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FULL PAPER

Synthesis of L-Tricholomic Acid Analogues and Pharmacological Characterization at Ionotropic Glutamate Receptors

Lucia Tamborini,[a] Federica Mastronardi,[a] Leonardo Lo Presti,[b] Birgitte Nielsen,[c] Carlo De Micheli,[a] Paola Conti[a] and Andrea Pinto*[d]

Abstract: The synthesis of analogues of the natural compound L-tricholomic acid and of its *threo* diastereoisomer was accomplished in order to explore their affinity for glutamate ionotropic receptors. In this study, fourteen new unnatural amino acids, characterized by a 3-hydroxy-Δ^2-isoxazoline or 3-hydroxy-Δ^2-pyrazoline-skeleton, were obtained exploiting, as key reaction, a 1,3-dipolar cycloaddition or an intramolecular cyclization.

Introduction

L-Tricholomic acid [Figure 1] is a natural compound extracted from the poisonous mushrooms *Tricholoma muscarium*, *Amanita strobiliformis* and *Ustilago maydis*.[1-4] It is a partially rigidified analogue of the endogenous ligand L-glutamate (L-Glu), in which the distal carboxylic group is bioisosterically replaced by the 3-hydroxy-isoxazoline ring. L-Glu is the main excitatory neurotransmitter in the central nervous system (CNS), where it is involved in the modulation of many physiological processes such as learning, memory, and synaptic plasticity.[5] On the other hand, L-Glu plays a crucial role in acute and chronic neurodegenerative diseases (*i.e.*, cerebral ischemia, traumatic brain injury, spinal injury, epilepsy, ALS, Parkinson's, Alzheimer's and Huntington's diseases).[6] Once released into the synaptic cleft, L-Glu operates through through both ionotropic and metabotropic L-Glu receptors (iGluRs and mGluRs, respectively).[7]

We previously prepared L-tricholomic acid together with its three non-natural isomers, and we demonstrated that L-tricholomic acid is an agonist at the ionotropic glutamate receptors AMPA and KA (IC_{50} = 0.95 μM and 0.29 μM, respectively), whereas its D-enantiomer interacts selectively with the NMDA receptors (K_i = 0.67 μM) and both the L-*threo* and D-*threo* diastereoisomers are weak and non-selective iGluR ligands.[8]

It is well known that the homologation of the amino acidic chain of glutamatergic ligands could lead to a modification of the selectivity profile towards different Glu receptors and, interestingly, also to a shift of the pharmacological profile from an agonist to a partial agonist or even an antagonist.[9] Our interest in the design of selective ligands for glutamate receptors arises from the fact that the glutamatergic system has proved to be an important target for the therapy of acute and chronic diseases of the mammalian central nervous system. The availability of highly selective ligands for the different receptor subtypes represents a primary target to understand the physiological role played by the different subtypes and to design new neuroprotective agents with reduced side effects. Therefore, we decided to synthesize new enantiomerically pure amino acids **1**-**4** (Figure 1), which represent higher homologues of the model compound tricholomic acid and its *threo* diastereoisomer. Compounds (α*S*,5*S*)-**3a-c**, (α*S*,5*R*)-**4a-c** (Figure 1) are also characterized by an increased molecular complexity obtained through the insertion of a bulky phenyl ring in the 3-position of the isoxazoline core, functionalized in the *ortho*, *meta* or *para* position with the second acidic function, *i.e.*, the carboxylic acid. Increasing the molecular complexity could lead to an increase of the binding affinity for one glutamate receptor subtype, thus obtaining more selective ligands.

Similarly, an increase of the molecular complexity was obtained in compounds (α*S**,5*R**)-**5a**, (α*S**,5*S**)-**5b**, (α*S**,5*R**)-**6a** and (α*S**,5*S**)-**6b**, in which the isoxazoline ring was substituted with a pyrazoline ring bearing a benzyl group at the N1.

Very recently, this strategy led us to the identification of novel subtype selective NMDA receptor antagonists.[10] In order to facilitate the synthetic pathway, these amino acids have been initially synthesized as racemates starting from D,L-serine, with the purpose of preparing and testing the single enantiomers only if any interesting biological activity would be observed. The *ortho*-substituted derivative resulted to be unstable in bases because it underwent intramolecular lactamization due to the reaction of the aromatic carboxylate with the N2 of the pyrazoline, forming a stable six-membered ring.

