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Stereoselective synthesis and preliminary evaluation of new D-3-heteroarylcarbonylalanines as ligands of the NMDA receptor

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Abstract—New *N*-heteroarylcarbonylalanines of the D-series were stereoselectively prepared from enoates derived from D-mannitol. These compounds were active in binding and functional assays of the NMDA sub-type of glutamate receptors. A pyridine derivative inhibited MK801 binding, protected neurons from excitotoxic damage and blocked NMDA-induced currents in neurons. A thiophene derivative positively modulated the NMDA receptor, possibly through the allosteric glycine site.
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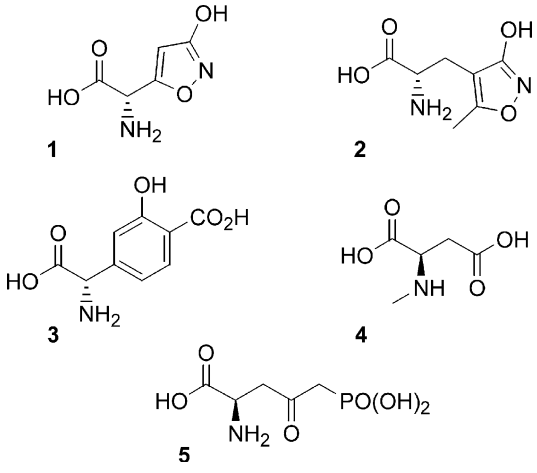
1. Introduction

(*S*)-Glutamic acid is the major excitatory aminoacid neurotransmitter in the vertebrate central nervous system, where it operates on two types of receptors (GluR): ionotropic (ligand-gated ion channels) and metabotropic (G-protein coupled).[1] Glutamatergic neurotransmission plays an important role in memory, learning, and developmental plasticity, as well as in pathological processes.[2] GluR hypoactivity has been implicated in schizophrenia, while excessive activity leads to acute and chronic neurodegenerative disorders.[3]

The interest in nonnatural aromatic α-aminoacids aiming at GluR started with the discovery of the strong hallucinogenic effect of ibotenic acid (**1**), a naturally occurring excitotoxin that is active as agonist at all sub-types of GluR. A structurally related compound, (*S*)-AMPA (**2**), is the prototype agonist for the AMPA sub-type of ionotropic GluR while (*S*)-4C3HPG (**3**) is a metabotropic receptor agonist. On the other hand, the NMDA sub-type of ionotropic GluR generally exhibits affinity for amino acids bearing the nonnatural



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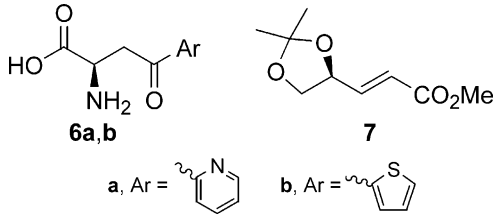
R-configuration, including the prototype agonist *N*-methyl-D-aspartate (**4**), and the antagonist MDL 100453 (**5**).[4] The peculiar stereoselectivity of NMDA receptors is also present at the coagonist glycine site, where D-serine is the main endogenous agonist in most regions of the brain.

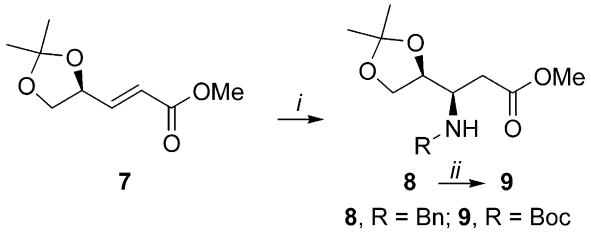
Considering the potential use of NMDA receptor ligands in therapeutics, we started a program aiming at the synthesis and pharmacological evaluation of new heteroaromatic amino acids related to known agonists and antagonists. As initial targets, we designed compounds **6a**,**b** (series D) as analogues of **5**, where a methylenephosphonate moiety has been substituted for a heteroaromatic group. Although **6a**,**b** have longer chains than the usual aromatic analogues such as **1**, **2**, and **3**, the presence of a carbonyl group introduces conformational restrictions that might favorably affect binding to the glutamate site. The role of the distal acidic group in NMDA antagonists is well established, but the effect of replacement with basic (pyridine, **6a**) or neutral (thiophene, **6b**) heteroaromatic groups has not been described.



