DOI: 10.1002/cmdc.200900258

4,4-Dimethyl- and Diastereomeric 4-Hydroxy-4-methyl-(2*S*)-Glutamate Analogues Display Distinct Pharmacological Profiles at Ionotropic Glutamate Receptors and Excitatory Amino Acid Transporters

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Subtype-selective ligands are of great interest to the scientific community, as they provide a tool for investigating the function of one receptor or transporter subtype when functioning in its native environment. Several 4-substituted (*S*)-glutamate (Glu) analogues were synthesized, and altogether this approach has provided important insight into the structure–activity relationships (SAR) for ionotropic and metabotropic glutamate receptors (iGluRs and mGluRs), as well as the excitatory amino acid transporters (EAATs). In this work, three 4,4-disubstituted Glu analogues **1**–**3**, which are hybrid structures of important 4-substituted Glu analogues **4**–**8**, were investigated at iGluRs and EAATs. Collectively, their pharmacological profiles add new and valuable information to the SAR for the iGluRs and EAAT1–3.

Introduction

(*S*)-Glutamate (Glu) functions as the major excitatory neurotransmitter in the central nervous system (CNS) by activating a plethora of glutamate receptors (GluRs).[1,2] The GluRs are divided into two major classes: the ionotropic Glu receptors (iGluRs) and the metabotropic Glu receptors (mGluRs). The iGluRs are ligand-gated ion channels and thus mediate fast excitatory responses of the neurotransmitter. Based on differential ligand affinities, the iGluRs have been further divided into three groups: the 2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)-propionic acid (AMPA) receptors (comprising the subunits iGluR1–4),[3] the kainic acid (KA) receptors (comprising subunits iGluR5–7 and KA1,2),[4] and the *N*-methyl-D-aspartic acid (NMDA) receptors (comprising subunits NR1A–H, 2A–D, 3A–C).[5] The mGluRs are G-protein-coupled receptors and thus produce a slower signal transduction through second-messenger systems.[6] Eight subtypes, mGluR1–8, have been identified and they are clustered into three groups on the basis of: involved second-messenger systems, pharmacology, and amino acid sequence homologies (group I: subtypes mGluR1,5; group II: subtypes mGluR2,3; group III: subtypes mGluR4,6–8).[7] Termination of the excitatory signal by removal of Glu from the synaptic cleft is mediated by the excitatory amino acid transporters (EAATs).[8,9] Five EAAT subtypes have been identified of which EAAT1–4 are present in the CNS,[10] whereas EAAT5 is found exclusively in the retina.[11]

Glutamatergic neurotransmission is believed to be involved in key neurophysiological processes such as memory and learning, control of motor functions, and neuronal plasticity and development. Therefore, neurological and psychiatric diseases such as depression,[12] anxiety,[13] mood disorders,[14] addiction,[15,16] migraine,[17] and schizophrenia[18] may be directly related to malfunctioning glutamatergic neurotransmission.[8,19,20] Moreover, excessive Glu signaling is neurotoxic and leads to neuronal death.[21] On this basis it has been suggested that neurotoxic states and neurodegenerative diseases such as Alzheimer's disease,[22] Huntington's disease,[23] amyotrophic lateral sclerosis,[24] cerebral stroke,[18] and epilepsy[25] may be the result of a malfunctioning glutamatergic neurotransmitter system.

Introduction of a substitution at the 4-position of Glu has led to several new compounds which have disclosed fundamental aspects of the SAR of the iGluRs,[4,26–28] mGluRs,[29] and EAATs.[30] In this context we focus on the finding that (4*R*)-methyl-Glu (**5**) is a selective and potent agonist at iGluR5,[31] and displays differentiated function at transporter subtype EAAT1 (substrate) versus subtypes EAAT2,3 (inhibitor).[26,32] In addition, the diastereomeric 4-hydroxy analogues **7** and **8** show distinct pharmacological profiles at the EAATs; **7** is inactive, whereas **8** is a substrate at EAAT1–3.[26] To continue the investigation and further development of the SAR for the iGluRs and EAATs, we present herein the rational design and pharmacological evaluation of 4,4-disubstituted Glu analogues **1**–**3**,

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which are hybrid structures of previously described 4-substituted Glu analogues **4**–**8** (Figure 1). Furthermore, the 4-hydroxy Glu analogues **7** and **8** were also re-synthesized according to published procedures and characterized in binding assays at the iGluRs.