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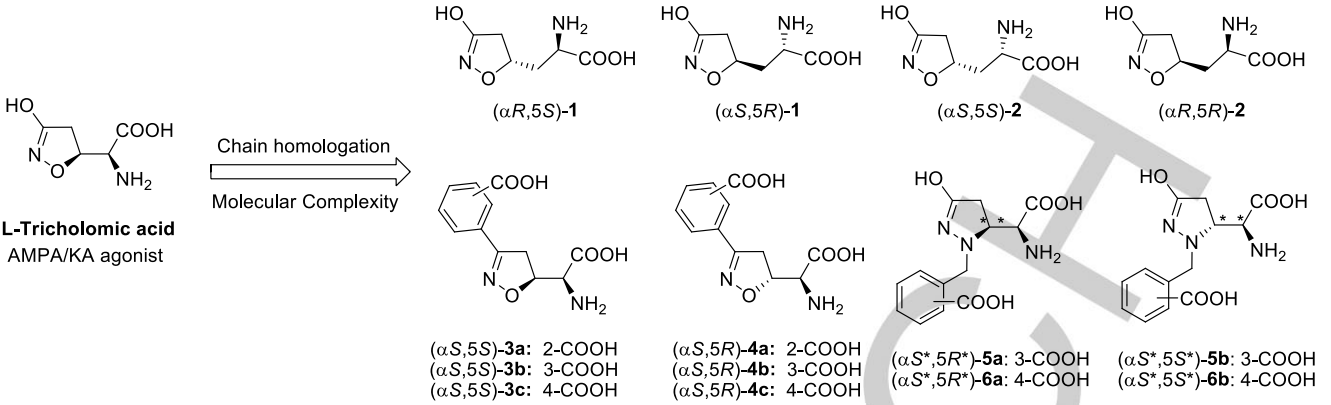
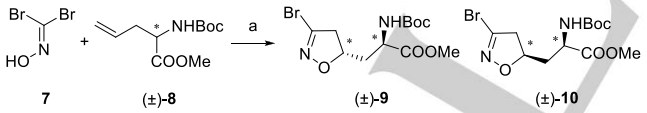


Figure 1. Structures of the model compound L-tricholomic acid and target compounds.

Results and Discussion

To synthesize the target compounds **1-2**, we exploited the 1,3-dipolar cycloaddition reaction using the racemic dipolarophile (±)-**8** and bromoformonitrile oxide, generated *in situ* by treating its precursor dibromoformaldoxime **7** with a base, with the idea to separate the single enantiomers of the formed cycloadducts by semi-preparative chiral HPLC (Scheme 1). When the reaction was complete, the two diastereoisomers (±)-**9** and (±)-**10** were efficiently separated by flash chromatography. The relative configuration of the new cycloadducts was initially assigned by comparison of the chemical shifts and the coupling constants with the ones of some previously reported analogues.[11] In particular, the signals corresponding to the two hydrogen atoms in 3'-position were peculiar. The outcome of the cycloaddition reaction, which gave as the major products the 5-substituted isoxazolines, is in line with theoretical prediction on the basis of the Frontier orbital theory where the dominant interaction involves the LUMO of the 1,3-dipole and the HOMO of the dipolarophile.[12]



Scheme 1. Reagents and conditions: a) $NaHCO_3$, EtOAc, rt, 48 h.

In order to separate the two pairs of enantiomers (+)-**9**/(–)-**9** and (+)-**10**/(–)-**10** by chiral HPLC, a number of different stationary phases in combination with various eluent solutions and flow rates have been explored. The best conditions for the separation of the two racemic mixture were obtained using a *tris*-(3,5-dimethylphenyl)carbamoyl amylose as chiral stationary phase. The separation of the enantiomers (+)-**9**/(–)-**9** and (+)-**10**/(–)-**10** was then efficiently performed, obtaining the single enantiomers in 99% ee.

Compound (+)-**9** has been crystallized using a mixture EtOAc/*n*-hexane and, due to the anomalous scattering of the bromine atom, its absolute configuration was unequivocally determined by single-crystal X-ray diffraction analysis and resulted to be (+)-(α*S*,5*R*)-**9**, confirming our initial speculation on the basis of the ^{1}H NMR. Figure 2 shows the asymmetric unit of compound (+)-**9** with the atom-numbering scheme and a photograph of the crystal used for the analysis.