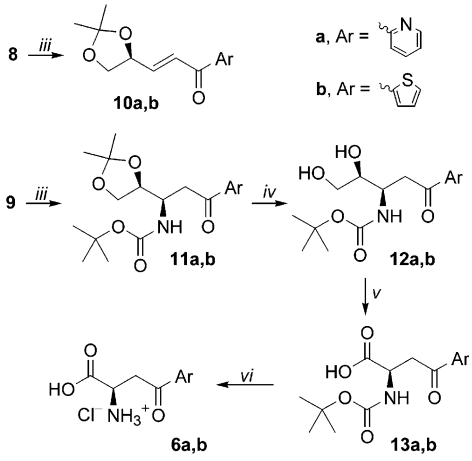
A synthetic approach was designed to obtain these compounds in enantiomerically pure form, using in the key step a *syn*-stereoselective conjugate addition of benzylamine to enoate **7**,[5] prepared from D-mannitol,[6] to introduce the configuration required for D-aminoacids. A new transformation of β-aminoesters into β-aminoarylketones, previously described by our group, was also employed in the synthetic strategy.[7] Since the enantiomer of **7** is available from vitamin C,[8] the synthesis of the L-series analogues of **6a**,**b** is also possible using this approach.

The reaction between **7** and benzylamine led to our key intermediate, *syn*-**8**, in good to excellent stereoselection (*syn*/*anti* > 9.5/0.5 at −30 °C) (Scheme 1). Hydrogenolysis in MeOH of the *N*-benzyl group in **8**, followed by in situ addition of a $(Boc)_2O$/MeOH solution, furnished the *N*-Boc-aminoester **9** in excellent yield.

The reactional course of the addition of aryllithium reagents to *syn*-**8** and *syn*-**9** was modulated by the nature of R, as previously described by our laboratory.[7] While the unexpected arylketones **10a**,**b** were obtained from *syn*-**8**, the undesired elimination of benzylamine could be avoided when the *N*-Boc derivative *syn*-**9** was the substrate, leading to **11a**,**b** as the only product isolated, in reasonable to good yields (Scheme 2). The dimethylacetal group in compounds **11a**,**b** could be selectively



Scheme 1. Synthesis of *syn*-**8** and *syn*-**9**. Reagents and conditions: (i) $BnNH_2$ (3 equiv), 30 °C, 3 d, 80%, *syn*/*anti* > 90%; (ii) (a) H_2 (1 atm), MeOH, Pd/C 10% p/p, 1 h; (b) $(Boc)_2O$, 95%.



Scheme 2. Synthesis of heteroarylalanines **6a**,**b**. Reagents and conditions: (iii) ArLi (2.5 equiv), THF, −78 °C; (iv) HCl 1 M (2. equiv), MeOH/H_2O, 0 °C, neat; (v) (a) $NaIO_4$, THF/H_2O; (b) $NaClO_2$, NaH_2PO_4, H_2O_2, 10–15 °C; (vi) $HCl_{(g)}$/MeOH (dry), 0 °C.

hydrolyzed under mild acidic conditions, leading to the corresponding *N*-protected amino diols **12a**,**b**. In contrast with what was reported by Dondoni and collaborators for similar hydroxyketones,[9] we did not observe the presence of cyclic hemiketals as the result of the attack of the primary hydroxy group at the benzylic ketone group. The diols **12a**,**b** were oxidized with sodium periodate[6] and the resulting crude aldehydes were allowed to react with sodium chlorite.[10] The formation of a carboxylic function was confirmed by preparation of the methyl ester derivatives from **13a**,**b** with diazomethane. The target amino acids **6a**,**b** were obtained as hydrochlorides from **13a**,**b** after cleavage of the protecting Boc group under nonhumid acidic conditions. Similar synthetic sequences have been employed by other groups, without racemization of the α-stereogenic center.[11]

