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SYNTHESIS AND BIOLOGY OF THE RIGIDIFIED GLUTAMATE ANALOGUE, *TRANS*-2-CARBOXYAZETIDINE-3-ACETIC ACID (*t*-CAA)

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Abstract: Chemical approaches to the (-)- and (+)-*trans*-2-carboxyazetidine-3-acetic acids (-)-**1** and (+)-**1**, and their homologues (-)-**2** and (+)-**2**, compounds that represent rigidified analogues of glutamate (glu), are reported together with the complete biological characterization of (+)-**1** (*t*-CAA) at the known glu recognition sites. *t*-CAA was found to be an inhibitor of Na^+-dependent glu uptake and to act as a kainate receptor ligand.

The amino acid glutamate (glu) plays a pivotal role in biological processes ranging from memory and learning to neuronal degeneration. This major excitatory amino acid (EAA) acts through disparate glu receptors, which can be categorized into two distinct types, the so-called ionotropic receptors and the metabotropic receptors.[1] The ionotropic glu receptors, or iGluRs, are associated with integral cation-specific ion channels and include the NMDA, AMPA, and kainate (KA) subtypes. On the other hand, the metabotropic receptors are coupled to cellular effectors through GTP-binding proteins. The metabotropic glu receptors, or mGluRs, have been distinguished pharmacologically from the iGluRs by the use of the mGluR-selective agonist 1*S*,3*R*-ACPD generally through measurements involving phosphoinositide hydrolysis or Ca^{2+} mobilization. To date the use of expression cloning techniques has led to the identification of eight mGluR subtypes that have been placed into three major categories based on their molecular structure, signal transduction mechanisms, and pharmacological properties. Group 1 mGluRs (mGluR1 and 5) are coupled to phosphoinositide (PI) hydrolysis, whereas group 2 (mGluR2 and 3) and group 3 (mGluR4, 6, 7, and 8) are negatively linked to adenylyl cyclase activity. The group 1 receptors are more sensitive to quisqualic acid than they are to ACPD, the group 2 receptors are more sensitive to ACPD than quisqualic acid, and the group 3 receptors are most sensitive to 2-amino-4-phosphonobutyric acid (L-AP4).[2]

Diverse work from a number of laboratories has provided evidence that the mGluRs are involved in the mechanisms underlying synaptic plasticity, both during development and in adult life.[3] *N*-Methyl-D-aspartate (NMDA) receptors are believed to initiate a short-term potentiation (STP), which is then transformed and maintained as long-term potentiation (LTP) by the agency of mGlu receptors.[4,5] A substantive body of evidence is also accumulating that reveals a role for the various mGluR subtypes in hypoxic/ischemic cell death. Since both agonists and antagonists have been found under certain conditions to exhibit neuroprotective effects, it is likely that the various subtypes of mGluRs are differentially involved in neurodegenerative processes.[6]

In order to better characterize the roles of GluRs in physiological processes, there is an important need to identify novel, high affinity ligands which are family and subtype specific.[7-10] Herein we describe our chemical and biological efforts aimed at probing the effect of rigidifying the glu molecule by linking its nitrogen atom to its carbon backbone (Scheme 1). Previously, employing this type of rigidification strategy, we produced *t*-ADA and showed that it serves as a useful pharmacological tool.[7,8]

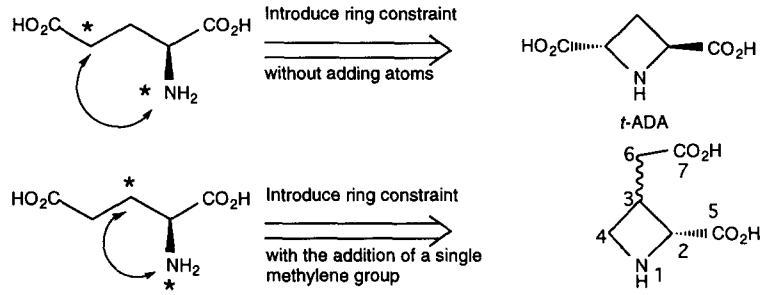
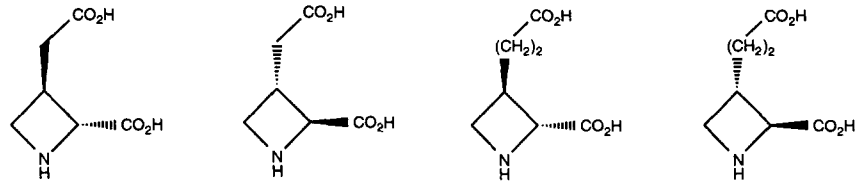