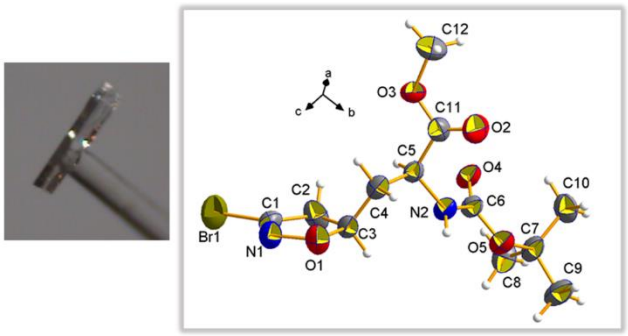


Figure 2. Diffraction-quality crystal and perspective view of the molecular structure of (+)-**9** at room temperature, with the atom numbering scheme. Thermal ellipsoids are drawn at the 30 % probability level.

Also enantiomer (–)-**10** was successfully crystallized using a 1:1 mixture dichloromethane/*n*-hexane and its absolute configuration was determined by single-crystal X-ray diffraction analysis and resulted to be (–)-(α*S*,5*S*)-**10** (Figure 3). CCDC 1560014 for [(+)-**9**] and 1560013 for [(–)-**10**] contain the full supplementary crystallographic data for this work. The latter can be obtained free of charge from the Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

Finally, compounds (α*S*,5*R*)-**9**, (α*R*,5*S*)-**9**, (α*R*,5*R*)-**10** and (α*S*,5*S*)-**10** were converted into the desired amino acids (α*S*,5*R*)-**1**, (α*R*,5*S*)-**1**, (α*R*,5*R*)-**2** and (α*S*,5*S*)-**2**, respectively, by alkaline hydrolysis of the ester function, replacement of the 3-Br substituent with a hydroxyl group through a nucleophilic substitution reaction and standard Boc-deprotection with trifluoacetic acid (Scheme 2).

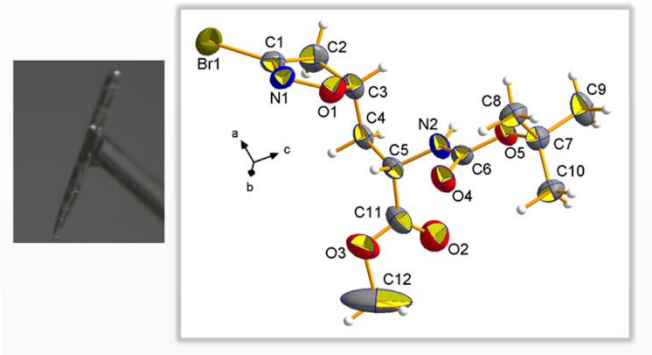
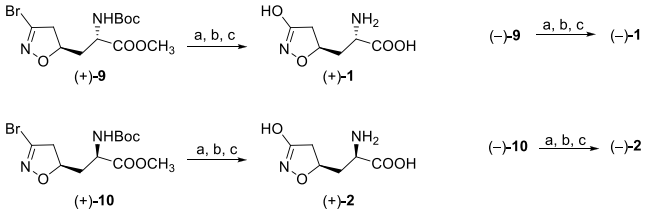
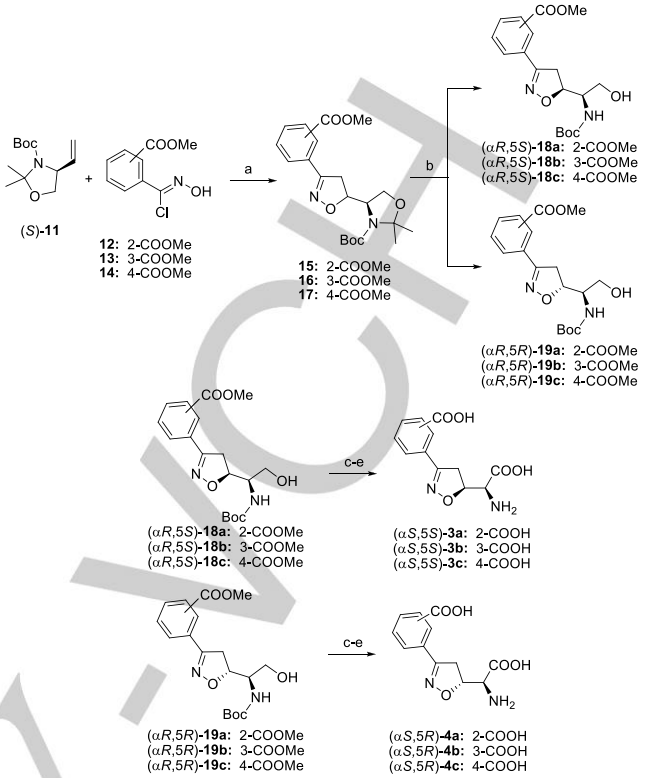


