N₄O₄ 215.1139, obsd. 215.1138.

(RS) 2-Amino-3-(5-hydroxy-2-methyl-2H-1,2,3-triazol-4-yl)propanoic acid (7a).: White solid (m.p. = 152.2-155.8 °C; from trituration with diethyl ether). Yield 98%. ¹H-NMR (300 MHz, D₂O): δ 3.22 (*d*, *J* = 5.9 Hz, 2H, -CH₂CH-), 3.90 (*s*, 3H, -NCH₃), 4.29 (*t*, *J* = 5.9 Hz, 1H -CH₂CH-); ¹³C-NMR (75 MHz, D₂O): δ 24.3 (-CH₂CH-), 41.3 (-NCH₃), 52.6 (-CH₂CH-), 125.8 (*C-d*), 156.2 (*C-e*), 171.5 (-COOH). MS (ES⁺): 187 (M+1), (ES⁻): 185 (M-1). ESI-HRMS (m/z) [M + H]⁺ calcd. for C₆H₁₁N₄O₃ 187.0826, obsd. 187.0825.

(RS) 2-Amino-3-(2-ethyl-5-hydroxy-2H-1,2,3-triazol-4-yl)propanoic acid (7b).: White solid, (m.p. = 196.0-199.3 °C (dec.), from trituration with diethyl ether). Yield 93%. ¹H-NMR (300 MHz, D₂O + MeOH): δ 1.32 (*t*, *J* = 7.3 Hz, 3H, -NCH₂CH₃), 3.04 (*dd*, *J* = 15.9, 7.7 Hz, 1H, -CH₂CH-), 3.14 (*dd*, *J* = 15.9, 4.7 Hz, 1H, -CH₂CH-), 3.94 (*dd*, *J* = 7.7, 4.7 Hz, 1H, -CH₂CH-), 4.12 (*q*, *J* = 7.3 Hz, 2H, -NCH₂CH₃); ¹³C-NMR (75 MHz, D₂O + MeOH): δ 14.0 (-NCH₂CH₃), 25.2 (-CH₂CH-), 48.9 (-NCH₂CH₃), 54.1 (-CH₂CH-)

126.9 (*C-d*), 158.1 (*C-e*), 173.6 (–COOH). MS (ES+): 201 (M+1), (ES–): 199 (M-1). ESI-HRMS (m/z) [M + H]⁺ calcd. for C₇H₁₃N₄O₃ 201.0982, obsd. 201.0980.

(*RS*) 2-Amino-3-(5-hydroxy-2-propyl-2*H*-1,2,3-triazol-4-yl)propanoic acid (7c): White solid, (m.p. = 233.2-235.4 °C; from trituration with diethyl ether). Yield 93% ¹H-NMR (300 MHz, D₂O + MeOH): δ 0.77 (*t*, *J* = 7.4 Hz, 3H, –NCH₂CH₂CH₃), 1.77 (*h*, *J* = 7.2 Hz, 2H, –NCH₂CH₂CH₃), 3.06 (*dd*, *J* = 15.9, 7.6 Hz, 1H, –CH₂CH–), 3.16 (*dd*, *J* = 15.9, 4.8 Hz, 1H, –CH₂CH–), 3.95 (*dd*, *J* = 7.6, 4.8 Hz, 1H, –CH₂CH–), 4.08 (*t*, *J* = 6.8 Hz, 2H, –NCH₂CH₂CH₃); ¹³C-NMR (75 MHz, D₂O + MeOH): δ 10.8 (–NCH₂CH₂CH₃), 23.2 (–NCH₂CH₂CH₃), 25.8 (–CH₂CH–), 54.7, 56.6 (–CH₂CH–, –NCH₂CH₂CH₃), 127.4 (*C-d*), 158.4 (*C-e*), 174.1 (–COOH). MS (ES+): 215 (M+1), (ES–): 213 (M-1). ESI-HRMS (m/z) [M + H]⁺ calcd. for C₈H₁₅N₄O₄ 215.1139, obsd. 215.1137.

(*RS*) 2-Amino-2-(5-hydroxy-2-methyl-2*H*-1,2,3-triazol-4-yl)acetic acid (8): A suspension of **22** (0.507 mmol; 0.120 g) in 6N hydrochloric acid (10 mL) was heated at reflux for 6 h and then concentrated *in vacuo*. The crude solid material was then triturated with ethyl acetate, dissolved in water (10 mL) and the resulting solution concentrated *in vacuo* to afford the hydrochloride of the title compound as a yellow solid. The hydrochloride of compound **8** was then converted to zwitterion using a Dowex 50W-X8 (200-400 mesh, capacity 1.7 meq/mL wet bed volume) ion-exchange resin to afford the title compound as a white solid. (m.p. = 125.3-127.0 °C. from trituration with diethyl ether). Yield 90 %. ¹H-NMR (300 MHz, D₂O + MeOH): δ 3.53 (*s*, 3H, –NCH₃), 4.10 (*s*, 1H, –CH); ¹³C-NMR (75 MHz, D₂O + MeOH): δ 40.9 (–NCH₃), 51.3 (–CH), 134.0 (*C-d*), 164.4 (*C-e*), 180.8 (–COOH). MS (ES+): 173 (M+1), (ES–): 171 (M-1). ESI-HRMS (m/z) [M + H]⁺ calcd. for C₅H₉N₄O₃ 173.0669, obsd. 173.0669.

General procedure for ester reduction to alcohol (11a-c and 12a-c): Sodium borohydride (20.00 mmol) was added portion wise over 5 min to a solution of corresponding ester (**9a-c** and **10a-c**) (1.00 mmol) in dry EtOH (60 mL) and the mixture was stirred under inert atmosphere at 30 °C overnight. The reaction mixture was poured into iced water (100 mL) and the ethanol evaporated *in vacuo*. The aqueous layer was extracted with diethyl ether (3 × 50 mL). The organic layers were collected, dried and concentrated *in vacuo*. The crude was purified by flash chromatography (eluent: DCM / MeOH 95:5 v/v).

(4-(Benzyloxy)-1-methyl-1*H*-1,2,3-triazol-5-yl)methanol (11a): White solid (m.p. = 74.5-75.1 °C; from trituration with diisopropyl ether). Yield 98%. ¹H-NMR (300 MHz, CDCl₃): δ 2.33 (*br s*, 1H, –CH₂OH), 4.01 (*s*, 3H, –NCH₃), 4.58 (*s*, 2H, –CH₂OH), 5.35 (*s*, 2H, –OCH₂Ph), 7.30-7.48 (*m*, 5H, *aromatic protons*); ¹³C-NMR (75 MHz, CDCl₃): δ 35.8 (–NCH₃), 51.4 (–CH₂OH), 72.3 (–OCH₂Ph), 119.3 (*C-d*), 128.3, 128.4, 128.5 (*aromatic carbons*), 136.5 (*Cipso*), 157.5 (*C-e*). MS (CI): 220 (M+1).

(4-(Benzyloxy)-1-ethyl-1*H*-1,2,3-triazol-5-yl)methanol (11b): Colorless oil. Yield 92%. ¹H-NMR (300 MHz, CDCl₃): δ 1.49 (*t*, *J* = 7.3 Hz, 3H, –NCH₂CH₃), 2.66 (*br s*, 1H, –CH₂OH), 4.32 (*q*, *J* = 7.3 Hz, 2H, –NCH₂CH₃), 4.55 (*s*, 2H, –CH₂OH), 5.31 (*s*, 2H, –OCH₂Ph) 7.28-7.42 (*m*, 5H, *aromatic protons*); ¹³C-NMR (75 MHz, CDCl₃): δ 15.4

($-\text{NCH}_2\text{CH}_3$), 44.6 ($-\text{NCH}_2\text{CH}_3$), 51.4 ($-\text{CH}_2\text{OH}$), 72.4 ($-\text{OCH}_2\text{Ph}$), 118.7 (*C-d*), 128.4, 128.5, 128.6 (*aromatic carbons*), 136.6 (*Cipso*), 157.5 (*C-e*). MS (ES⁺): 234 (M+1).