Figure 1. Generation of Rigidified Analogues of L-Glutamate

In continuation of the azetidine theme, we now chose to explore the effect of generating an azetidine bearing carboxylic acid functionality at the 2- and 3-positions. As illustrated in Figure 1, heuristically these molecules can be envisaged as arising from L-glu through the introduction of an additional methylene group into the starting amino acid at the positions starred. In particular, based on overlay comparisons with *t*-ADA, we chose to develop chemical approaches to the (-)- and (+)-*trans*-2-carboxyazetidine-3-acetic acids (-)-**1** and (+)-**1**, and the homologues (-)-**2** and (+)-**2**.



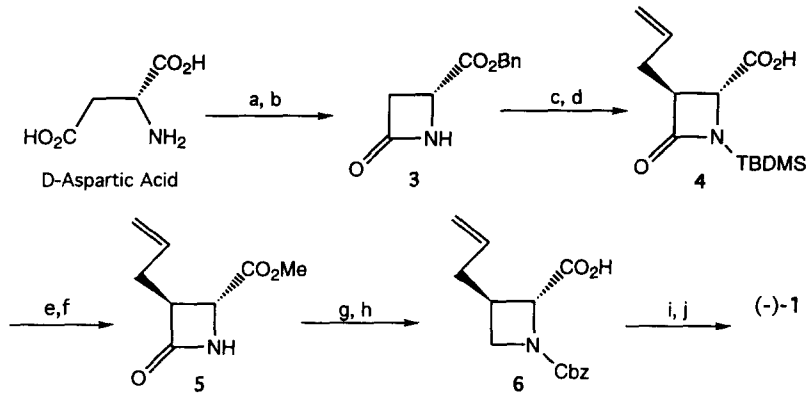
(-)-**1** (+)-**1** (-)-**2** (+)-**2**

Chemical Synthesis. D-Aspartic acid was used as the starting material in the preparation of (-)-**1** (Scheme 1). Accordingly, the D-acid was converted via its dibenzyl ester to the known β-lactam **3**,[11] which was *N*-silylated and subjected to hydrogenolysis to give the corresponding carboxylactam. This lactam was reacted with two equivalents of LDA, and the resulting dianion was treated with allyl bromide to provide **4**.[11] The acid **4** was esterified using diazomethane, and the *N*-silyl group was cleaved to provide **5**. Next, reduction of the lactam carbonyl group with simultaneous reduction of the carbomethoxy group was brought about with Dibal-H, the azetidine nitrogen was reprotected by carbobenzyloxylation, and the alcohol was oxidized to the acid **6**. The double bond of **6** was subjected to ozonolysis, the derived aldehyde was oxidized with O_2/PtO_2 to the *N*-protected azetidine diacid, and removal of the Cbz group was carried out under hydrogenolytic conditions to provide the desired azetidine diacid (-)-**1**. The (+)-isomer of **1** was prepared in an identical fashion starting from L-aspartic acid.

The one carbon homologue (-)-**2** was readily obtained from **5** through a four-step protocol involving Dibal-H reduction, hydroboration with 9-BBN followed by oxidative workup to afford **7**, Jones oxidation, and hydrogenolytic removal of the Cbz group. The homologue (+)-**2** was obtained in a similar fashion from *ent*-**5**.