Figure 3. Diffraction-quality crystal and the asymmetric unit of compound (–)-**10** at room temperature, with the atom numbering scheme. Thermal ellipsoids are drawn at the 30 % probability level.



Scheme 2. Reagents and conditions: a) 0.5N NaOH, dioxane, rt; b) 1N NaOH, 65 °C; c) 30% TFA in CH_2Cl_2, rt.

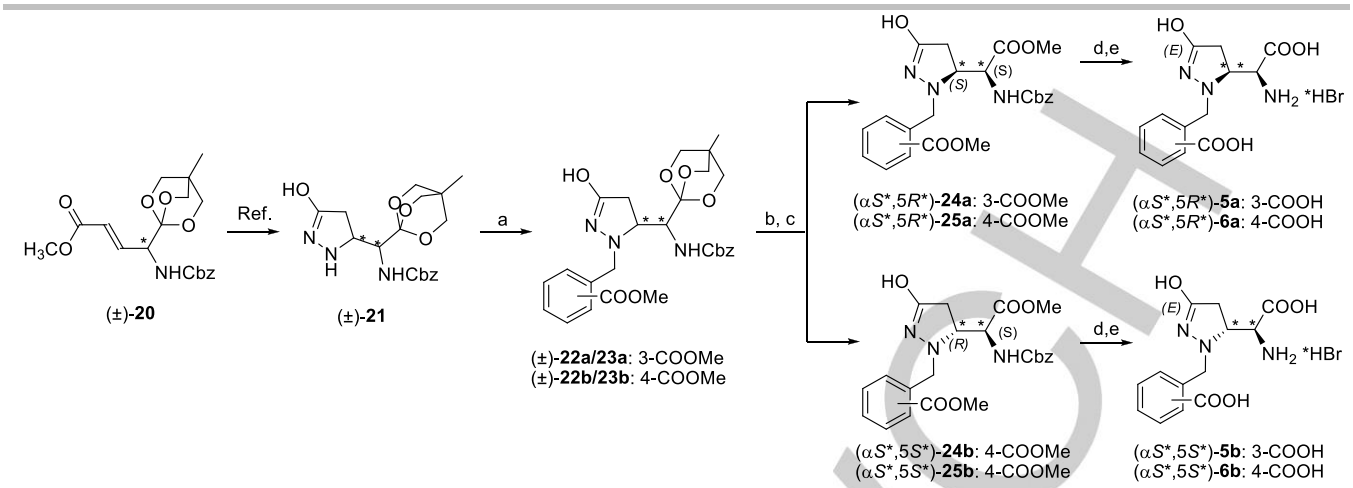


Scheme 3. Reagents and conditions: a) $NaHCO_3$, EtOAc, rt; b) 1:5 mixture H_2O/AcOH; c) PDC, DMF; d) 0.5N NaOH; e) 30% TFA in CH_2Cl_2.

The synthesis of compounds (α*S*,5*S*)-**3a-c** and (α*S*,5*R*)-**4a-c** was accomplished exploiting the 1,3-dipolar cycloaddition of the suitable nitrile oxide, generated *in situ* by treatment of its corresponding stable chloro oxime precursor **12-14** with a base, with (*S*)-3-(*tert*-butoxycarbonyl)-2,2-dimethyl-4-vinyloxazolidine (*S*)-**11** (Scheme 3), prepared starting from D-serine following a literature procedure.[8] Choro oximes **12**, **13** and **14** were prepared starting from the corresponding aldehydes following a reported protocol.[13] The acetonide of intermediates **15**-**17** was removed by treatment with a 1:5 solution H_2O/AcOH,[14] and, at this stage, it was possible to separate the couple of diasteroisomers by flash column chromatography. The primary alcohol of the isolated diastereoisomers was oxidized to carboxylic acid with PDC in DMF and in turn converted into the final amino acids (α*S*,5*S*)-**3a-c** and (α*S*,5*R*)-**4a-c** using standard deprotection conditions (Scheme 3).The relative configuration of derivatives **3a**/**4a**, **3b**/**4b**, and **3c**/**4c** was assigned by comparing their ^{1}H NMR spectroscopic signals with the signals of the model compound L-tricholomic acid.