(4-(Benzyloxy)-1-propyl-1H-1,2,3-triazol-5-yl)methanol (11c): White solid (m.p. = 74.5-75.1 °C; from trituration with diisopropyl ether). Yield 90%. ¹H-NMR (300 MHz, CDCl₃): δ 0.96 (*t*, *J* = 7.4 Hz, 3H, $-\text{NCH}_2\text{CH}_2\text{CH}_3$), 1.78-2.11 (*m*, 2H, $-\text{NCH}_2\text{CH}_2\text{CH}_3$), 2.20 (*s*, 1H, $-\text{OH}$), 4.26 (*t*, 2H, *J* = 7.4 Hz, $-\text{NCH}_2\text{CH}_2\text{CH}_3$), 4.56 (*s*, 2H, $-\text{CH}_2\text{OH}$), 5.37 (*s*, 2H, $-\text{OCH}_2\text{Ph}$), 7.28 - 7.50 (*m*, 5H, *aromatic protons*); ¹³C-NMR (75 MHz, CDCl₃): δ 11.2 ($-\text{NCH}_2\text{CH}_2\text{CH}_3$), 23.5 ($-\text{NCH}_2\text{CH}_2\text{CH}_3$), 51.1, 51.7 ($-\text{NCH}_2\text{CH}_2\text{CH}_3$, $-\text{CH}_2\text{OH}$), 72.7 ($-\text{OCH}_2\text{Ph}$), 119.0 (*C-d*), 128.4, 128.5, 128.6 (*aromatic carbons*), 136.7 (*Cipso*), 157.5 (*C-e*). MS (ES⁺): 248 (M+1).

(5-(Benzyloxy)-2-methyl-2H-1,2,3-triazol-4-yl)methanol (12a): White solid (m.p. = 54.9-55.6 °C; from trituration with diisopropyl ether). Yield 95%. ¹H-NMR (300 MHz, CDCl₃): δ 2.19 (*br s*, 1H, $-\text{CH}_2\text{OH}$), 3.99 (*s*, 3H, $-\text{NCH}_3$), 4.63 (*s*, 2H, $-\text{CH}_2\text{OH}$), 5.24 (*s*, 2H, $-\text{OCH}_2\text{Ph}$), 7.28 - 7.47 (*m*, 5H, *aromatic protons*); ¹³C-NMR (75 MHz, CDCl₃): δ 41.7 ($-\text{NCH}_3$), 54.5 ($-\text{CH}_2\text{OH}$), 72.2 ($-\text{OCH}_2\text{Ph}$), 128.0, 128.3, 128.5 (*aromatic carbons*), 131.6 (*C-d*), 136.3 (*Cipso*), 157.9 (*C-e*). MS (CI): 220 (M+1).

(5-(Benzyloxy)-2-ethyl-2H-1,2,3-triazol-4-yl)methanol (12b): Colorless oil. Yield 94 %. ¹H-NMR (300 MHz, CDCl₃): δ 1.48 (*t*, *J* = 7.3 Hz, 3H, $-\text{NCH}_2\text{CH}_3$), 2.26 (*br s*, 1H, $-\text{CH}_2\text{OH}$), 4.26 (*q*, *J* = 7.3 Hz, 2H, $-\text{NCH}_2\text{CH}_3$), 4.63 (*s*, 2H, $-\text{CH}_2\text{OH}$), 5.24 (*s*, 2H, $-\text{OCH}_2\text{Ph}$), 7.28-7.49 (*m*, 5H, *aromatic protons*); ¹³C-NMR (75 MHz, CDCl₃): δ 14.7 ($-\text{NCH}_2\text{CH}_3$), 50.1 ($-\text{NCH}_2\text{CH}_3$), 54.7 ($-\text{CH}_2\text{OH}$), 72.2 ($-\text{OCH}_2\text{Ph}$), 128.2, 128.4, 128.6 (*aromatic carbons*), 131.3 (*C-d*), 136.5 (*Cipso*), 157.8 (*C-e*). MS (ES⁺): 234 (M+1).

(5-(Benzyloxy)-2-propyl-2H-1,2,3-triazol-4-yl)methanol (12c): Colorless oil. Yield 93%. ¹H-NMR (300 MHz, CDCl₃): δ 0.91 (*t*, *J* = 7.3 Hz, 3H, $-\text{NCH}_2\text{CH}_2\text{CH}_3$), 1.84-1.98 (*m*, 2H, $-\text{NCH}_2\text{CH}_2\text{CH}_3$), 1.98 (*s*, 1H, $-\text{CH}_2\text{OH}$), 4.17 (*t*, 2H, *J* = 7.1 Hz, $-\text{NCH}_2\text{CH}_2\text{CH}_3$), 4.64 (*s*, 2H, $-\text{CH}_2\text{OH}$), 5.25 (*s*, 2H, $-\text{OCH}_2\text{Ph}$), 7.28-7.48 (*m*, 5H, *aromatic protons*); ¹³C-NMR (75 MHz, CDCl₃): δ 11.2 ($-\text{NCH}_2\text{CH}_2\text{CH}_3$), 23.2 ($-\text{NCH}_2\text{CH}_2\text{CH}_3$), 54.8, 56.7 ($-\text{CH}_2\text{OH}$, $-\text{NCH}_2\text{CH}_2\text{CH}_3$), 72.3 ($-\text{OCH}_2\text{Ph}$), 128.4, 128.7 (*aromatic carbons*), 131.3 (*C-d*), 136.5 (*Cipso*), 157.8 (*C-e*). MS (ES⁺): 248 (M+1).

General procedure for synthesis bromide derivatives (13a-c and 14a-c): NBS (1.50 mmol) was added portion wise over 30 min under inert atmosphere to a cooled (−10 °C) solution of appropriate alcohol (**11a-c** or **12a-c**, 1.00 mmol) and triphenylphosphine (1.10 mmol) in dry DCM (30 mL). The reaction mixture was stirred at −10 °C for 1 h and then directly loaded into a flash chromatography column (eluent: DCM) to afford compound as a colorless oil.

4-(Benzyloxy)-5-(bromomethyl)-1-methyl-1H-1,2,3-triazole (13a): Colorless oil. Yield 81%. ¹H-NMR (300 MHz, CDCl₃): δ 3.91 (*s*, 3H, $-\text{NCH}_3$), 4.36 (*s*, 2H, $-\text{CH}_2\text{Br}$), 5.32 (*s*, 2H, $-\text{OCH}_2\text{Ph}$), 7.19-7.43 (*m*, 5H, *aromatic protons*); ¹³C-NMR (75 MHz, CDCl₃): δ 16.8 ($-\text{CH}_2\text{Br}$), 35.6 ($-\text{NCH}_3$), 71.8 ($-\text{OCH}_2\text{Ph}$), 115.9 (*C-d*), 128.1, 128.2, 128.4 (*aromatic carbons*), 136.2 (*Cipso*), 157.5 (*C-e*). MS (CI): 282/284 (M+1).

4-(Benzyloxy)-5-(bromomethyl)-1-ethyl-1*H*-1,2,3-triazole (13b): Colorless oil. Yield 65%. ¹H-NMR (300 MHz, CDCl₃): δ 1.58 (*t*, *J* = 7.3 Hz, 3H, –NCH₂CH₃) 4.32 (*q*, *J* = 7.3 Hz, 2H, –NCH₂CH₃), 4.43 (*s*, 2H, –CH₂Br), 5.42 (*s*, 2H, –OCH₂Ph) 7.27-7.52 (*m*, 5H, *aromatic protons*); ¹³C-NMR (75 MHz, CDCl₃): δ 15.0 (–NCH₂CH₃), 16.9 (–CH₂Br), 44.4 (–NCH₂CH₃), 72.0 (–OCH₂Ph), 115.4 (*C-d*), 128.3, 128.4, 128.6 (*aromatic carbons*), 136.6 (*Cipso*), 157.9 (*C-e*). MS (ES+): 296/298 (M+1).