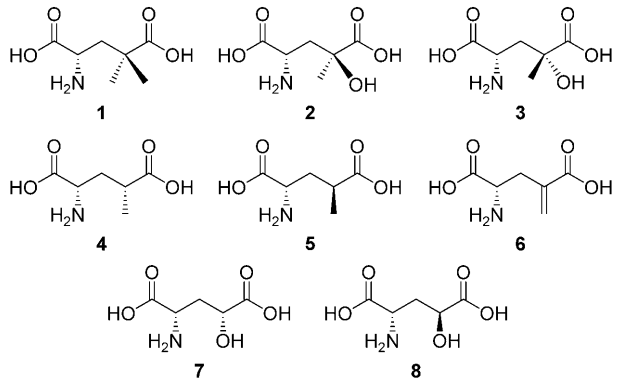


Figure 1. Chemical structures of 4,4-dimethyl- and diastereomeric 4-hydroxy-4-methyl-Glu analogues **1**–**3**, which are hybrid structures of the published Glu analogues **4**–**8**.

Results and Discussion

The enantioselective synthesis of Glu analogues **1**–**3** was carried out using a chemoenzymatic approach with stereoselective transamination as the key step, reported elsewhere.[33,34] The scope and limitations of this enzymatic procedure have been explored extensively by us with the aim of synthesizing a large variety of Glu analogues.[26,28,30,33–36] With compounds **1**–**3** in hand, we first investigated them in a binding assay at native AMPA, KA, and NMDA receptors and subsequently at the cloned rat homomeric KA receptor subtypes iGluR5–7 (Table 1). The 4,4-dimethyl-Glu analogue **1** was shown to be a low-affinity ligand at native AMPA, KA (the radioligand binding assay predominantly reflects binding affinities to KA1,2-containing subtypes), and NMDA receptors, and only displayed medium-range nanomolar affinity for iGluR5 with a 28- and 7-fold preference over iGluR6 and iGluR7, respectively. Diastereomers **2** and **3** also displayed low affinity toward native AMPA, KA, and NMDA receptors, but interestingly, the binding affinities for these two compounds at homomeric iGluR5–7 were highly distinct: Whereas **2** displayed low nanomolar affinity for iGluR5, high nanomolar affinity for iGluR6 and medium-range nanomolar affinity for iGluR7, its C4 diastereomer **3** is a low-affinity ligand at all three cloned KA subtypes, iGluR5–7. The observed binding affinities of **3** are accredited to contamination (< 1%) with diastereomer **2**.

The syntheses of diastereomeric 4-hydroxy Glu analogues **7** and **8** were carried out according to published procedures.[37] The two analogues were first investigated in binding assays at native iGluRs (Table 1). The 2,4-*syn* diastereomer **7** displayed preference for the NMDA receptors ($K_i = 1.3$ μM) over both AMPA and KA receptors (> 100- and 30-fold, respectively). In detailed studies on cloned homomeric iGluR5–7, the Glu analogue showed weak affinity for iGluR5 and negligible affinity for iGluR6,7. The 2,4-*anti* diastereomer **8** was carried through the same assays. At native iGluR receptors, it displayed low- to medium-range affinity for AMPA, KA, and NMDA receptors and in studies on cloned homomeric iGluR5–7.

Subsequently, hybrid analogues **1**–**3** were evaluated as potential inhibitors and/or substrates at HEK293 cells stably expressing the EAAT1–3 subtypes in the FLIPR membrane potential blue (FMP) assay (Table 2).[41] Whereas **1** and **3** were completely inactive at all three EAAT subtypes, analogue **2** inhibited Glu transport at all three subtypes with medium-range micromolar affinities ($K_i = 88$, 48, and 130 μM, respectively).

Modeling study

Glu is a highly flexible molecule which may adopt nine staggered conformations.[42] Among these, it is well documented that Glu binds in a *folded conformation* to the iGluRs,[28,43] and in an *extended conformation* to the mGluRs[44] (Figure 2). At the EAATs, several studies have shown that inhibitors, which are analogues of Glu, bind in the *folded conformation*;[26,30,45] whereas the conformational requirements for EAAT substrates are somewhat opaque, they are thought to involve an *extended-like conformation*.[26,30]

Table 1. Binding affinities of Glu analogues **1**–**3** and **7**–**8** at native AMPA, KA, and NMDA receptors and at cloned homomeric iGluR5–7.