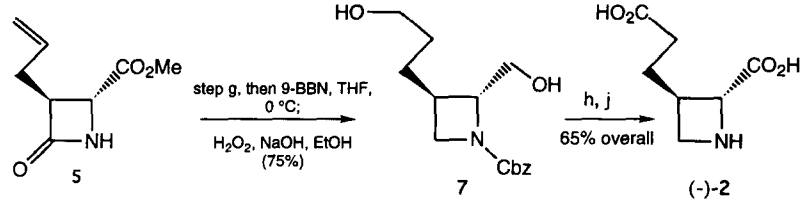
Pharmacological Testing. Methods: Receptor binding experiments using [^{3}H]α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) (47 Ci/mmol) and [^{3}H]3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP) (58 Ci/mmol) (all from New England Nuclear, USA) were performed as previously described using rat cerebral cortical membranes.[12] Receptor binding experiments using [^{3}H]glu (the specific activity was 50 Ci/mmol, Amersham) to membranes prepared from baby hamster kidney (BHK) cells expressing mGluR1a were performed as reported.[13] Sodium dependent [^{3}H]glu-uptake into rat cortical synaptosomes was performed according to the method by Fletcher and Johnston.[14] Calcium chloride dependent glu uptake was measured as

Scheme 1. Asymmetric Synthesis of (-)-*trans*-2-Carboxyazetidine-3-acetic Acid [(-)-**1**].



Reagents and conditions. a. BnOH, *p*-TsOH, PhH, reflux, 12 h; aq Na_2CO_3 (88%); b. TMSCl, Et_3N, Et_2O, 0 °C, 1h; *t*-BuMgCl (2 equiv), 0 °C, overnight; aq 2 N HCl saturated with NH_4Cl (75%); c. TBDMSCl, Et_3N, THF, DMAP (cat), rt, 2 d (83%); H_2, 10% Pd/C, MeOH (95%); d. LDA (2 equiv), ether, 0 °C, 0.5 h; allyl bromide, 0 °C, 2 h; 1 N $KHSO_4$ to adjust pH to 2-3 (94%); e. CH_2N_2, Et_2O, 0 °C, 1 h; f. 1 N HCl, MeOH, H_2O (61% overall); g. DIBAL-H, THF, reflux, 3 h; $ClCO_2Bn$, Et_3N, 0 °C, 2 h (38% overall); h. Jones oxidation (83%); i. O_3, CH_2Cl_2, MeOH, -78 °C; Me_2S, -78 °C to rt, overnight; O_2/PtO_2, H_2O, acetone, 40 °C, 4 h (82% overall); j. H_2, 5 % Pd/C, MeOH, H_2O (60%).

Scheme 2. Synthesis of the Homologous Diacid (-)-**2**.[a]



[a] Reagents and conditions correspond to those of Scheme 1.

described previously using [^{3}H]L-AP4 (specific activity 44 Ci/mmol, Tocris Neuramin, U.K.) binding to rat cortical membranes.[15] [^{3}H]L-AP4 binding was also used to label mGluR4a when expressed in BHK cells.[16]

Chinese hamster ovary (CHO) cells stably expressing mGluR1a, mGluR5a, mGluR2, or mGluR6 were cultured as described previously[17] and used for measurements of phosphoinositide (PI) hydrolysis or cAMP formation. For measurements of PI hydrolysis, cells expressing mGluR1a or mGluR5a were cultured in 24 well plates and then labelled overnight with 1 μCi/mL of [^{3}H]*myo*-inositol (specific activity 17 Ci/mmol, Amersham). CHO cells expressing mGluR2 or mGluR6 were cultured in 96-well plates. Measurements of PI hydrolysis and of forskolin-induced cAMP formation were performed as described previously.[18] Measurements of intracellular Ca^{2+} concentrations were performed on primary cultures of cerebellar neurons using Fura-2 fluorescent dye as described previously.[19]

The effect of (+)-**1** on the interstitial levels of glu was measured in anesthetized male Sprague-Dawley rats (200-500 g, Moellegaard, Denmark). A microdialysis probe was inserted into the nucleus accumbens as previously described,[20] and the levels of glu were determined using HPLC analysis.[21]

Results: In preliminary experiments employing cerebellar granule cell cultures and assaying second messenger effects, only azetidine (+)-**1** (or *t*-CAA) was found to exhibit biological activity. Therefore, all further binding and functional experiments were conducted with only (+)-**1**.