4-(Benzyloxy)-5-(bromomethyl)-1-propyl-1*H*-1,2,3-triazole (13c): Colorless oil. Yield 60%. ¹H NMR (300 MHz, CDCl₃): δ 1.0 (*t*, *J* = 7.4 Hz, 3H, –NCH₂CH₂CH₃), 1.91-2.07 (*m*, 2H, –NCH₂CH₂CH₃), 4.24 (*t*, *J* = 7.4 Hz, 2H, –NCH₂CH₂CH₃), 4.43 (*s*, 2H, –CH₂Br), 5.42 (*s*, 2H, –OCH₂Ph), 7.28-7.53 (*m*, 5H, *aromatic protons*); ¹³C NMR (75 MHz, CDCl₃): δ 11.3 (–NCH₂CH₂CH₃), 17.0 (–CH₂Br), 23.2 (–NCH₂CH₂CH₃), 50.1 (–NCH₂CH₂CH₃), 72.1 (–OCH₂Ph), 115.6 (*C-d*), 128.4, 128.5, 128.7 (*aromatic carbons*), 136.6 (*Cipso*), 157.9 (*C-e*). MS (ES+): 310 / 312 (M+1).

4-(Benzyloxy)-5-(bromomethyl)-2-methyl-2*H*-1,2,3-triazole (14a): Colorless oil. Yield 93%. ¹H-NMR (300 MHz, CDCl₃): δ 4.02 (*s*, 3H, –NCH₃), 4.46 (*s*, 2H, –CH₂Br), 5.27 (*s*, 2H, –OCH₂Ph), 7.30-7.52 (*m*, 5H, *aromatic protons*); ¹³C-NMR (75 MHz, CDCl₃): δ 20.4 (–CH₂Br), 42.1 (–NCH₃), 72.3 (–OCH₂Ph), 128.0, 128.4, 128.7 (*aromatic carbons*), 128.8 (*C-d*), 136.3 (*Cipso*), 158.0 (*C-e*). MS (CI): 282/284 (M+1).

4-(Benzyloxy)-5-(bromomethyl)-2-ethyl-2*H*-1,2,3-triazole (14b): Colorless oil. Yield 66%. ¹H-NMR (300 MHz, CDCl₃): δ 1.50 (*t*, *J* = 7.3Hz, 3H, –NCH₂CH₃), 4.27 (*q*, *J* = 7.3 Hz, 2H, –NCH₂CH₃), 4.48 (*s*, 2H, –CH₂Br), 5.28 (*s*, 2H, –OCH₂Ph) 7.32-7.48 (*m*, 5H, *aromatic protons*); ¹³C-NMR (75 MHz, CDCl₃): δ 14.6 (–NCH₂CH₃), 20.6 (–CH₂Br), 50.3 (–NCH₂CH₃), 72.2 (–OCH₂Ph), 128.1, 128.3, 128.4, (*C-d*), 128.6 (*aromatic carbons*), 136.3 (*Cipso*), 157.9 (*C-e*). MS (ES+): 296 / 298 (M+1).

4-(Benzyloxy)-5-(bromomethyl)-2-propyl-2*H*-1,2,3-triazole (14c): Colorless oil. Yield 66%. ¹H NMR (300 MHz, CDCl₃): δ 0.84 (*t*, *J* = 7.4 Hz, 3H, –NCH₂CH₂CH₃), 1.77-1.92 (*m*, 2H, –NCH₂CH₂CH₃), 4.11 (*t*, *J* = 7.4 Hz, 2H, –NCH₂CH₂CH₃), 4.40 (*s*, 2H, –CH₂Br), 5.20 (*s*, 2H, –OCH₂Ph), 7.24-7.43 (*m*, 5H, *aromatic protons*); ¹³C NMR (75 MHz, CDCl₃): δ 11.4 (–NCH₂CH₂CH₃), 20.8 (–CH₂Br), 23.4 (–NCH₂CH₂CH₃), 57.2 (–NCH₂CH₂CH₃), 72.6 (–OCH₂Ph), 128.4, 128.7, 128.8 (*C-d*), 128.9 (*aromatic carbons*), 136.7 (*Cipso*), 158.1 (*C-e*). MS (ES+): 310 / 312 (M+1).

General procedure for synthesis of compounds 17a-c and 18a-c: Sodium hydride (60% w/w, 1.20 mmol) was added portion wise to an ice cooled solution of diethyl-2-acetamidomalonate (1.10 mmol) in dry THF (35 mL) kept under inert atmosphere. The reaction mixture was stirred at rt for 1 h, then a solution of appropriate bromide derivatives (**13a-c** or **14a-c**, 1.00 mmol) in dry THF (10 mL) was added over 15 min. The resulting mixture was stirred at rt for 36 h, and then poured into a saturated solution of NH₄Cl (80 mL). The THF was evaporated under reduced pressure and the aqueous layer was extracted with diethyl ether (3 × 50 mL). The organic layers were collected, dried and concentrated *in vacuo* to afford the compound as a white solid (**15a-c** and **16a-c**). This latter was used in the next step without any further purification and characterized *via* mass spectroscopy.

Pd/C (10% w/w, 90 mg) was added to a solution of the solid material rising from the previously step (**15a-c** or **16a-c**, 0.900 g) in dry THF (30 mL). The resulting mixture was vigorously stirred under hydrogen (1 atm) at rt for 1 h. The resulting mixture was then filtered over celite and the filtrate was concentrated *in vacuo*. The crude material was purified by flash chromatography (eluent: DCM / 2-propanol 95:5 v/v) to afford compounds **17a-c** and **18a-c**.

Diethyl 2-acetamido-2-((4-(benzyloxy)-1-methyl-1H-1,2,3-triazol-5-yl)methyl)malonate

(15a). The crude material was purified by flash chromatography (first petroleum ether / EtOAc 7:3 v/v next 6:4 v/v) to afford the compound as a white solid. MS (CI): 419 (M+1).

Diethyl 2-acetamido-2-((4-(benzyloxy)-1-ethyl-1H-1,2,3-triazol-5-yl)methyl)malonate

(15b). The crude material was purified by flash chromatography (first petroleum ether / EtOAc 7:3 v/v next 6:4 v/v) to afford the compound as a white solid. MS (CI): 433 (M+1).

Diethyl 2-acetamido-2-((4-(benzyloxy)-1-propyl-1H-1,2,3-triazol-5-yl)methyl)malonate

(15c). The crude material was purified by flash chromatography (eluent starting from petroleum ether / EtOAc 9:1 v/v to 7:3) to afford the compound as a white solid. MS (CI): 447 (M+1).

Diethyl 2-acetamido-2-((5-(benzyloxy)-2-methyl-2H-1,2,3-triazol-4-yl)methyl)malonate

(16a). The crude material was purified by flash chromatography (first petroleum ether / EtOAc 7:3 v/v next 6:4 v/v) to afford the compound as a white solid. MS (CI): 419 (M+1).

Diethyl 2-acetamido-2-((5-(benzyloxy)-2-ethyl-2H-1,2,3-triazol-4-yl)methyl)malonate

(16b). The crude material was purified by flash chromatography (first petroleum ether / EtOAc 7:3 v/v next 6:4 v/v) to afford the compound as a white solid. MS (CI): 433 (M+1).

Diethyl 2-acetamido-2-((5-(benzyloxy)-2-propyl-2H-1,2,3-triazol-4-yl)methyl)malonate

(16c). The crude material was purified by flash chromatography (eluent petroleum ether / acetone 8:2 v/v) to afford the compound as a colorless oil. MS (CI): 447 (M+1).

