Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry Letters 15 (2005) 2279–2283
Bioorganic & Medicinal Chemistry Letters

Analogues of the neuroprotective tripeptide Gly-Pro-Glu (GPE): synthesis and structure–activity relationships

Sergio A. Alonso De Diego,[a] Pilar Muñoz, Rosario González-Muñiz,[a] Rosario Herranz,[a] Mercedes Martín-Martínez,[a] Edurne Cenarruzabeitia,[c] Diana Frechilla,[c] Joaquín Del Río,[c] M. Luisa Jimeno[b,*] and M. Teresa García-López[a,*]

[a]*Instituto de Química Médica, Juan de la Cierva, 3, 28006 Madrid, Spain*
[b]*Centro de Química Orgánica Manuel-Lora Tamayo (CSIC), Juan de la Cierva, 3, 28006 Madrid, Spain*
[c]*Departamento de Farmacología-Unidad Asociada al CSIC, Universidad de Navarra, Irunlarrea, 1, 31080 Pamplona, Spain*

Received 23 December 2004; revised 23 February 2005; accepted 3 March 2005

Abstract—A series of GPE analogues, including modifications at the Pro and/or Glu residues, was prepared and evaluated for their NMDA binding and neuroprotective effects. Main results suggest that the pyrrolidine ring puckering of the Pro residue plays a key role in the biological responses, while the preference for *cis* or *trans* rotamers around the Gly-Pro peptide bond is not important.

It has been reported that the tripeptide H-Gly-Pro-Glu-OH (GPE) (**1**), naturally cleaved from the N-terminal sequence of insulin-like growth factor (IGF-1)[1,2] shows neuroprotective properties, both in vitro and in different animal models of neurodegenerative diseases, such as Huntington and Parkinson diseases and hypoxia-mediated ischemic brain injury.[3–5] This tripeptide displays a different range of biological actions compared to the remaining IGF-1 fragment, des(1-3) IGF-1, known as truncated IGF-1. Thus, truncated IGF-1 acts as a potent neurotrophic factor via the IGF-1 receptor while GPE neither binds to this receptor nor has any growth promoting effect.[6] Although the mode of action of this tripeptide remains unclear, there is evidence that it plays a neuromodulatory role in the CNS. Initial structural considerations suggested that GPE could interact with one or more glutamate receptor types and, in fact, it was demonstrated that it binds to the *N*-methyl-D-aspartate (NMDA) receptor but not to the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) or kainate (KA) receptors.[1] This pioneering study indicated that while the C-terminal glutamate is necessary for NMDA receptor binding, the N-terminal glycine potentiates this cross-reaction. It has also been shown that GPE potentiates the potassium-evoked release of acetylcholine and dopamine through an unknown mechanism and via the NMDA receptor, respectively.[1,5–7]

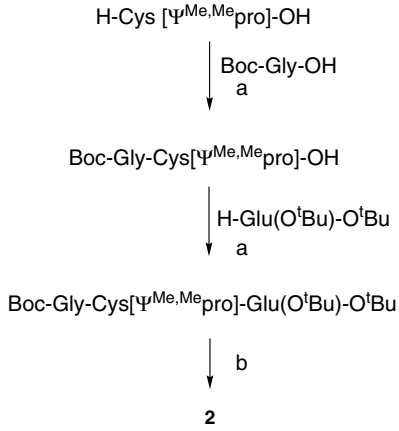
Our current interest in exploring novel strategies for the further development of effective neuroprotective drugs directed our attention to the search of non-peptide GPE mimics, which could be used as suitable pharmacological tools for gaining insight into the mode of action of this tripeptide. To this aim, we have followed the general structure/conformation/activity approach towards peptidomimetics based on the structure of the native peptide.[8] Because of the unique conformational properties of proline,[9] we considered that this amino acid residue should play a key role in the biological activity of GPE, as it happens with bioactive peptides containing Xaa-Pro residues.[10] In particular, unlike other peptide bonds which essentially adopt the *trans* form, the Xaa-Pro amide bond exists, in unfolded proteins and short peptides, as a mixture of *cis* and *trans* isomers[11] which, in the case of the Gly-Pro dipeptide residue of GPE, corresponds to a 19% *cis* content in 90/10 H_2O/D_2O solution, as indicated our preliminary ^1H NMR conformational analysis.[12] A number of alkylprolines[13,14] and pseudoprolines (ΨPro's)[15,16] derived from cysteine, serine and threonine (Xaa[Ψ^{R1R2}pro]) have been used to alter the *cis*/*trans* ratio of the peptide bond N-terminal to prolyl residues and, hence, to study the