The in vitro selectivity of azetidine (+)-**1** for glu receptors and transporters is shown in Table 1. In binding studies of the ionotropic glu receptors, *t*-CAA showed the highest affinity for KA receptors (0.7 μM)

Table 1. In vitro characterization of *t*-CAA.

Receptor/Transporter	IC_{50} [μM]
Ionotropic Glutamate Receptors	
AMPA ([^{3}H]AMPA)	13
NMDA ([^{3}H]CPP)	49
([^{3}H]TCP)	>660
Kainate ([^{3}H]kainate)	0.7
Metabotropic Glutamate Receptors	
mGluR1a ([^{3}H]glutamate)	12
mGluR4a ([^{3}H]L-AP4)	315
Glutamate Uptake Sites	
Na^+-dependent ([^{3}H]glutamate)	5
$CaCl_2$-dependent ([^{3}H]L-AP4)	33

The values (IC_{50}'s) are the mean of 2-3 experiments which were performed in triplicate as described in the Methods section.

and AMPA receptors (13 μM), but was less potent at NMDA receptors. At metabotropic receptors *t*-CAA displaced potently the binding of [^{3}H]glu to mGluR1a receptors (group 1) but had little effect on the binding of [^{3}H]L-AP4 to mGluR4a (group 3 receptors). The ability of *t*-CAA to act at metabotropic glu receptors (mGluRs) was tested on CHO cell lines expressing two mGluRs coupled to phosphoinositide hydrolysis (mGluR1a, mGluR5), as well as on mGluRs coupled to the inhibition of cAMP formation (mGluR2 and mGluR6) expressed in CHO cells. As shown in Table 2, *t*-CAA activated both PI-coupled (group 1) receptors with an EC_{50} of 210 μM for mGluR1a and 105 μM for mGluR5, thus being 5- to 7-fold less potent than *t*-ACPD (the EC_{50} values for ACPD were 47 μM and 15 μM for cells transfected with mGluR1a and mGluR5a, respectively). The (-)- isomer of *t*-CAA was found to be significantly less active. In addition, *t*-CAA acted at mGluR1a as a partial agonist, inducing a maximal effect corresponding to 52% of the maximal effect induced by *t*-ACPD or glu. *t*-CAA also produced a weak antagonistic action at mGluR2 receptors (group 2) and was without effect at mGluR6 receptors (group 3).

Table 2. Effects of *t*-CAA on second messenger formation in CHO cells expressing mGluR1a, mGluR5a, mGluR2, or mGluR6 receptors.[a]

mGluR Subtype	Agonist Effects		Antagonist Effects	
	EC_{50} (μM)	% max response	IC_{50} (μM)	% Inhibition
mGluR1a	210	52	--	--
mGluR5a	105	95	--	--
mGluR2	>600	0	>600	40
mGluR6	>300	17	>300	0

[a]Measurements of PI hydrolysis (mGluR1a and mGluR5a) and of cAMP formation (mGluR2 and mGluR6) were performed as described in Methods. *t*-CAA was applied alone (agonist effects) or 5 min prior to the agonist (antagonist effects). For mGluR1a and mGluR5a the maximal responses obtained with 1 mM glutamate were 546 ± 18% and 445 ± 10% of basal PI hydrolysis, respectively. For mGluR2 and mGluR6 maximal inhibitions of cAMP formation induced by 10 μM forskolin obtained with 1 mM glutamate were 92 ± 4% and 94 ± 2%, respectively. Antagonism was tested using as agonists 20 μM *t*ACPD for mGluR2 and 1 μM L-AP4 for mGluR6. The values are means of 2-3 experiments performed in duplicate.