Diethyl 2-acetamido-2-((4-hydroxy-1-methyl-1H-1,2,3-triazol-5-yl)methyl)malonate

(17a). White solid (m.p. = 181.4-182.4 °C; from trituration with diisopropyl ether). Yield 69% over two steps. ¹H-NMR (300 MHz, DMSO): δ 1.16 (t, *J* = 7.1 Hz, 6H –OCH₂CH₃), 1.93 (s, 3H, –NHCOCH₃), 3.46 (s, 2H, –CH₂C–), 3.68 (s, 3H, –NCH₃), 4.04-4.23 (m, 4H –OCH₂CH₃), 8.34 (s, 1H, –NHCOCH₃), 9.95 (br s, 1H, –OH), ¹³C-NMR (75 MHz, DMSO): δ 13.7 (–OCH₂CH₃), 22.1 (–NHCOCH₃), 25.6 (–CH₂C–), 35.1 (–NCH₃), 62.0 (–OCH₂CH₃), 65.3 (–CH₂C–), 112.7 (C-d), 157.1 (C-e), 166.8 (–COOEt), 170.1 (–NHCOCH₃). MS (CI): 329 (M+1).

Diethyl 2-acetamido-2-((1-ethyl-4-hydroxy-1H-1,2,3-triazol-5-yl)methyl)malonate

(17b). Colourless oil. Yield 67% over two steps. ¹H-NMR (300 MHz, CDCl₃): δ 1.27 (t, *J* = 6.8 Hz, 6H, –OCH₂CH₃), 1.44 (t, *J* = 7.0 Hz, 3H, –NCH₂CH₃), 2.01 (s, 3H, –NHCOCH₃), 3.72 (s, 2H, –CH₂C–), 4.15 (q, *J* = 7.0 Hz, 2H, –NCH₂CH₃), 4.21-4.37 (m, 4H, –OCH₂CH₃), 6.91 (s, 1H, –NHCOCH₃), 7.81 (vvbr s, 1H, –OH), ¹³C-NMR (75 MHz,

CDCl₃): δ 14.0 (–OCH₂CH₃), 15.4 (–NCH₂CH₃), 23.1 (–NHCOCH₃), 26.2 (–CH₂C–), 44.2 (–NCH₂CH₃), 63.3 (–OCH₂CH₃), 65.3 (–CH₂C–), 112.9 (*C-d*), 157.3 (*C-e*), 167.1 (–COOEt), 170.2 (–NHCOCH₃). MS (ES⁺): 343 (M+1).

Diethyl 2-acetamido-2-((4-hydroxy-1-propyl-1*H*-1,2,3-triazol-5-yl)methyl)malonate

(17c). White solid (m.p. = 167.3 - 169.7 °C; from trituration with diisopropyl ether). Yield 69% over two steps. ¹H-NMR (300 MHz, CDCl₃): δ 0.90 (*t*, *J* = 7.4 Hz, 3H, –NCH₂CH₂CH₃), 1.27 (*t*, *J* = 7.1 Hz, 6H, –OCH₂CH₃), 1.75-1.91 (*m*, 2H, –NCH₂CH₂CH₃), 2.01 (*s*, 3H, –NHCOCH₃), 3.72 (*s*, 2H, –CH₂C–), 4.07 (*t*, *J* = 7.3 Hz, 2H, –NCH₂CH₂CH₃), 4.23-4.35 (*m*, 4H, –OCH₂CH₃), 6.89 (*s*, 1H, –NHCOCH₃); ¹³C-NMR (75 MHz, CDCl₃): δ 11.1 (–NCH₂CH₂CH₃), 14.0 (–OCH₂CH₃), 23.1 (–NCH₂CH₂CH₃), 23.6 (–NHCOCH₃), 26.2 (–CH₂C–), 50.5 (–NCH₂CH₂CH₃), 63.3 (–OCH₂CH₃), 65.3 (–CH₂C–), 113.0 (*C-d*), 157.5 (*C-e*), 167.1 (–COOEt), 170.1 (–NHCOCH₃). MS (ES⁺): 357 (M+1).

Diethyl 2-acetamido-2-((5-(benzyloxy)-2-methyl-2*H*-1,2,3-triazol-4-yl)methyl)malonate

(18a). White solid (m.p. = 124.5-125.4 °C; from trituration with diisopropyl ether). Yield 70% over two steps. ¹H-NMR (300 MHz, DMSO): δ 1.16 (*t*, *J* = 7.0 Hz, 6H –OCH₂CH₃), 1.86 (*s*, 3H, –NHCOCH₃), 3.34 (*s*, 2H, –CH₂C–), 3.81 (*s*, 3H, –NCH₃), 4.14 (*q*, *J* = 7.0 Hz, 4H –OCH₂CH₃), 7.96 (*s*, 1H, –NHCOCH₃), 10.17 (*s*, 1H, –OH), ¹³C-NMR (75 MHz, DMSO): δ 14.6 (–OCH₂CH₃), 23.0 (–NHCOCH₃), 27.5 (–CH₂C–), 42.0 (–NCH₃), 62.5 (–OCH₂CH₃), 65.6 (–CH₂C–), 126.2 (*C-d*), 158.3 (*C-e*), 167.9 (–COOEt), 170.2 (–NHCOCH₃). MS (CI): 329 (M+1).

Diethyl 2-acetamido-2-((5-(benzyloxy)-2-ethyl-2*H*-1,2,3-triazol-4-yl)methyl)malonate

(18b). White solid (m.p. = 110.7-111.9 °C; from trituration with diisopropyl ether). Yield 33% over two steps. ¹H-NMR (300 MHz, CDCl₃): δ 1.27 (*t*, *J* = 7.1 Hz, 6H, –OCH₂CH₃), 1.41 (*t*, *J* = 7.3 Hz, 3H, –NCH₂CH₃), 2.02 (*s*, 3H, –NHCOCH₃), 3.65 (*s*, 2H, –CH₂C–), 4.14 (*q*, *J* = 7.3 Hz, 2H, –NCH₂CH₃), 4.25 (*q*, *J* = 7.1 Hz, 4H, –OCH₂CH₃), 6.67 (*s*, 1H, –NHCOCH₃), 9.84 (*br s*, 1H, –OH), ¹³C-NMR (75 MHz, CDCl₃): δ 14.1 (–OCH₂CH₃), 14.6 (–NCH₂CH₃), 23.1 (–NHCOCH₃), 27.6 (–CH₂C–), 49.8 (–NCH₂CH₃), 62.9 (–OCH₂CH₃), 66.2 (–CH₂C–), 126.1 (*C-d*), 157.7 (*C-e*), 167.6 (–COOEt), 169.9 (–NHCOCH₃). MS (ES⁺): 343 (M+1).

Diethyl 2-acetamido-2-((5-(benzyloxy)-2-propyl-2*H*-1,2,3-triazol-4-yl)methyl)malonate

(18c). White solid (m.p. = 147.1-149.7 °C; from trituration with diisopropyl ether). Yield 60% over two steps. ¹H-NMR (300 MHz, CDCl₃): δ 0.86 (*t*, *J* = 7.4 Hz, 3H, –NCH₂CH₂CH₃), 1.27 (*t*, *J* = 7.1 Hz, 6H, –OCH₂CH₃), 1.83 (*h*, *J* = 7.2 Hz, 2H, –NCH₂CH₂CH₃), 2.02 (*s*, 3H, –NHCOCH₃), 3.65 (*s*, 2H, –CH₂C–), 4.06 (*t*, *J* = 7.0 Hz, 2H, –NCH₂CH₂CH₃), 4.25 (*q*, *J* = 7.1 Hz, 4H, –OCH₂CH₃), 6.67 (*s*, 1H, –NHCOCH₃), 8.70 (*vv br s*, 1H, –OH), ¹³C-NMR (75 MHz, CDCl₃): δ 11.1 (–NCH₂CH₂CH₃), 14.0 (–OCH₂CH₃), 23.0 (–NCH₂CH₂CH₃), 23.1 (–NHCOCH₃), 27.5 (–CH₂C–), 56.4 (–NCH₂CH₂CH₃), 62.9 (–OCH₂CH₃), 66.2 (–CH₂C–), 126.0 (*C-d*), 157.7 (*C-e*), 167.6 (–COOEt), 169.9 (–NHCOCH₃). MS (ES⁺): 357 (M+1).