Keywords: Gly-Pro-Glu (GPE); Neuroprotection; Structure–activity relationships.
* Corresponding authors. Tel.: +34 915622900; fax: +34 915644853; e-mail: iqmgl37@iqm.csic.es

0960-894X/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2005.03.015

relationship between *cis*/*trans* conformers and peptide bioactivity.[17,18] In this context, the easily synthetically accessible 2,2-dimethyl-thiazolidine-4(*R*)-carboxylic acid, Cys[$\Psi^{Me,Me}$pro] is a useful tool to induce up to 100% the *cis* conformation,[19] while the (*S*)-α-methylproline (P^{Me}) induces preferentially the *trans* form.[20] On this basis, we have replaced the Pro residue in GPE with Cys[$\Psi^{Me,Me}$ pro] and P^{Me}, respectively. On the other hand, it is known that the presence of the sulfur atom of the thiazolidine moiety alters the ring puckering of Cys[Ψ^{R1R2}pro] as compared to proline,[16,21] which may result in significant changes in the conformational properties of peptides incorporating thiazolidine proline surrogates. Therefore, to determine the influence of the proline ring puckering on the neuroprotective effect of GPE, we have also prepared the corresponding analogues containing Cys[$\Psi^{H,H}$pro] and L-5,5-dimethylproline (dmP).[21,22] To further explore the spatial requirements of GPE for this effect and to establish the influence of the length of the Glu side chain, stereoisomeric GPE analogues incorporating one or both chiral residues with D-configuration and analogues containing aspartic or homoglutamic (Hgl) acid have also been synthesized.[23] Here, we report the synthesis of the GPE analogues **2**–**12** (Fig. 1), and their in vitro biological effects, in comparison to the native neuroprotective tripeptide GPE (**1**).

The synthesis of the target tripeptides was performed in solution using BOP/DIEA activation in dichloromethane for the coupling reactions. Boc- and ^{t}Bu-protected Gly and Glu were, respectively, used for the synthesis of the ΨPro-containing tripeptides **2** and **3** since the Cys-derived thiazolidine ring system is stable under the Boc/^{t}Bu cleavage conditions.[24] As indicated in Scheme 1, Cys[$\Psi^{Me,Me}$pro], readily obtained by cyclocondensation of Cys with acetone,[16] was directly coupled with Boc-Gly-OH via BOP/DIEA activation. Similar coupling of the resulting ΨPro dipeptide with H-Glu-(O^{t}Bu)-O^{t}Bu, followed by concomitant removal of the Boc and ^{t}Bu protecting groups led to compound **2** in 25% overall yield. Inspection of the ^{1}H NMR spectrum of this 2-C dimethylated ΨPro containing tripeptide showed that the content of the *cis* population of the Gly-Cys [$\Psi^{Me,Me}$pro] amide bond is practically quantitative in 90/10 H_2O/D_2O,[25] indicating a drastic increase in this conformer in comparison to GPE. In the case of the Cys[$\Psi^{H,H}$pro] analogue **3**, the fully protected C-terminal dipeptide was firstly prepared by coupling the commercial Fmoc-ΨPro derivative with H-Glu(O^{t}Bu)-O^{t}Bu (Scheme 2). Fmoc deprotection with piperidine followed by coupling with Boc-Gly-OH and subsequent removal of the acid labile Boc and ^{t}Bu groups provided compound **3** in 56% total yield. As expected, the ^{1}H NMR spectrum of this compound revealed the presence of *cis* and *trans* conformers around the Gly-[$\Psi^{H,H}$pro] peptide bond in a 23:77 ratio, similar to that of Gly-Pro in GPE.[25,26]



Scheme 1. Reagents: (a) BOP, DIEA, CH_2Cl_2; (b) 15% TFA, CH_2Cl_2.