Finally, the 3-hydroxy-pyrazoline derivatives were synthesized through a condensation/intramolecular cyclization between hydrazine and the α,β-unsatured ester (±)-**20**, in turn obtained starting from D,L-serine.[15a] The key intermediate (±)-**21** was obtained as a 1:1 mixture of two racemic diasteroisomers with relative configuration (α*S**,5*S**) and (α*S**,5*R**), inseparable at this stage. Treatment of intermediate (±)-**21** with methyl-3-(bromomethyl)benzoate or methyl-4-(bromomethyl)benzoate in presence of K_2CO_3 under microwave irradiation yielded intermediates (±)-**22a/23a** and (±)-**22b/23b**, respectively (Scheme 4).

The couples of diastereoisomers (±)-**22a/23a** and (±)-**22b/23b** were separated by column chromatography after conversion into the corresponding methyl esters (±)-**24a,b** and (±)-**25a,b**. The assignment of the relative configuration to the diastereoisomers was based on the comparison of the ^{1}H NMR spectra of each diastereoisomer with that of previously reported analogues, whose relative configuration was unambiguously assigned by X-ray analysis.[15b] The final amino acids (α*S**,5*R**)-**5a**, (α*S**,5*S**)-**5b**, (α*S**,5*R**)-**6a** and (α*S**,5*S**)-**6b** were obtained after alkaline hydrolysis of the methyl esters followed by cleavage of the Cbz protecting group with hydrobromic acid in a solution of acetic acid. Binding affinities of the synthesized amino acids were determined at native AMPA, KA, and NMDA receptors (rat synaptosomes). The data reported in Table 1 show that the carbon homologation carried out on the model compound tricholomic acid and its unnatural isomers has produced some changes in the affinity profile for iGluRs. In fact, while amino acid (α*R*,5*S*)-**1** is a ligand endowed with a good affinity (K_i = 6.0 μM) and selectivity for the NMDA receptors, its enantiomer is a non-selective ligand, characterized by low affinity for both the KA and NMDA receptors. Concerning stereoisomers (α*R*,5*R*)-**2** and (α*S*,5*S*)-**2**, we observed that while amino acid (α*R*,5*R*)-**2** possesses affinity for both the AMPA (K_i = 12 μM) and NMDA (K_i = 6.2 μM) receptors, compound (α*S*,5*S*)-**2** shows no affinity for any of the iGluRs. The obtained data parallel the results observed for the majority of the NMDA antagonists reported in the literature, in which the interaction with NMDA receptor complex is usually enantioselective and resides in the (*R*)-enantiomer.



Scheme 4. a) Methyl-3-(bromomethyl)benzoate or methyl-4-(bromomethyl)benzoate, K_2CO_3, NaI, CH_3CN, 85 °C, mw; b) PPTS, MeOH, H_2O; c) K_2CO_3, MeOH; d) 0.5N NaOH, dioxane; e) 33% HBr in AcOH.

Binding data showed for all the other compounds a general decrease in affinity. None of the amino acids, **3a-c** and **4a-c,** revealed affinity for AMPA or KA receptors at concentration up to 100 μM. Replacing the isoxazoline ring with the pyrazoline nucleus did not improve the binding affinity: among derivatives **5a,b** and **6a,b** only (α*S**,5*R**)-**5a** is able to interact with AMPA or KA receptors with affinities in the mid-micromolar range. Notably, in this case the binding preference depends on the relative stereochemistry since (α*S**,5*R**)-**5a** is a non-selective AMPA/KA ligand whereas its diasteroisomer (α*S**,5*S**)-**5b** is devoid of any affinity for iGluRs.