2-(5-(Benzyloxy)-2-methyl-2H-1,2,3-triazol-4-yl)acetonitrile (19): Compound **14a** (5.73 mmol; 1.61 g) was added to a solution of NaCN (11.46 mmol; 0.562 g) in EtOH / water 9:1 v/v (50 mL). The reaction was stirred at rt for 24 h, and then poured into water (60 mL). EtOH was evaporated *in vacuo* and the aqueous layer was extracted with DCM (3 × 50 mL). The organic layers were collected, dried and concentrated *in vacuo*. The crude material was purified by flash chromatography (eluent: from petroleum ether / EtOAc 9:1 v/v to 7:3) to afford the title compound as a colorless oil. Yield 53%. ¹H-NMR (300 MHz, DMSO): δ 3.99 (s, 5H, -NCH₃ overlapped -CH₂CN), 5.25 (s, 2H, -OCH₂Ph), 7.28-7.51 (m, 5H, aromatic protons); ¹³C-NMR (75 MHz, DMSO): δ 12.4 (-CH₂CN), 41.8 (-NCH₃), 71.8 (-OCH₂Ph), 117.2 (-CH₂CN), 121.9 (C-d), 127.9, 128.2, 128.4 (aromatic carbons), 136.2 (Cipso), 156.9 (C-e). MS (CI): 229 (M+1).

Ethyl 2-(5-(benzyloxy)-2-methyl-2H-1,2,3-triazol-4-yl)acetate (20): Dry EtOH (4.00 eq; 17.5 mmol; 1.02 mL), TMSCl (4.00 eq; 17.5 mmol; 2.23 mL) and **19** (3.73 mmol; 0.850 g) were subsequently added to a dry flask kept under inert atmosphere at rt. The reaction mixture was stirred for 24 h, and then quenched with water (5 mL). Na₂CO₃ (14.9 mmol; 2.25 g) was then added portion wise to the reaction mixture. The obtained suspension was extracted with EtOAc (3 × 20 mL), the organic layers were collected, dried and concentrated *in vacuo*. The resulting crude material was purified by flash chromatography (eluent: petroleum ether / EtOAc 9:1 v/v) to afford the title compound as a colorless oil. Yield 84%. ¹H-NMR (300 MHz, CDCl₃): δ 1.23 (t, J = 7.1 Hz, 3H, -OCH₂CH₃), 3.61 (s, 2H, -CH₂COOEt), 4.00 (s, 3H, -NCH₃), 4.16 (q, J = 7.1 Hz, 2H, -OCH₂CH₃), 5.23 (s, 2H, -OCH₂Ph), 7.28-7.46 (m, 5H, aromatic protons); ¹³C-NMR (75 MHz, CDCl₃): δ 14.1 (-OCH₂CH₃), 29.7 (-CH₂COOEt), 41.7 (-NCH₃), 61.1 (-OCH₂CH₃), 72.0 (-OCH₂Ph), 125.7 (C-d), 127.8, 128.1, 128.5 (aromatic carbons), 136.5 (Cipso), 158.4 (C-e), 169.9 (-CH₂COOEt). MS (ES⁺): 276 (M+1).

(E/Z) Ethyl 2-(5-(benzyloxy)-2-methyl-2H-1,2,3-triazol-4-yl)-2-(hydroxyimino)acetate (21): A solution of **20** (3.27 mmol; 0.900 g) in dry EtOH (15 mL) was slowly dropped into a solution of sodium hydride (60% w/w in mineral oil, 4.00 eq, 13.1 mmol, 0.503 g) in dry EtOH, stirred and kept under inert atmosphere. The reaction mixture was stirred at 0 °C for 30 min and then EtONO (4.00 eq; 13.1 mmol; 12.4 mL) was added. Then the reaction mixture was stirred rt for 20 h, and then glacial acetic acid was added to reach an acidic pH. The ethanol was evaporated *in vacuo* and the residue dissolved in water (50 mL) and the resulting solution was extracted with EtOAc (3 × 30 mL). The combined organic layers were collected, dried and concentrated *in vacuo*. The crude material was purified by flash chromatography (eluent: petroleum ether / EtOAc 7:3 v/v) to afford the title compound (mixture 1:1 of Z and E isomers) as a yellow solid. Yield 44%. ¹H-NMR (300 MHz, CDCl₃): δ 1.27, 1.32 (t, 3H, J = 7.1, -OCH₂CH₃), 4.02, 4.11 (s, 3H, -NCH₃), 4.38, 4.29 (q, 2H, J = 7.1, -OCH₂CH₃), 5.23, 5.27 (s, 2H, -OCH₂Ph), 7.27-7.47 (m, 5H, aromatic protons), 9.72 (s, 1H, -NOH); ¹³C-NMR (75 MHz, CDCl₃): δ 14.1, 14.2 (-OCH₂CH₃), 42.4, 42.5 (-NCH₃), 62.3, 62.4 (-OCH₂CH₃), 72.5, 72.6 (-OCH₂Ph), 121.3, 124.1 (C-d), 127.8, 128.0, 128.3, 128.4, 128.5, 128.6 (aromatic carbons), 135.8, 136.2 (Cipso), 141.0, 143.9 (-C=NOH), 158.3, 159.0 (C-e), 162.4, 174.4 (-COOEt). MS (ES⁺): 305 (M+1).

Ethyl 2-amino-2-(5-(benzyloxy)-2-methyl-2H-1,2,3-triazol-4-yl)acetate (22).: Pd/C (10% w/w, 24 mg) and a saturated solution of hydrochloric acid in dry EtOH were added to a solution of **21** (0.789 mmol, 240 mg) in dry EtOH (35 mL) kept under inert atmosphere. The resulting mixture was vigorously stirred at rt under hydrogen for 40 min, then filtered over celite. The filtrate was concentrated *in vacuo* to give a crude solid material that was then triturated with diisopropyl ether, dissolved in water (10 mL) and the resulting solution concentrated *in vacuo* to afford the hydrochloric salt of the title compound as a yellow solid (m.p. = 63.2-64.1 °C; from trituration with diisopropyl ether). Yield 93%. ¹H-NMR (300 MHz, D₂O + MeOH): δ 1.21 (t, 3H, *J* = 7.1, -OCH₂CH₃), 3.97 (s, 3H, -NCH₃), 4.29 (q, 2H, *J* = 7.1, -OCH₂CH₃), 5.38 (s, 1H, -CH-); ¹³C-NMR (75 MHz, D₂O + MeOH): δ 13.7 (-OCH₂CH₃), 42.2 (-NCH₃), 47.8 (-CH-), 65.0 (-OCH₂CH₃), 123.2 (*C-d*), 156.8 (*C-e*), 168.0 (-COOEt). MS (ES⁺): 201 (M+1)

5.2 *In vitro* pharmacology.

5.2.1 Native receptor binding assays.—Affinities for native AMPA, KA and NMDA receptors in rat cortical synaptosomes were determined using 5 nM [³H]AMPA (57.5 Ci/mmol),⁵³ 5 nM [³H]KA (43.8 Ci/mmol),⁵⁴ and 2 nM [³H]CGP 39653 (41.9 Ci/mmol),⁵⁵ respectively, with minor modifications as previously described.⁵⁶ Rat brain membrane preparations used in these receptor binding experiments were prepared according to a method previously described.⁵⁷

5.2.2 Recombinant receptor binding assays.—*Sf9* cells were cultured and infected with recombinant baculovirus of rat AMPA receptors (GluA1_o-4_o) and membranes prepared and used for binding as previously detailed.^{58, 59} The affinities of the compounds for GluA1_o, GluA2(*R*)_o, GluA3_o and GluA4_o were determined from competition experiments with 2-5 nM (*RS*)-[³H]AMPA. Italic letters in parentheses indicate the RNA-edited isoforms of the subunits. Ligand concentration-response curves were analyzed using GraphPad Prism v6 (GraphPad Software Inc., San Diego, CA) to determine the IC₅₀, n_H and K_i (n = 3 - 4 experiments at 12-15 ligand concentrations conducted in triplicate).