For the synthesis of the GPE analogues containing P^{Me}, dmP and D-Pro **4**–**7**, the N^{α}-Boc proline derivatives, Boc-AA_1-OH, were coupled with L-or D-glutamic acid dibenzyl esters H-AA_2(OBzl)-OBzl (Scheme 3). Removal of the N-terminal Boc group followed by coupling with Z-Gly-OH led to the fully protected tripeptides **13**–**16**, which upon hydrogenolysis in the presence of 10%Pd/C gave compounds **4**–**7** (63–70% total yield). Although *cis* and *trans* conformers around the Gly-P^{Me} and Gly-dmP peptide bond were observed in the ^{1}H NMR spectra of tripeptides **4** and **5**, respectively, the *trans* content increased by 9% in compound **4**, com-

H-Gly-Pro-Glu-OH (GPE)	**1**	H-Gly-D-Pro-D-Glu-OH	**7**
H-Gly-Cys[$\Psi^{Me,Me}$pro]-Glu-OH	**2**	H-Gly-Pro-D-Glu-OH	**8**
H-Gly-Cys[$\Psi^{H,H}$pro]-Glu-OH	**3**	H-Gly-Pro-Asp-OH	**9**
H-Gly-P^{Me}-Glu-OH	**4**	H-Gly-Pro-D-Asp-OH	**10**
H-Gly-dmP-Glu-OH	**5**	H-Gly-Pro-Hgl-OH	**11**
H-Gly-D-Pro-Glu-OH	**6**	H-Gly-Pro-D-Hgl-OH	**12**

Cys[$\Psi^{Me,Me}$pro] =

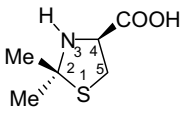
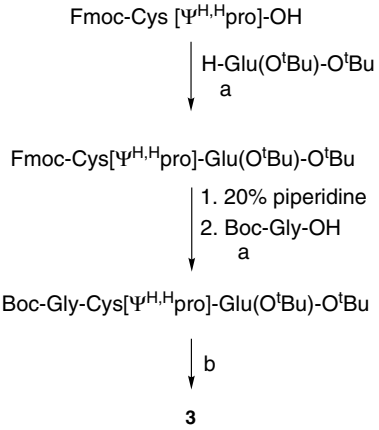
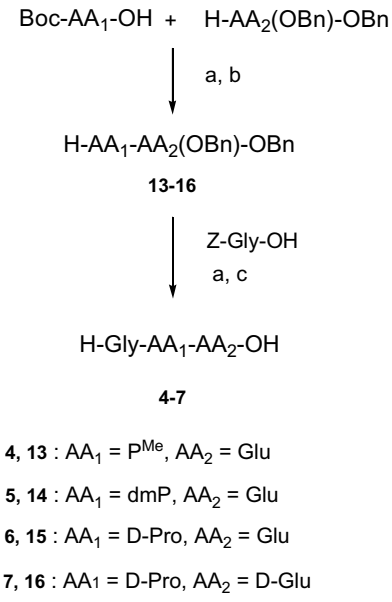


Figure 1. GPE analogues studied.



Scheme 2. Reagents: (a) BOP, DIEA, CH_2Cl_2; (b) 15% TFA, CH_2Cl_2.

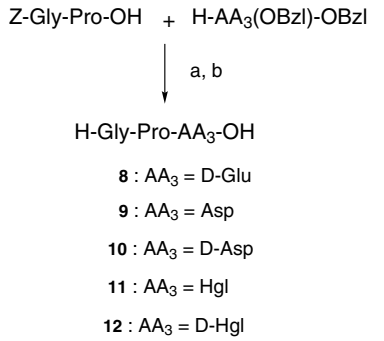


Scheme 3. Reagents: (a) BOP, DIEA, CH_2Cl_2; (b) HCl, EtOAc; (c) 10% Pd/C, H_2, MeOH.

pared to the model compound GPE, whereas the *cis* content increased by 50% in compound **5** with respect to this model.[27]

Finally, the GPE analogues modified at the glutamate residue **8**–**12** were prepared by coupling of the commercially available Z-Gly-Pro-OH with the corresponding acidic amino acid dibenzyl ester followed by hydrogenolysis, under the conditions indicated in Scheme 4 (total yields ranging from 70% to 85%).