		Native receptors (IC_{50} [μM])[a]			Cloned homomeric receptors (K_i [nM])[b]		
Compd	Ref.	[^{3}H]AMPA	[^{3}H]KA[c]	NMDA[d]	iGluR5$(Q)_{1b}$	iGluR6$(V,C,R)_a$	iGluR7$_a$
1		> 100	7.4 [5.13 ± 0.05]	> 100	168 ± 10	4670 ± 70	1090 ± 100
2		24 [4.62 ± 0.02]	0.75 [6.12 ± 0.05]	> 100	17.9 ± 1.5	800 ± 137	182 ± 25
3		> 100	16 [4.79 ± 0.02]	> 100	1180 ± 85	55 600 ± 4100	7840 ± 1800
4	[28,29,38,39]	26.6	0.032	5.9	0.66	17.0	5.7
5	[38]	14.6	0.33	9.6	–	–	–
6	[29,40]	0.15	0.23	1.2	270	450	–
7		> 100	31 [28;31]	1.3 [1.0;1.5]	4175 ± 920	21 416 ± 1854	14 938 ± 2794
8		35 [31;39]	9.1 [8.5;9.8]	4.0 [3.3;4.8]	3740 ± 720	7432 ± 432	28 134 ± 7533

[a] In rat brain synaptosomes; data are the mean of at least three independent experiments; values given in brackets are pIC_{50} or $pK_i \pm$ SEM. [b] Rat clones of the receptors and radioligand: [^{3}H](2*S*,4*R*)-4-methyl-Glu (SYM-2081 = [^{3}H]**4**); data are reported as the mean ± SEM of at least three competition experiments at 16 drug concentrations performed in triplicate; –: no data available. [c] In this assay, the binding to native KA1,2-containing iGluR subtypes is predominant. [d] Radioligand: [^{3}H]CGP-39653.

Table 2. Pharmacological characteristics of Glu analogues at human EAAT1–3 in the FMP assay.

Compd	Ref.	K_i [μM][a] EAAT1	EAAT2	EAAT3
1		>3000	>3000	>3000
2		88 [4.1±0.05]	48 [4.3±0.04]	130 [3.9±0.05]
3		>3000	>3000	>3000
4	[26, 32]	***13***	13	6.6
5	[32]	>1000	>1000	–
6		–	–	–
7	[26]	***~1000***	***~1000***	***~1000***
8	[26]	***140***	***67***	***81***

[a] Values given in brackets are pK_i±SEM; ***bold italics*** indicate K_M values for substrates.

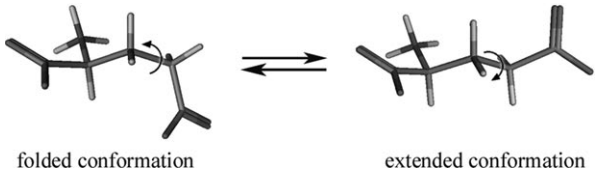


Figure 2. Glu folded conformation when crystallized in iGluR5 (PDB code: 1TXF) and extended conformation when crystallized in mGluR1 (PDB code: 1EWK).

To address the structural origin of the distinct pharmacological profiles observed for **1**–**3** at the iGluRs and EAATs, we calculated conformational energies and thereby depicted the biologically relevant conformations. In detail, the Glu analogues were submitted to a stochastic conformational search (see Experimental Section for further details). For 4,4-dimethyl-Glu analogue **1**, the global low-energy conformation was found to equal the folded conformation, with its extended conformation located at an energy level ~1.5 kcal mol^{-1} higher (Figure 3).

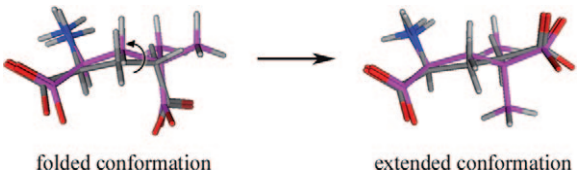


Figure 3. Folded and extended conformations of **1** (purple) superimposed onto Glu folded conformation (atom-type coloring; PDB code: 1TXF) and Glu extended conformation (atom-type coloring; PDB code: 1EWK).

The global low-energy conformation of **2** matched the folded conformation (Figure 4) and displayed a favorable intramolecular hydrogen bond between the ammonium and hydroxy groups. Finally, the global low-energy conformation of **3** was found to equal the extended conformation (Figure 4). The converse conformations of **2** and **3** which may be obtained by rotation of the C3–C4 bond were significantly higher in energy due to the repulsive van der Waals interactions (α-ammonium and the 4-methyl groups, Figure 4).