Compounds **6a**,**b** were initially screened in binding assays of [^{3}H]MK-801 (dizocilpine), a noncompetitive antagonist of the NMDA receptor that binds to and blocks the channel pore mostly when the receptor is in its active conformation. In rat brain homogenates, **6a** inhibited the binding of [^{3}H]MK-801 in a concentration-dependent manner with IC_{50} around 300 μM

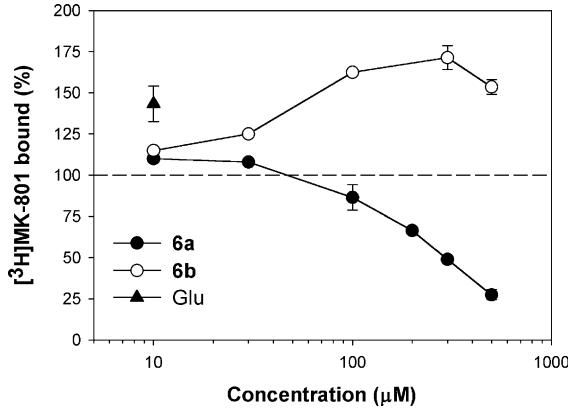
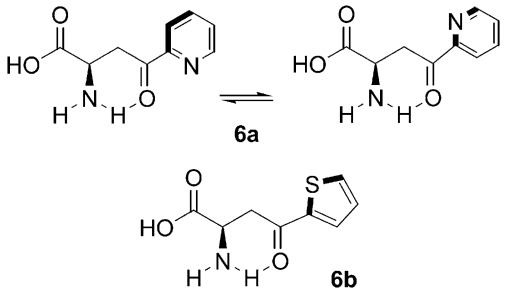


Figure 1. Modulation of [^{3}H]MK-801 binding to rat cerebral cortical membranes. Data are means and SEM from triplicate measurements in 1–4 experiments.



Scheme 3. Conformational equilibrium in **6a** and **6b** by molecular modeling (PM3).

($n=2$). The effect of **6a** was not modulated by addition of glutamate (0.1–100 μM) or glycine (0.01–10 μM), suggesting a noncompetitive mechanism. In contrast, **6b** increased the binding of [^{3}H]MK-801, suggesting a positive allosteric modulation similar to that seen with L-glutamate (Fig. 1). Neither **6a** nor **6b** (50–500 μM) significantly affected the binding of [^{3}H]kainic acid, showing selectivity for NMDA versus kainate receptors.

Data from neuroprotection assays performed with cultured rat cortical neurons agreed with the binding results. Compound **6a** inhibited delayed neuronal death induced by a brief application of glutamate (200 μM) and glycine (10 μM), by 18.4% ± 3.9% at 50 μM, and by 60.3% ± 9.4% at 500 μM ($P<0.05$, $n=3$, ANOVA). In the same assay, **6b** was not significantly protective at 50–500 μM ($n=3$).

In whole-cell patch-clamp recordings performed on cultured hippocampal neurons, **6a** (200 μM) inhibited NMDA-evoked currents by 14.3 ± 3.1% ($n=10$). Compound **6b** (200 μM) also produced a small but significant inhibition of NMDA-evoked currents, reducing them by 12.5 ± 2.2% at −60 mV ($n=5$). These inhibitory effects reached an apparent steady state in less than one second after switching from the solution containing agonist alone to that containing agonist plus **6a** or **6b**. Like NMDA, neither of the two novel compounds produced whole-cell currents in hippocampal neurons when applied alone ($n=10$), thus ruling out an agonist effect on AMPA, kainate, or $GABA_A$ receptors, which are abundant in this preparation. However, a brief (5 s) application of **6b** (200 μM) in the presence of NMDA (10 μM, without glycine) induced robust currents, similar in amplitude and time course to those elicited by glycine (10 μM) with NMDA.