Displacement of L-[^{3}H]glutamate from rat brain synaptic membranes by all these GPE analogues was determined and the results were compared to those of GPE and (2*R*)-2-amino-5-phosphonopentanoic acid [(*R*)-AP5], which were included in the same assay (Table 1). No displacement was found using concentrations up to 100 μM of compounds **2**, **3**, **6**–**12** (data not shown). However, analogues **4** and **5** incorporating the P^{Me} and dmP residues, respectively, displayed a noticeable affinity for GluRs which favourably compares to that of GPE. Particularly, in the case of compound **5**,



Scheme 4. Reagents: (a) BOP, DIEA, CH_2Cl_2; (b) 10% Pd/C, H_2, MeOH.

the affinity is similar to that exhibited by (*R*)-AP5, one of the most potent selective competitive NMDA antagonists.[28]

The highly *trans*-populated P^{Me} containing compound **4** and its dmP analogue **5**, in which the Gly-dmP bond was found to mainly adopt a *cis* conformation, showed very similar binding affinities. These results seem to suggest that the Gly-Pro amide conformation is not crucial for the interaction of GPE with the NMDA receptor complex. It is interesting to note that neither the Cys[$\Psi^{Me,Me}$pro] derivative **2** (>98% *cis* conformation) nor the Cys[$\Psi^{H,H}$ pro] analogue **3** (23:77 *cis*/*trans* ratio) were able to bind the NMDA receptor. The presence of the sulfur atom, that could alter the ring puckering and the hydrophobicity of the Cys[$\Psi^{Me,Me}$ pro] and Cys[$\Psi^{H,H}$pro] residues, as compared to proline, could serve to explain the loss of affinity of pseudoprolines **2** and **3** with respect to GPE and to compounds **4** and **5**.[16] Anyway, the lack of affinity of the stereoisomeric GPE analogues **6**–**8** also indicate that the spatial requirements for interacting GPE with the NMDA receptor are strict. At this point, it is worth pointing out that, a survey of literature reports on the structure–activity relationships of ligands interacting with the glutamate binding site of the NMDA receptor reveals the preference for the D-configuration, but a few exceptions have been described for both agonists and antagonists including D-Glu which interacts much less potently with NMDA than does L-Glu.[28–30] This is also the case of H-Gly-Pro-D-Glu-OH (**8**) which, in contrast to its diastereoisomer containing L-Glu, GPE, did not inhibit the L-[^{3}H]glutamate binding at concentrations up to 10^{-4} M. On the other hand, it has also been found that the α-carboxyl group and the side chain acidic function are separated by four or six atoms in most of the competitive NMDA antagonists.[28,30] However, homologation of the Glu residue in GPE to give the Hgl derivative **11**, bearing a side chain of four carbon atoms linking the two acidic groups, led to the loss of affinity. In summary, the studies on the structure–activity described here show the scarce tolerance of GPE to structural modifications. This makes its mode of interaction with the NMDA receptor an intriguing question.

Compounds **4** and **5**, which were effective in displacing L-[^{3}H]glutamate from rat membranes, were further investigated to evaluate their neuroprotective effects in cultured hippocampal neurons exposed to NMDA 100 μM, or oxygen–glucose deprivation (OGD) following described procedures.[31,32] As shown in Figure 2A,

Table 1. Displacement of L-[^{3}H]glutamate from rat brain synaptic membranes

Compound	K_i (μM)
L-Glutamate	0.87 ± 0.21
(R)-AP5	3.83 ± 1.76
GPE (H-Gly-Pro-Glu-OH) (**1**)	31.24 ± 15.65
H-Gly-P^{Me}-Glu-OH (**4**)	7.96 ± 1.83
H-Gly-dmP-Glu-OH (**5**)	3.79 ± 0.53

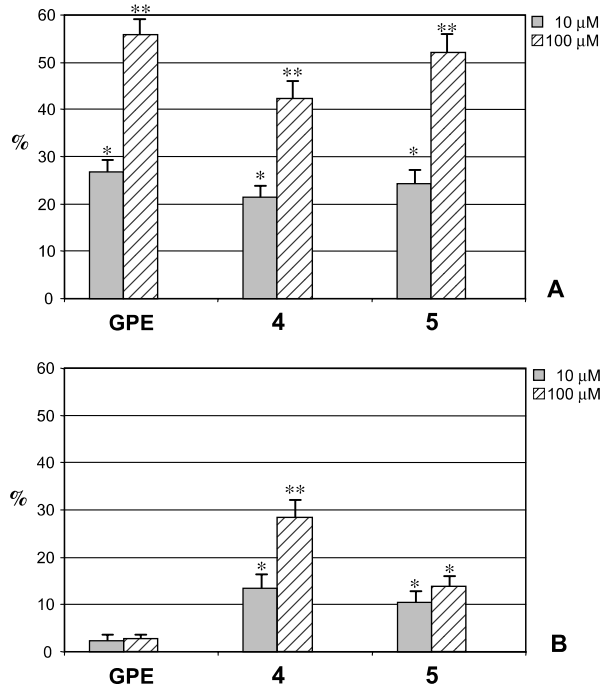


Figure 2. Percentage of protection of rat hippocampal neurons from NMDA (100 μM) excitotoxicity (A) and oxygen–glucose deprivation (B). Cell survival was estimated by measuring the activity of mitochondrial dehydrogenase on the tetrazolium derivative MTT. Values are the mean ± SEM of 8–13 experiments. Asterisks indicate difference from control (*$P < 0.05$, **$P < 0.01$, Student's *t*-test).