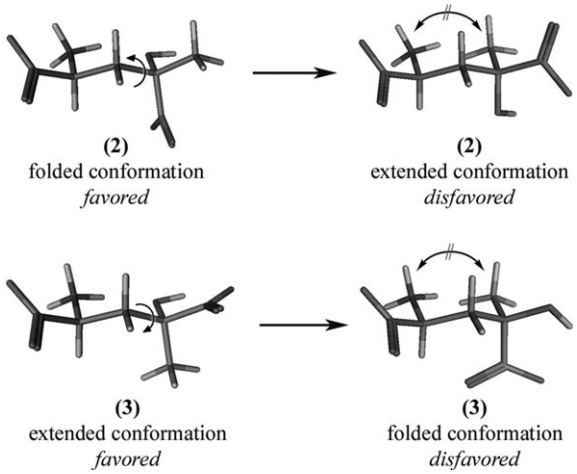


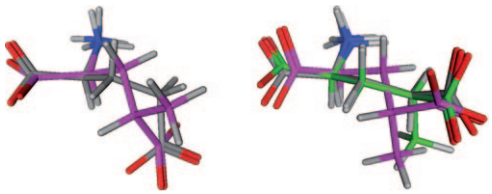
Figure 4. Low-energy folded conformation of **2** (upper left) and its enforced disfavored extended conformation (upper right); low-energy extended conformation of **3** (lower left) and its enforced disfavored folded conformation (lower right).

SAR study at iGluRs

A correlation of results from the in silico conformational analysis (Table 3) with respective pharmacological profiles at the iGluRs (Table 1) provides new insight into the SAR. Even though **1** may adopt the folded conformation (Figure 3), this Glu analogue is *not* a high-affinity ligand at the iGluRs (Table 1). This finding is best explained by the fact that the one 4-methyl group which is oriented in the same plane as the ammonium group induces a steric repulsion with the residue Glu 753. This residue is conserved throughout the AMPA and KA receptors and is critical for the ability of the ligand to bind to the receptor through the formation of a salt bridge to the ammonium group.[43] Hence, substitution of this methyl group for a hydroxy group, compound **2**, not only favors the folded conformation, but because the hydroxy group is smaller in size, more space is left for the important ligand–ammonium group/Glu 753 interaction. These in silico predictions are in excellent agreement with compound **2** being a high-affinity ligand at the KA receptor subtype iGluR5. The observed preference for this subtype over KA1, 2 and iGluR6, 7 is likely due to the larger iGluR5 receptor pocket volume.[4] Interestingly, 4-hydroxy Glu analogue **8** also favorably adopts the folded conformation, but displays much weaker affinity for KA1, 2 and iGluR5 receptors (12- and 200-fold, respectively). We believe this observation is due to differences in energies of desolvation of **2** and **8**, the latter being more soluble in water.

Finally, the C4-diastereomer, compound **3**, shows poor affinity for the iGluRs which is in accordance with its folded conformation being energetically disfavored. On the other hand, as the extended conformation is favored, this compound could be a potential mGluR ligand. This is also supported by the fact that its corresponding demethylated analogue **7** prefers to adopt the extended conformation and is a known, albeit weak, mGluR ligand.[32]

Table 3. Conformational analyses of Glu analogues **1**–**8** by in silico methods.[a]



Compd	2,4-Relative Stereochemistry		$\Delta\Delta G$ [kcal mol^{-1}][b] Folded (iGluR agonist)	Folded-like	Extended (mGluR agonist)	Extended-like
1	n.a.[c]	Me, Me	0	–	+1.5	–
2	*anti*	OH, Me	0[d]	–	–	–
3	*syn*	OH, Me	–	–	0[d]	–
4[26]	*syn*	Me	0	–	–	+2.7
5	*anti*	Me	–	0	+1.0	–
7	*syn*	OH	+4.3	–	0[d]	+4.7
8	*anti*	OH	0[d]	+4.4	+6.1	+8.9

[a] Structures at left: Glu (folded, atom-type coloring) and **5** (folded-like, purple); structures at right: Glu (extended, atom-type coloring), **5** (extended, green), and **4** (extended-like, purple). [b] Global low-energy conformation set to 0 in all cases; $\Delta\Delta G$ calculated to '+' indicates higher energy conformations; –: indicates $\Delta\Delta G$ calculated for this conformation is >40 kcal mol^{-1}, which excludes this conformation from the SAR study. [c] Not applicable. [d] An intramolecular hydrogen bond between the ammonium and hydroxy groups is observed.