These data suggest that the two novel D-amino acids **6a**,**b** interact with NMDA receptors in distinct manners. Results from three different assays suggest that **6a** is a weak noncompetitive antagonist of NMDA receptors, since it inhibits the binding of MK-801 in an agonist-independent manner, protects neurons from glutamate injury (mediated by activation of NMDA receptors), and partially blocks NMDA-evoked whole-cell currents. The main activity of **6b** was a positive modulation of the NMDA receptor, as evidenced by the increased binding of MK-801 and increased channel activation observed in electrophysiological experiments. The target of **6b** for its agonist-like effect is likely to be the glycine site, because receptor activation appeared only in the absence of glycine. In the presence of saturating glycine (10 μM), **6b** actually inhibited currents activated by a low concentration of NMDA (10 μM). This weak inhibition may suggest that **6b** competed with glycine, being an agonist with slightly lower intrinsic efficacy. Alternatively, **6b** could be a full agonist at the glycine site, with an additional inhibitory action through other site(s).

Semi-empirical calculations using PM3 hamiltonian (MOPAC) showed that compounds **6a**,**b** prefer a cyclic structure, maintained by hydrogen bond between the hydrogen attached at the nitrogen and the carbonyl group at the benzylic position, placing the aromatic moieties near the carboxy group and almost coplanar with the carbonyl group at the six-membered ring. In the thiophene derivative, a *transoid* relationship between the oxygen at the carbonyl group and the sulfur atom is preferred whereas in the pyridine derivative, *cisoid* and *transoid* conformers (O, N relationship) are almost isoenergetic (Scheme 3). These conformational trends will be useful in the design of new analogues and the synthesis and pharmacological evaluation of derivatives of **6** is under way in our laboratories.

2. Experimental section

Enoate **7** and adduct *syn*-**8** were prepared as previously described, using D-mannitol as starting material.[5]

2.1. Procedure for addition of 2-lithium-pyridine to 9

n-BuLi 1.4 M (1.9 mL; 2.7 mmol) was diluted with 9 mL hexane and cooled at −78 °C. A solution of 2-bromopyridine (0.23 mL; 2.4 mmol) in THF (2 mL) was then added slowly. After 10 min, a solution of *syn*-**9** (0.324 g; 2.7 mmol) in THF (1 mL) was added drop-wise. The reaction was completed in 15 min, then quenched

with AcOH 20% v/v in THF. The medium was neutralized with $NaHCO_3$ 10%, the aqueous phase was separated, and extracted with AcOEt. The final organic layer was dried under anhydrous Na_2SO_4 and the solvent was removed by evaporation under vacuum. After flash chromatography, **11a** was obtained as a yellow oil (0.211 g; 58% yield).

2.2. Procedure for addition of 2-lithium-thiophene to 9

To a solution of thiophene (0.4 mL; 5 mmol) in THF (10 mL) cooled at −78 °C was added *n*-BuLi 1.4 M (3.8 mL; 5.3 mmol) drop-wise. After 1 h, the medium was set at −15 °C for 1 h more, then cooled at −78 °C again. A solution of *syn*-**9** (0.606 g; 2.0 mmol) in THF (5 mL) was added drop-wise. Performing the same procedure described above, **11b** was obtained as a yellow oil (0.571 g; 80% yield).

2.3. General procedure for hydrolysis of the ketal group in 11a,b

To a solution of **11a**,**b** in 7 mL MeOH/H_2 O 3:1 (v/v) was added 2 mol equiv of HCl 1 M. The medium was cooled at 0–5 °C without agitation. After 16 h, the reaction mixture was neutralized with $NaHCO_3$ satd and then it was extracted with EtOAc (6×10 mL). The organic layer was dried under anhydrous Na_2SO_4 and the solvent was removed by evaporation under vacuum. The diols **12a**,**b** were obtained as amorphous solids (92% and 77% yield, respectively).