the neuronal death caused by NMDA administration was significantly prevented on pretreatment with the tripeptide analogues **4** and **5**, at the two concentrations assayed, 10 and 100 μM. These neuroprotective effects appeared to be of similar potency to that of GPE at the same concentrations in this in vitro assay. Although neuroprotective effects of i.v. infusion of exogenous GPE have been described in hypoxic-ischemic rats,[33] this tripeptide was not able to increase significantly the neuronal survival after OGD injury. However, compounds **4** and **5** showed neuroprotective activity, which was particularly significant in the case of the P^{Me} containing analogue **4**, with a protection value of 28.5%.

In conclusion, our findings suggest that the pyrrolidine ring puckering of the Pro residue is a key determinant for the NMDA binding and the neuroprotective effects of GPE. Contrastingly, the preference for *cis* or *trans* rotamers around the Gly-Pro peptide bond has no influence on these biological responses. The above results offer interesting perspectives for further investigating on GPE analogues incorporating other proline surrogates. This work is now in progress.

Acknowledgements

This work has been supported by the Spanish Ministry of Education and Science (SAF2003-07207-C02). S.A.A.D.D. holds a predoctoral fellowship from this Ministry for Associated Units to CSIC.

References and notes

1. Sara, V. R.; Carlsson-Skwirut, C.; Bergman, T.; Jörnvall, H.; Roberts, P. J.; Crawford, M.; Hakansson, L. N.; Civalero, I.; Nordberg, A. *Biochem. Biophys. Res. Commun.* **1989**, *165*, 766.
2. Yamamoto, H.; Murphy, L. J. *J. Endocrinol.* **1995**, *146*, 141.
3. Alexi, T.; Hughes, P. E.; Van Roon-Mom, W. M. C.; Faull, R. L. M.; Williams, C. E.; Clark, R. G.; Gluckman, P. D. *Exp. Neurol.* **1999**, *159*, 84.
4. Guan, J.; Krishnamurthi, R.; Waldvogel, H. J.; Faull, R. L. M.; Clark, R.; Gluckman, P. *Brain Res.* **2000**, *859*, 286.
5. Sizonenko, S. V.; Sirimanne, E. V.; Williams, C. E.; Gluckman, P. D. *Brain Res.* **2001**, *922*, 42.
6. Sara, V. R.; Carlsson-Skwirut, C.; Drakemberg, K.; Giacobini, M. B.; Hakansson, L.; Mirmiran, M.; Nordberg, A.; Olson, L.; Reinecke, M.; Stahlbom, P. A.; Nordqvist, A. C. S. *Ann. N.Y. Acad. Sci.* **1993**, *692*, 183.
7. Nilson-Hakansson, L.; Civalero, I.; Zhang, X.; Carlsson-Skwirut, C.; Sara, V. R.; Nordberg, A. *NeuroReport* **1993**, *4*, 111.
8. Hruby, V. J. *Nature Rev. Drug Discovery* **2002**, *1*, 847.
9. Schmid, F. X.; Mayr, L. M.; Mücke, M.; Schönbrunner, E. R. *Adv. Prot. Chem.* **1993**, *44*, 25.
10. Yaron, A.; Naider, F. *Crit. Rev. Biochem. Mol. Biol.* **1993**, *28*, 31.
11. Wedemeyer, W. J.; Welker, E.; Scheraga, H. A. *Biochemistry* **2002**, *41*, 14637, and references cited therein.
12. Conformer population ratio was determined by integration and/or deconvolution of the Gly α-CH_2 protons (*cis*: 3.95 and 3.74 ppm; *trans*: 4.03 and 3.98 ppm).
13. Beausoleil, E.; Lubell, W. D. *J. Am. Chem. Soc.* **1996**, *118*, 12902.
14. Beausoleil, E.; Sharma, R.; Michnick, S. W.; Lubell, W. *J. Org. Chem.* **1998**, *63*, 6572.
15. Haack, T.; Mütter, M. *Tetrahedron Lett.* **1992**, *33*, 1589.
16. Keller, M.; Sager, C.; Dumy, P.; Schutkowski, M.; Fischer, G. S.; Mütter, M. *J. Am. Chem. Soc.* **1998**, *120*, 2714.
17. An, S. S. A.; Lester, C. C.; Peng, J. L.; Li, Y. J.; Rothwarf, D. M.; Welker, E.; Thannhauser, T. W.; Zhang, L. S.; Tam, J. P.; Scheraga, H. A. *J. Am. Chem. Soc.* **1999**, *121*, 11558.
18. Wittelsberger, A.; Keller, M.; Scarpellino, L.; Patiny, L.; Acha-Orbea, H.; Mütter, M. *Angew. Chem., Int. Ed.* **2000**, *39*, 1111.
19. Dumy, P.; Keller, M.; Ryan, D. E.; Rohwedder, T.; Wöhr; Mütter, M. *J. Am. Chem. Soc.* **1997**, *119*, 918.
20. Delaney, N. G.; Madison, V. J. *J. Am. Chem. Soc.* **1982**, *104*, 6635.
21. An, S. S. A.; Lester, C. C.; Peng, J.-L.; Li, Y.-Jin.; Rothwarf, E. W.; Thannhauser, T. W.; Zhang, L. S.; Tam, J. P.; Scheraga, H. A. *J. Am. Chem. Soc.* **1999**, *121*, 11558.
22. Xia, Q.; Ganem, B. *Tetrahedron Lett.* **2002**, *43*, 1597.
23. During the preparation of this manuscript the D-Glu-containing GPE analogue was also described by others: Trotter, N. S.; Brimble, M. A.; Harris, P. W. R.; Callis, D. J.; Sieg, F. *Bioorg. Med. Chem.* **2005**, *13*, 501.
24. Wöhr, T.; Wahl, F.; Nefzi, A.; Rohwedder, T.; Sato, T.; Sun, X.; Mütter, M. *J. Am. Chem. Soc.* **1996**, *118*, 9218.
25. Although traces of the *trans* isomer cannot be excluded in compound **2** (1–2%), no evidence for this form was found either by H^1 or ^{13}C NMR. The *cis*/*trans* ratio of compound **3** was determined in 90/10 H_2O/D_2O by integration of the Glu α-H signals (δ_{cis}: 4.62 ppm and δ_{trans}: 4.56 ppm).
26. Kern, D.; Schutkowski, M.; Drakenberg, T. *J. Am. Chem. Soc.* **1997**, *119*, 8403.