SAR study for EAAT1–3

In earlier studies, 4-methyl-Glu **4** was shown to be a substrate at EAAT1, whereas it is an inhibitor at EAAT2, 3, and 4-hydroxy Glu **8** was shown to be a substrate at all three EAAT subtypes, EAAT1–3.[26] However, their structural hybrid, compound **2**, is an inhibitor of all three subtypes, EAAT1–3. Given that the low-energy conformation of compound **2** is the folded conformation and the presence of the extended conformation is negligible (Table 3), this supports our previous findings that an EAAT substrate must be able to adopt an extended conformation.[26] Our finding that its C4-diastereomer, compound **3**, is not an inhibitor at EAAT1–3, correlates well with results from the in silico study (Table 3), which indicate that a folded conformation is energetically disfavored. However, the fact that its low-energy conformation is the extended conformation and the observation that it is not an EAAT substrate, provides new insight into the SAR for EAAT substrate transport activity. Finally 4,4-dimethyl analogue **1** is neither an inhibitor nor a substrate for EAAT1–3. Correlating this finding with the fact that it readily adopts the folded conformation gives new insight into the EAAT inhibitory pharmacophore.

Conclusions

In summary, we have described the pharmacological evaluation of Glu analogues **1**–**3**, which are hybrid structures of previously reported Glu analogues **4**–**8**. We investigated the three analogues at the iGluRs and EAAT1–3, and to complete the study we furthermore characterized diastereomeric 4-hydroxy Glu analogues **7** and **8** at the iGluRs. By means of an in silico study, we addressed the observed differences in their pharmacological profiles, which provide new insight into the SAR for iGluR and EAAT ligands. Most remarkable is the contribution to the understanding of the SAR for EAAT substrates and inhibitors: extended ligand conformations tend to be substrates, whereas folded conformations tend to be non-transportable inhibitors.

Experimental Section

In silico study

The modeling study was performed with the software package MOE (Molecular Operating Environment, v2006.08, Chemical Computing Group, 2006) using the built-in mmff94x force field and the GB/SA continuum solvent model.

*General procedure for compounds **1**–**8***: The γ-carboxylate group was protonated, and the compound was submitted to a stochastic conformational search (standard setup). All conformations that enclosed intramolecular hydrogen bond(s) were discarded. Superimpositions of selected conformations were carried out using the built-in function in MOE, by fitting the ammonium group and the two carboxylate groups.

Binding affinities at native and homomeric iGluRs

Binding affinities for **1**–**3**, **7**, and **8** at native AMPA, KA, and NMDA receptors (rat synaptosomes) were determined according to published experimental procedures[46] using radioligands [^{3}H]AMPA, [^{3}H]KA (representing predominantly subtypes KA1, 2), and [^{3}H]CGP-39653, respectively. Determination of binding affinities for **1**–**3**, **7**, and **8** at cloned rat homomeric subtypes iGluR5–7 were carried out following the procedures described earlier, using [^{3}H]SYM-2081 as the radioligand.[28]

Functional characterization at EAAT1–3

Compounds **1**–**3** were characterized in the FLIPR membrane potential blue (FMP) assay, which was carried out essentially as described previously.[41]

Chemistry

(2*S*)-4,4-Dimethylglutamic acid (1). This Glu analogue was prepared according to published procedures.[34] Elemental analysis calcd for $C_7H_{13}NO_4 \cdot 0.5\,H_2O$: C 45.65, H 7.66, N 7.60, found: C 45.90, H 7.54, N 7.56.

(2*S*,4*S*)-4-Hydroxy-4-methylglutamic acid (2). This Glu analogue was prepared according to published procedures.[33] Analytical and NMR spectroscopic data were identical to what we reported earlier.[34] Additional analytical data: *de* = 97% (determined by NMR); el-

emental analysis calcd for $C_6H_{11}NO_5 \cdot 0.5\,H_2O$: C 38.71, H 6.50, N 7.52, found: C 38.82, H 6.39, N 7.24.

(2*S*,4*R*)-4-Hydroxy-4-methylglutamic acid (3). This Glu analogue was prepared according to published procedures.[33] Analytical and NMR spectroscopic data were identical to what we reported earlier.[34] Additional analytical data: *de* > 98% (determined by NMR); elemental analysis calcd for $C_6H_{11}NO_5 \cdot 0.25\,H_2O$: C 39.67, H 6.38, N 7.71 found: C 39.20, H 6.46, N 7.80.

(2*S*,4*R*)-4-Hydroxyglutamic acid (7) and (2*S*,4*S*)-4-Hydroxyglutamic acid (8). These Glu analogues were prepared according to published procedures. All analytical and spectral data were identical to what we reported earlier.[34,37]

Acknowledgements

We thank the Carlsberg Foundation, the Lundbeck Foundation, GluTarget, and the Danish Research Council for financial support. Dr. S. G. Amara is thanked for her generous gift of cDNAs for the human EAATs.

Keywords: EAATs · glutamic acid · kainic acid · structure–activity relationships · subtype-selective ligands

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Received: June 30, 2009
Revised: August 11, 2009
Published online on September 3, 2009