In primary cultures of cerebellar granule cells the application of (+)-*t*-CAA (100 μM for 2 min) resulted in a fast transient increase of intracellular calcium concentration, as measured by Fura-2 fluorescence (data not shown). This increase was blocked by 10 μM NBQX, an inhibitor of non-NMDA glu receptors, suggesting that *t*-CAA acts as an agonist at these receptors. In cultured granule cells similar NBQX-sensitive increases of

$[Ca^{2+}]_i$ are induced by AMPA and KA and result from depolarization-activated Ca^{2+} influx through voltage-sensitive Ca^{2+} channels. The agonist action of *t*-CAA at non-NMDA receptors is in agreement with the ability of this compound to inhibit the binding of [^{3}H]AMPA and [^{3}H]KA.

The effect of *t*-CAA on Na^+ and Ca^{2+}/Cl^- -dependent glu uptake into cortical synaptosomes was subsequently examined (Table 1). *t*-CAA inhibited Na^+-dependent [^{3}H]glu uptake with a potency (5 μM) that is similar to that of L-*trans*-pyrrolidine-2,4-dicarboxylate (4.6 μM). Lower potency was observed at the Ca^{2+}/Cl^- -dependent glu uptake sites. When *t*-CAA was perfused into the nucleus accumbens of rats via a microdialysis probe, a highly significant increase in the interstitial levels of glu was observed. The 1800% increase in the levels of glu observed during perfusion with 500 μM *t*-CAA is similar to that observed upon perfusion with a similar concentration of L-*trans*-pyrrolidine-2,4-dicarboxylate.

Molecular Modeling Studies. Molecular modeling studies were performed on *t*-CAA using the QUANTA96 molecular modeling package,[22] and energy calculations and molecular dynamics simulations were performed using the CHARMM program[23] with its associated CHARMM23 parameter set. The two carboxylic acid groups were set to be deprotonated and the amine group to be protonated, as expected under physiological conditions. The dielectric constant was set to 80. An adopted-basis Newton-Raphson algorithm, implemented in the CHARMM program, was used in energy minimization, and the energy was minimized in 1000 steps, or until convergence, defined as an energy gradient tolerance ≤ 0.001 kcal mol^{-1} $Å^{-1}$. High temperature molecular dynamics simulations (T = 1000 K) showed that the 4-membered ring in *t*-CAA, although more rigid than the corresponding 5- and 6-membered rings, can still adopt two puckered forms. For the structure adopting puckered form A, a systematic conformational search revealed that there are three conformational clusters with respect to the torsion angle C2-C3-C6-C7 (corresponding to the torsion angle 1 in glu). The three local minima (A1, A2, and A3) have a conformational energy of 43.96, 45.58, and 44.30 kcal/mol, respectively. Similarly, for the structure adopting puckered form B, three local minima (B1, B2, and B3) were found possessing a conformational energy of 45.52, 46.34, and 45.62 kcal/mol, respectively. Structural overlay showed that conformation A3 of *t*-CAA closely resembles the g$^+$a glu-like conformation (RMS = 0.36 Å), which is believed to be relevant to both KA receptor recognition, as well as one of the likely conformations recognized by the mGluRs (Figure 2).[24] Conformation B2 closely resembles the ag$^+$-like conformation of glu (RMS = 0.40 Å), which has been proposed to be the likely conformation recognized by the glu-transporter (Figure 2). The modeling results appear to be entirely consistent with the biological results found for *t*-CAA, thus providing further structural information for the rational design of selective glu ligands.

Figure 2. Molecular modeling studies of *t*-CAA.

Summary. The present work details the biological activity of a new azetidine diacid derivative (+)-**1**, or *t*-CAA, a rigidified analogue of glu. Of the various biological measurements performed with this compound, *t*-CAA would appear to be a potent KA receptor agonist (0.7 μM) as well as a potent inhibitor of Na^+-dependent glu uptake (5 μM). Although this compound does possess some activity at the metabotropic receptors, and in fact comparatively good binding affinity (12 μM) for mGluR1a, this activity is less relative to the other activities. In view of the structural resemblance that (+)-**1** bears to kainic acid and to L-*trans*-pyrrolidine-2,4-dicarboxylate, the activities found for (+)-**1** are not surprising.[24] The present work provides further support for the g^+a conformation of glu as being the conformation relevant to kainic acid receptor recognition, while the ag^+ conformation is required for recognition at the Na^+-dependent glu transporter.

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