5.2.3 Two-electrode voltage-clamp electrophysiology for AMPA receptors.—Surgical procedures were conducted under the approval of the Danish Ministry of Justice Animal Experiments Inspectorate. Mature female *Xenopus laevis* were anaesthetized using 0.1% ethyl 3-aminobenzoate methanesulfonate (tricaine) and ovaries were surgically removed. The ovarian tissue was dissected and treated with 2 mg/mL collagenase in nominally Ca²⁺-free Barth's medium (in mM: 88 NaCl, 1 KCl, 0.33 Ca(NO₃)₂, 0.41 CaCl₂, 0.82 MgSO₄, 2.4 NaHCO₃, 10 HEPES, pH 7.4) for 2 h at rt. On the second day, oocytes were injected with 25-50 nL of (≈ 1 mg/mL) rat GluA2(*Q*)_i cRNA and incubated in Barth's medium with 0.10 mg/mL gentamicin (Sigma Chemical) and 1 % penicillin-streptomycin (Life Technologies, Paisley, UK) at 17 °C. Oocytes were typically used for recordings from 3 to 10 days post-injection and receptor responses were measured using two-electrode voltage-clamp electrophysiology (GeneClamp 500B, Axon Instruments, Union City, CA) with both microelectrodes filled with 3 M KCl. Recordings were made while the oocytes were continuously superfused with frog Ringer's solution (in mM: 115 NaCl, 2 KCl, 1.8 BaCl₂, 5 HEPES, pH 7.6). Compounds were dissolved in frog Ringer's solution and added

by bath application. Recordings were made at rt. Efficacy measurements were made in the presence of 100 μ M cyclothiazide in order to block receptor desensitization. To determine the maximum response, oocytes were stimulated with 1 mM (*S*)-glutamate plus 100 μ M cyclothiazide ($n = 10$ oocytes). Agonist concentration-response curves were analyzed using GraphPad Prism v6 (GraphPad Software Inc., San Diego, CA) to determine the EC_{50} using a four-parameter logistic equation ($n = 6$ oocytes).

5.2.4 Fluo-4/ Ca^{2+} assay.—The functional properties of (*S*)-glutamate and compounds **6a-c**, **7a-d** and **8** were characterized at stable GluA1 $_i$ -, GluA2(*Q*) $_i$ -, GluA3 $_i$ - and GluA4 $_i$ -HEK293 cell lines in the Fluo-4/ Ca^{2+} assay essentially as previously described.⁶⁰ The cells were split into poly-D-lysine-coated black 96-well plates with clear bottom (BD Biosciences, Bedford, MA). 16-24 hours later the medium was aspirated, and the cells were incubated in 50 μ L assay buffer [Hanks Buffered Saline Solution (HBSS) containing 20 mM HEPES, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 2.5 mM probenecid, pH 7.4] supplemented with 6 μ M Fluo4/AM (Molecular Probes, Eugene, OR) at 37 °C for 1 hour. The buffer was aspirated, the cells were washed with 100 μ L assay buffer, and then 100 μ L assay buffer supplemented with 100 μ M cyclothiazide was added to the wells (in the antagonist experiments, this buffer was supplemented with various antagonist concentrations). Then the 96-well plate was assayed in a FlexStation3 Benchtop Multi-Mode Microplate Reader (Molecular Devices) measuring emission at 520 nm [in fluorescence units (FU)] caused by excitation at 485 nm a total of 90 sec before and after addition of 33 μ L agonist solution (the agonist was dissolved in assay buffer). The experiments were performed in duplicate at least three times for each compound at each cell line.

5.2.5 Two-electrode voltage-clamp electrophysiology for NMDA receptors.—

The cDNAs encoding rat NMDA receptor subunits GluN1-1a (Genbank accession number [U11418](#) and [U08261](#); hereafter GluN1), GluN2A (D13211), GluN2B (U11419), GluN2C (M91563), and GluN2D (L31611) were provided by Dr. S. Heinemann (Salk Institute, La Jolla, CA), Dr. S. Nakanishi (Osaka Bioscience Institute, Osaka, Japan), and Dr. P. Seeburg (University of Heidelberg). For expression in *Xenopus laevis* oocytes, the rat GluN2B open reading frame was edited without changing the amino acid sequence to remove a T7 RNA polymerase termination site located in the C-terminal domain.⁶¹ *Xenopus* oocytes were obtained from Rob Weymouth (Xenopus 1, Dexter, MI). Oocyte incubation, cRNA synthesis, and injections were performed essentially as previously described.⁶² Briefly, the oocytes were injected with cRNAs encoding GluN1 and GluN2 in a 1:2 ratio, and maintained at 17 °C in Barth's solution containing (in mM) 88 NaCl, 1 KCl, 2.4 $NaHCO_3$, 0.82 $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.91 $CaCl_2$, 10 HEPES (pH 7.5 with NaOH) supplemented with 100 IU/mL penicillin, 100 μ g/mL streptomycin and 50 μ g/mL gentamycin (Invitrogen, Life Technologies, Paisley, UK). Two-electrode voltage-clamp recordings were performed at rt 2-6 days post-injection with the extracellular recording solution comprised of (in mM) 90 NaCl, 1 KCl, 10 HEPES, 0.5 $BaCl_2$ and 0.01 EDTA (pH 7.4 with NaOH) at a holding potential of -40 mV as previously described.⁶² NMDA receptor ligands were dissolved in extracellular recording solution and applied to the oocyte by gravity-driven perfusion. Concentration-response data measured using two-electrode

voltage-clamp electrophysiology were fitted to the Hill equation to obtain EC₅₀ values for individual oocytes as previously described.⁶²

5.3 Crystal Structures.

5.3.1 Expression and purification.—The rat GluA2-ABD (segment S1 residues 413–527 and segment S2 residues 653–797, numbering according to UNP P19491, connected by a Gly-Thr linker) was expressed and purified essentially as previously described⁶³ using 50 mM L-Asp in the buffers except for the final step. After removal of purification tag two expression remnants (Gly-Ala) residues are present in the N-terminus.

5.3.2 Crystallization and data collection.—Both ligands were crystallized in complex with GluA2-ABD using the hanging drop vapour diffusion method at 6 °C. The drops contained 1 µL of the protein-ligand solution and 1 µL of reservoir solution, and the reservoir volume was 0.5 mL. The protein-ligand solutions were as follows: GluA2-ABD in complex with **6b**: 4.8 mg/mL GluA2-ABD, 4.8 mM **6b**, 10 mM HEPES pH 7.0, 20 mM NaCl, 1 mM EDTA. GluA2-ABD in complex with **7a**: 4.5 mg/mL GluA2-ABD, 7.5 mM **7a**, 10 mM HEPES pH 7.0, 20 mM NaCl, 1 mM EDTA. Crystals for diffraction studies appeared within a couple of days, and were obtained with the following reservoir conditions: GluA2-ABD with **6b**: 20% PEG4000, 0.3 M lithium sulfate, 0.1 M phosphate-citrate, pH 4.5. GluA2-ABD with **7a**: 10% PEG4000, 0.1 M lithium sulfate, 0.1 M phosphate-citrate, pH 4.5. Crystals were flash cooled in liquid nitrogen after briefly soaking in cryo buffer consisting of the reservoir solution containing 10 % glycerol. X-ray data of the GluA2-ABD in complex with **6b** and **7a** were collected at the BioMAX beamline (MAXIV, Lund, Sweden) at a wavelength of 0.9800 Å.