2.4. General procedure for oxidative cleavage of diol group in 12a,b

A suspension of $NaIO_4$ (190 mg; 1.1 mmol) in THF was added to a solution of crude **12a**,**b** in THF at rt. After 1 h, the medium was diluted with 10 mL H_2O, then extracted with CH_2Cl_2 (6×10 mL). The organic layer was dried under anhydrous Na_2SO_4 and the solvent was removed by evaporation under vacuum. The crude aldehyde was dissolved in MeCN (1 mL), followed by addition of a solution of $NaH_2PO_4{\cdot}H_2O$ (80 mg; 0.5 mmol) in 0.5 mL H_2O and later 0.1 mL H_2O_2 30% (1 mmol). The resultant medium was cooled at 0 °C, then a solution of $NaClO_2$ (125 mg; 1.1 mmol) in H_2O (1 mL) was added drop-wise for 15 min. After 3 h at 10–15 °C, the reaction mixture was acidified with HCl 10%, then extracted with EtOAc (6×10 mL). After usual work-up, the crude oil was purified by flash chromatography (AcOEt) furnishing **13a** (0.202 g; 76%) and **13b** (0.175 g; 71%) as amorphous solids.

2.5. Procedure for *N*-Boc deprotection on 13a,b

An anhydrous methanolic solution of *N*-Boc-amino acids **13a** (0.177 g) and **13b** (0.070 g) was cooled in ice bath and bubbled with HCl gas for 15 min. After 1 h, the solvent was removed by evaporation under vacuum, furnishing the solid hydrochlorides **6a** (0.152 g; 94%) and **6b** (0.050 g; 90%). ^{1}H NMR (200 MHz, D_2O) **6a**: δ 3.97 (2H, m); 4.53 (1H, dd, J = 4.1 and 6.3 Hz,); 8.10 (1H, m); 8.55 (2H, m); 8.55 (1H, d, J = 5.0 Hz), **6b**: δ 3.72 (1H, d, J = 5.0 Hz); 3.84 (1H, d, J = 5.0 Hz,); 4.56 (1H, t, J = 5.0 Hz); 7.23 (1H, m); 7.95 (2H, m).

2.6. Binding assays

About 100 μg of protein from adult Wistar rat brain cortical membranes were incubated in 0.5 mL of 5 mM Tris–HCl buffer (pH 7.2) and 2 nM [^{3}H]MK-801 for 1 h at 25 °C.[12] After separation of bound and free ligands by rapid filtration, the glass fiber filters were rapidly washed twice with 4 mL of ice-cold Tris–HCl buffer. Specific binding was defined as total binding minus binding measured in the presence of 3 mM ketamine. Similar procedures were used in the binding assay of 5 nM [^{3}H]kainic acid. Nonspecific binding was measured in the presence of 500 μM kainic acid.

2.7. Neuroprotection assay

Neocortical neurons were isolated from Wistar rat fetuses at 18–20 days of gestation and maintained in culture in 24-well plates for 14–16 days. Cells were washed with exposure saline (in mM: NaCl 165, KCl 5, $CaCl_2$ 2, D-glucose 30, HEPES 5, tetrodotoxin 15×10^{-5}, NaOH 2; pH 7.35) and the excitotoxic insult was started by adding glutamate 200 μM plus glycine 10 μM for 5 min. Compounds were added to the exposure saline 15 min before glutamate. After 5 min of glutamate exposure, cells were washed with culture medium, compounds were readded (without Glu or Gly) and the plate returned to the incubator. Four hours later, cell damage was evaluated by a kinetic spectrophotometric assay of lactate dehydrogenase in the culture supernatant.[13] Results from three experiments, each with triplicate cultures, were evaluated by ANOVA.

2.8. Electrophysiology

Hippocampal neurons were isolated from Wistar rat fetuses and maintained in culture for 23–41 days. Whole-cell membrane currents were recorded by the patch-clamp technique with membrane potential held at −60 mV and using a fast-switching motorized parallel tube array system for solution application.[14] The agonists NMDA (10 μM) and glycine (10 μM) were applied in assay saline (see above), with or without the test compounds, in short pulses (5–15 s). Data were analyzed with pCLAMP 6 software (Axon Instruments, USA).

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