27. The *cis*/*trans* ratio of compounds **4** (10:90) and **5** (69:31) was determined by integration of the Me and Me_2 of P^{Me} and dmP, respectively ($\mathbf{4}_{cis}$: 1.55 ppm; $\mathbf{4}_{trans}$: 1.57 ppm; $\mathbf{5}_{cis}$: 1.50 ppm; $\mathbf{5}_{trans}$: 1.43 ppm), in the ^1H NMR spectra in 90:10 H_2O/D_2O.
28. Braüner-Osborne, H.; Egebjerg, J.; Nielsen, E. O.; Madsen, U.; Krogsgaard-Larsen, P. *J. Med. Chem.* **2000**, *14*, 2609, and references cited therein.
29. Ornstein, P. L.; Klimkowski, V. J. In *Excitatory Amino Acid Receptors: Design of Agonists and Antagonists*; Krogsgaard-Larsen, P., Hansen, J. J., Eds.; Ellis Horwood: Chichester, 1992; pp 183–200.
30. Tikhonova, I. G.; Baskin, I. I.; Palyulin, V. A.; Zefirov, N. S.; Bachurin, S. O. *J. Med. Chem.* **2002**, *45*, 3836, and references cited therein.
31. Snider, B. J.; Moss, J. L.; Revilla, F. J.; Lee, C. S.; Wheeler, V. C.; MacDonald, M. E.; Choi, D. W. *Neuroscience* **2003**, *120*, 617.
32. De Cristobal, J.; Cárdenas, A.; Lizasoain, I.; Fernández-Tomé, P.; Lorenzo, P.; Moro, M. A. *Stroke* **2002**, *33*, 261.
33. Guan, J.; Thomas, G. B.; Lin, H.; Mathai, S.; Bachelor, D. C.; George, S.; Gluckman, P. D. *Neuropharmacology* **2004**, *47*, 892, and references cited therein.