5.3.3 Structure determination.—The diffraction data were processed with XDS⁶⁴ and scaled using SCALA⁶⁵ within CCP4.⁶⁶ The structures were solved with molecular replacement using PHASER⁶⁷ in CCP4 and the structure of GluA2-ABD with 2-Me-Tet-AMPA as search model (PDB code 1M5B, chain A). The structures were first refined with AUTOBUILD⁶⁸ and then in PHENIX.⁶⁹ The program MAESTRO [version 10.1.013, Schrodinger, LLC, New York, NY, 2015] was used to generate coordinate files for **6b** and **7a**. The energy minimization and conformational search were performed in water. The parameter files for **6b** and **7a** for refinements in PHENIX were obtained using eLBOW,⁷⁰ keeping the geometry obtained from MAESTRO. The structures were manually adjusted in COOT⁷¹ and refined in PHENIX with individual isotropic B-factors, NCS, TLS and riding H atoms. GluA2-ABD domain closures were calculated using the DynDom server⁷² relative to the apo structure of GluA2-ABD flop (PDB code 1FTO, chain A). Figures 4 and 5 were prepared in PyMOL [version 2.0.3, The PyMOL Molecular Graphics Systems, V.S., LLC].

Supplementary Material

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ABBREVIATION USED

Glu	glutamate
AMPA	(S)-2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid
NMDA	N-methyl-D-aspartic acid
KA	kainic acid
GluRs	glutamate receptors
iGluRs	ionotropic glutamate receptors
mGluRs	metabotropic glutamate receptors
ABD	agonist binding domain
SAR@	significant agonist response
MeTetAMPA	2-methyl-tetrazole-AMPA
BnTetAMPA	benzyl-tetrazole-AMPA

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Figure 1.
 Structures of Glutamic acid, Kainic acid, NMDA, selective AMPA analogues and hydroxy-1,2,3-triazole analogues **6a-c**, **7a-c** and **8**.

Figure 2.

Normalized, averaged concentration-response data at homomeric rat GluA2(Q)₁ receptors expressed in *Xenopus* oocytes. Data are given as means $\pm$ SEM values of the pooled data. Responses from each oocyte were normalized to the maximum response of each oocyte before averaging. The top of the curve is fixed to 100% and the bottom to 0%. EC₅₀ = 65 ± 6 μ M, Hill slope = 1.07 ± 0.08 (n = 6 oocytes). *Inset.* Representative two-electrode voltage-clamp recording ($V_h = -60$ mV) with duplicate stimulations (0.3 – 1000 μ M) followed by one stimulation at 3000 μ M and one final stimulation of 1000 μ M Glu.

Figure 3.

(A) Representative two-electrode voltage-clamp recording of responses from recombinant GluN1/2D receptors expressed in *Xenopus* oocytes. Responses were activated by 100 μ M compound 8 as indicated by the grey bar, and control responses were activated by co-application of 300 μ M Glu plus 100 μ M Gly. The horizontal scale bars indicate 30 sec and the vertical scale bars indicate 200 nA. (B) Summary of responses to 100 μ M compound 8 alone as percentage of control at recombinant GluN1/2A-D receptors. Data are given as mean $\pm$ SEM values from 4-6 oocytes. (C, D) Concentration-response data for compound 8 at recombinant NMDA receptor subtypes measured using two-electrode voltage-clamp recordings in the continuous presence of either 300 μ M Glu (C) or 100 μ M Gly (D). Responses are normalized to maximal activation by 300 μ M Glu plus 100 μ M Gly. Data are given as mean $\pm$ SEM values from 4 oocytes.

Figure 4. Structure of GluA2-ABD with **6b** (PDB ID: 6Q54). The GluA2-ABD dimer is shown in cartoon representation with chain A in beige and chain B in orange. A grey arrow indicates D1-D2 domain closure. Molecule **6b** is shown in cyan sticks representation.

Figure 5.

Structures of GluA2-ABD with **6b** (PDB ID: 6Q54) and **7a** (PDB ID: 6Q60). (A) Simple PHENIX omit 2Fo-Fc map around **6b**, Arg506 and Met729, carved at 1.6 Å and contoured at 1 sigma (chain A). GluA2-ABD residues within 4 Å of **6b** are shown in beige sticks representation. Molecule **6b** is shown in cyan sticks representation. (B) Hydrogen-bonding network (up to 3.2 Å; black dashed lines) among binding-site residues, **6b** and water molecules (red spheres). (C) Comparison of binding mode of **6b** (chain A), AMPA (grey; PDB code 1FTM, chain A) and Glu (yellow; PDB code 1FTJ, chain A). The structures were overlaid on lobe D1 residues. Only water molecules (within 4 Å of the ligands) are shown for clarity and colored according to the respective ligands. (D) Simple PHENIX omit 2Fo-Fc map around **7a**, Arg506 and Met729, carved at 1.6 Å and contoured at 1 sigma (chain A). Molecule **7a** is shown in salmon sticks representation. (E) Hydrogen bonding network among binding site residues, **7a** and water molecules. (F) Zoom on the hydrogen bonding network of W3 and W4 in the GluA2-ABD structures with **6b** (cyan), **7a** (magenta) and AMPA (grey).

Scheme 1.

Synthesis of hydroxy-1,2,3-triazole Glu analogues **6a-c** and **7a-c**: *i*) NaBH₄, EtOH abs; *ii*) NBS, (Ph)₃P, dry DCM, -10°C; *iii*) diethyl-2-acetamidomalonate, NaH, dry THF; *iv*) H₂, Pd/C 10% w/w, dry THF; *v*) a) 6N HCl, reflux; b) strong acidic cation exchange resin (elution with water until neutral pH. Finally the compound was eluted with 10% NH₃ solution).

Scheme 2.

Synthesis of compound **8**: *i*) NaCN, EtOH / H₂O 9:1 v/v; *ii*) TMSCl, abs EtOH, 50 °C; *iii*) NaH, dry ethanol, EtONO; *iv*) a) H₂, Pd/C, dry ethanol; b) HCl gas, dry ethanol; *v*) a) 6N HCl, reflux; b) strong acidic cation exchange resin (elution with water until neutral pH, then recover of the desired compound by elution with 10% w/w NH₃ solution).

Table 1.Receptor binding at native α GluRs in rat brain synaptosomes^a.

Compound	[³ H]AMPA IC ₅₀ (μ M)	[³ H]KA IC ₅₀ (μ M)	[³ H]CGP39653 K _i (μ M)
(S)-Glu ⁴²	0.34	0.38	0.20
AMPA ²⁰	0.039	>100	>100
NMDA ⁴²	>100	>100	6.2
KA ⁴³	4.0	0.007	>100
6a ⁴¹	1.4	> 100	> 100
6b	0.98 [6.03 $\pm$ 0.09]	> 100	> 100
6c	5.2 [5.30 $\pm$ 0.09]	> 100	> 100
7a ⁴¹	3.1	> 100	> 100
7b	> 100	> 100	> 100
7c	> 100	> 100	> 100
8	> 100	> 100	34 [4.49 $\pm$ 0.09]

^aAMPA, KA and NMDA receptors were studied using [³H]AMPA, [³H]KA and [³H]CGP 39653 as radioligands, respectively. Data are mean values of three individual experiments performed in triplicate. For **6b** and **6c**: pIC₅₀ values with SEM in brackets. For **8**: pK_i values with SEM in brackets.

Table 2.

Functional properties of Glu and six hydroxy-1,2,3-triazole Glu analogues at GluA1-, GluA2(*Q*)-, GluA3- and GluA4-HEK293 cell lines in the Ca²⁺/Fluo-4 assay.

Compound	GluA1 _i EC ₅₀ [pEC ₅₀ ± SEM]	GluA2 _i (<i>Q</i>) EC ₅₀ [pEC ₅₀ ± SEM]	GluA3 _i EC ₅₀ [pEC ₅₀ ± SEM]	GluA4 _i EC ₅₀ [pEC ₅₀ ± SEM]
Glu	33 [4.48 ± 0.07]	57 [4.25 ± 0.03]	24 [4.63 ± 0.05]	4.8 [5.31 ± 0.02]
6a	SAR@ 300	SAR@ 300	SAR@ 300	55 [4.25 ± 0.03]
6b	SAR@ 100	SAR@ 300	SAR@ 100	35 [4.46 ± 0.07]
6c	SAR@ 1000	SAR@ 1000	SAR@ 300	51 [4.29 ± 0.07]
7a	SAR@ 1000	SAR@ 1000	SAR@ 1000	~100 [~4.0]
7b	>1000 ^a	>1000 ^a	>1000 ^a	>1000 ^a
7c	>1000 ^a	>1000 ^a	>1000 ^a	SAR@ 1000

SAR@, significant agonist response >5% of the maximal response induced by (*S*)-Glu at concentrations indicated,

^a tested both as agonist and antagonist (using Glu EC₈₀). All compounds were tested at concentrations up to 1000 μM, and an assay buffer containing 10 mM Ca²⁺ and 100 μM cyclothiazide was used in the experiments. EC₅₀ values and concentrations for which significant agonist responses were observed (in those cases for which a complete concentration-response curves could not be obtained) are given in μM. pEC₅₀ ± SEM are shown in brackets.