Conclusions

We have synthesized a series of analogues of the natural compound L-tricholomic acid in the attempt to identify potential selective ligands for iGluR subtypes. The rationale was based on the use of classical medicinal chemistry strategies, widely applied in the design of glutamatergic ligands, *i.e.*, homologation of the amino acidic chain and increase of the molecular complexity. The key step for the synthesis of compounds **1-4** was a cycloaddition reaction performed in good yields starting from the appropriate dipoles and dipolarophiles. Compounds **1** and **2** have been obtained as single enantiomers by exploiting a semi-preparative chiral HPLC separation on the protected precursors, whereas enantiomerically pure **3a-c** and **4a-c** have been synthesised starting from the chiral dipolarophile. The absolute stereochemistry for compounds **1** and **2** has been unequivocally assigned by X-ray crystallography of the protected precursors, whereas, for all the other amino acids the configuration was deduced by comparison of the ^{1}H NMR spectra. Unfortunately, pharmacological investigation at native iGluRs did not highlight any ligand endowed with a worth noting affinity or selectivity for a specific receptor. We can speculate that the distance between the α-amino acidic group and the distal carboxylate may not be optimal for the interaction with the residues of the binding cavity. Unfortunately, also in the series of amino acids **5a,b** and **6a,b** we did not observe the expected increase of potency associated to an increased molecular complexity.

Table 1. Receptor binding affinities at native iGluRs (rat synaptosomes)[a]

Compound	[^{3}H]AMPA IC_{50} (μM)	[^{3}H]KAIN IC_{50} (μM)	[^{3}H]CGP39653 Ki (μM)
(α*S*,5*R*)-**1**	> 100	74 [4.13±0.01]	18 [4.74±0.03]
(α*R*,5*S*)-**1**	> 100	> 100	6.0 [5.22±0.03]
(α*R*,5*R*)-**2**	12 [4.96±0.15]	> 100	6.2 [5.21±0.03]
(α*S*,5*S*)-**2**	> 100	> 100	> 100
(α*S*,5*S*)-**3a**	> 100	> 100	> 100
(α*S*,5*R*)-**4a**	> 100	> 100	> 100
(α*S*,5*S*)-**3b**	> 100	> 100	> 100
(α*S*,5*R*)-**4b**	> 100	> 100	> 100
(α*S*,5*S*)-**3c**	> 100	> 100	> 100
(α*S*,5*R*)-**4c**	> 100	> 100	> 100
(α*S**,5*R**)-**5a**	83 [4.08±0.02]	22 [4.67±0.09]	> 100
(α*S**,5*S**)-**5b**	> 100	> 100	> 100
(α*S**,5*R**)-**6a**	> 100	> 100	> 100
(α*S**,5*S**)-**6b**	> 100	> 100	> 100
L-tricholomic acid	0.95 [6.02±0.01]	0.29 [6.55±0.06]	41 [4.40±0.07]

[a] Data are given as mean [mean pIC_{50} ± SEM or mean pK_i ± SEM] of three independent experiments

Supporting Information Summary

All the experimental procedures, compound characterizations, X-ray diffraction analyses, binding assays are available in the Supporting Information.

Keywords: amino acids • cycloaddition • L-glutamic acid• receptors • L-tricholomic acid

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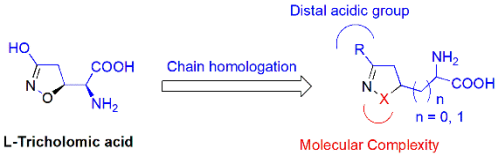
FULL PAPER

Fourteen analogues of the natural compound L-tricholomic acid and of its *threo* diastereoisomer have been designed and synthesized to explore their affinity for glutamate ionotropic receptors. The new derivatives, characterized by a 3-hydroxy-Δ^2-isoxazoline or 3-hydroxy-Δ^2-pyrazoline-skeleton, were obtained exploiting, as key reaction, a 1,3-dipolar cycloaddition or an intramolecular cyclization reaction. Binding affinities of the synthesized amino acids were determined at native AMPA, KA, and NMDA receptors and the results could help to design new selective glutamate receptor ligands.



Glutamate Receptor Ligands

*Lucia Tamborini, Federica Mastronardi, Leonardo Lo Presti, Birgitte Nielsen, Carlo De Micheli, Paola Conti and Andrea Pinto**

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