Table 3.

Binding of 4-hydroxy-1,2,3-triazole Glu analogues to AMPA receptor subtypes.

Ligand	GluA1 _o		GluA2(R) _o		GluA3 _o		GluA4 _o		GluA2 _o -ABD	
	K _i (μM)	n _H	K _i (μM)	n _H	K _i (μM)	n _H	K _i (μM)	n _H	K _i (μM)	n _H
(S)-2	0.022	n.d.	0.017	n.d.	0.021	n.d.	0.040	n.d.	n.d.	n.d.
5a	0.0052	n.d.	0.0039	n.d.	0.0022	n.d.	0.0027	n.d.	n.d.	n.d.
5b	7.7	n.d.	0.75	n.d.	0.39	n.d.	0.20	n.d.	n.d.	n.d.
6a	15.7 ± 1.1 [#]	1.02 ± 0.17	1.87 ± 0.72 [#]	0.95 ± 0.09	4.98 ± 0.58	0.95 ± 0.25	4.08 ± 1.66	0.89 ± 0.07	n.d.	n.d.
6b	9.02 ± 2.27 ^{##}	0.62 ± 0.04	0.71 ± 0.22 ^{##}	0.99 ± 0.04	1.91 ± 0.44	0.99 ± 0.03	9.47 ± 1.95	0.72 ± 0.03	0.32 ± 0.03 ^{**}	1.01 ± 0.15
6c	54.1 ± 8.6 ^{###}	0.81 ± 0.07	3.25 ± 0.47 ^{###}	1.55 ± 0.65	11.9 ± 2.9	0.75 ± 0.09	≈ 100	n.d.	n.d.	n.d.
7a	73 ± 34	0.97 ± 0.17	4.38 ± 2.18 [*]	0.80 ± 0.06	4.89 ± 0.51	0.82 ± 0.05	11.6 ± 2.1	0.91 ± 0.24	1.97 ± 0.17 [*]	0.92 ± 0.05
7b	> 100	n.d.	> 100	n.d.	≈ 100	n.d.	> 100	n.d.	n.d.	n.d.
7c	> 100	n.d.	> 100	n.d.	> 100	n.d.	> 100	n.d.	n.d.	n.d.

Shown are means ± SEM from n = 3 experiments conducted in triplicate at 12-15 ligand concentrations. n.d., not determined.

^{*} GluA2 not statistically significantly different from GluA2-ABD ($p > 0.05$, t-test).^{**} GluA2 not statistically significantly different from GluA2-ABD ($p > 0.05$, t-test).^{#, ##, ###} K_i at GluA1 statistically significantly different from GluA2 ($p < 0.05$, t-test).

Table 4.

Concentration-response data for compound **8** at recombinant NMDA receptors measured using two-electrode voltage-clamp electrophysiology.

8 + Glu					8 + Gly				
	EC₅₀ (μM)	Relative I_{max} (%)	n_H	n		EC₅₀ (μM)	Relative I_{max} (%)	n_H	n
GluN1/2A	-	38 ± 1 at 300 μM	-	4	GluN1/2A	-	No response at 300 μM	-	3
GluN1/2B		52 ± 1 at 300 μM		4	GluN1/2B	108 ± 13	22 ± 2	1.4	4
GluN1/2C	90 ± 4	107 ± 2	1.7	4	GluN1/2C	-	22 ± 2 at 300 μM	-	4
GluN1/2D	35 ± 1	98 ± 1	1.6	4	GluN1/2D	58 ± 1	58 ± 1	1.4	3

Data for compound **8** + Glu were obtained in the presence of 300 μM Glu, and data for compound **8** + Gly were obtained in the presence of 300 μM Gly. Relative I_{max} is the fitted maximal response relative to control responses activated by 100–300 μM glutamate plus 100–300 μM glycine in the absence of compound **8**. Low activity prevented determination of potencies in some cases (indicated by -). Data are mean ± SEM, n_H is the Hill coefficient, and n is the number of oocytes.

Table 5.

Crystal data, data collection, and refinement statistics of GluA2-ABD structures.

Crystal Data		
Ligand	Compound 6b	Compound 7a
Space group	$P2_12_12$	$P2_12_12$
Unit cell dimensions (Å)	98.33, 121.74, 47.13	98.34, 122.25, 47.35
Molecules in a.u. ^a	2	2
Data collection and processing		
Resolution (Å)	45.59-1.40 (1.48-1.40) ^b	44.15-1.55 (1.63-1.5)
Unique reflections	112,206 (16,195)	83,759 (12,103)
Average multiplicity	6.8 (6.7)	6.8 (6.7)
Completeness (%)	100 (99.9)	100 (100)
Wilson B-factor (Å ²)	12.3	13.3
R _{merge} (%) ^c	5.3 (45.8)	8.7 (36.8)
I/σI	8.3 (1.6)	4.7 (1.6)
CC1/2	0.999 (0.935)	0.997 (0.922)
Refinement		
Numbers of:		
Amino-acid residues (chain A/B)	264 / 263	264 / 263
Compound 6b/7a	2	2
Sulfate/glycerol/chloride/lithium/citrate/PEG ^d /PGE ^e /water	10 / 4 / 3 / 3 / 1 / 1 / 2 / 703	4 / 12 / 3 / 2 / - / - / - / 751
R _{work} ^f (%) / R _{free} ^g (%)	14.4 / 16.3	14.8 / 17.5
Average B-values (Å ²) for:		
Amino-acid residues (chain A/B)	21.6 / 20.9	24.1 / 21.9
Compound 6b/7a	15.0	15.1
Sulfate/glycerol/chloride/lithium/citrate/PEG/PGE/water	38.8 / 49.2 / 53.4 / 9.1 / 17.2 / 46.3 / 49.7 / 29.1	46.9 / 64.3 / 50.8 / 12.7 / - / - / - / 30.6
RMS deviation bonds length (Å)/angles (deg)	0.010 / 1.1	0.009 / 1.0
Ramachandran outliers/ favored (%) ^h	0 / 99.5	0 / 99.2
Rotamer outliers (%) / Cβ outliers (%) / Clash Score	0.6 / 0 / 1.58	0.65 / 0 / 2.04

^a a.u.: Asymmetric unit of the crystal.^b Values in parentheses correspond to the outermost resolution shell.^c $R_{\text{merge}} = \sum_{\text{hkl}} \sum_i |I_{\text{hkl}} - I_{\text{hkl}}| / \sum_{\text{hkl}} \sum_i I_{\text{hkl}}$, I_{hkl} is the intensity of an individual measurement of the reflection with Miller indices hkl, I_{hkl} is the intensity from multiple observations.^d di(hydroxyethyl)ether.^e triethylene glycol.^f $R_{\text{work}} = \sum_{\text{hkl}} (|F_{\text{o,hkl}}| - |F_{\text{c,hkl}}|) / |F_{\text{o,hkl}}|$, where $|F_{\text{o,hkl}}|$ and $|F_{\text{r,hkl}}|$ are the observed and calculated structure factor amplitudes, respectively.

σ^2_{Rfree} is equivalent to R_{work} , but calculated with 5 % of the reflections omitted from the refinement process.

h The Ramachandran plot was calculated according to MolProbity.⁴⁵

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