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New Gly-Pro-Glu (GPE) analogues: Expedite solid-phase synthesis and biological activity

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Abstract—A suitable solid-phase approach, based on Fmoc/^{t}Bu methodology and on the use of 2-chlorotrityl resin, allowed a rapid and efficient preparation of new GPE analogues. Most of the synthesized tripeptides displayed glutamate receptor binding affinity comparable to that of GPE, but only a few derivatives showed significant neuroprotective activity.

It is thought that the tripeptide Gly-Pro-Glu (GPE) is endogenously formed, along with the des(1-3)IGF-1 fragment, from the acid protease-mediated metabolism of IGF-1(insulin-like growth factor type 1).[1–3] Unlike IGF-1 and des(1-3)IGF-1, GPE is not able to bind to IGF-1 receptors, but it displays remarkable CNS activities. It was reported that GPE stimulates acetylcholine and dopamine release,[4,5] and protects different types of neurons from diverse induced injuries (hypoxia-ischemia, glutamate, quinolinic acid, etc.).[5–8] Moreover, GPE shows neuroprotective properties in different animal models of neurodegenerative processes, such as Huntington's, Parkinson's, and Alzheimer's diseases.[5,6,9] While, it seems that GPE facilitates the release of dopamine through interaction with NMDA receptors,[4] other bioactivities of GPE were elicited via as yet unknown non NMDA-receptor mediated pathways. Because of its structural simplicity, GPE can be considered a promising starting point (lead) for the development of non-peptide analogues, able to mimic its biological actions. Therefore, peptidomimetics based on the GPE structure could serve, not only as pharmacological tools for a better understanding of the GPE actions, but as new neuroprotective agents. To achieve this task, and before any attempt to reduce the peptide character of GPE, it is essential to acquire a deep knowledge on the role of each residue within the peptide sequence. In this sense, some recent contributions, by us and others, describe a small number of modifications at Gly, Pro, and Glu residues.[10–14] Variations of the N-terminal residue include the replacement of Gly by Ala, D-Ala, β-Ala, Aib, and two α,α-cycloalkyl amino acids, as well as alkylated derivatives at the N-terminal amino group. From these GPE analogues, L-Ala and *N*,*N*-dimethyl Gly-containing derivatives have been described to show neuroprotective activity comparable to that of the parent peptide GPE (striatal cell assay, injury promoted by okadaic acid).[10]

Most of the changes made at the C-terminal Glu residue resulted in inactive compounds, although the replacement of the C-terminal α-carboxylate by a *N*,*N*-dimethylcarboxamide prevented the neuronal cell death with potency similar to that of GPE.[11,12]

Keywords: GPE analogues; Solid-phase synthesis; Neuroprotective activity.
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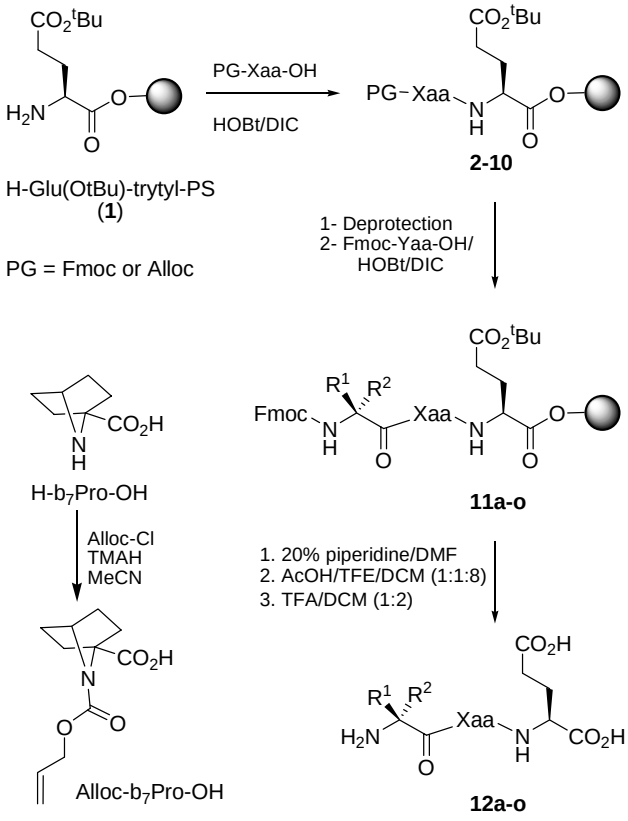
In our previous contribution to this subject, we have demonstrated that α-Me-L-Pro and 5,5-dimethyl-L-Pro GPE analogues rescued neuronal cultures from death induced by NMDA treatment or oxygen–glucose deprivation.[14]

In all the above contributions, the GPE derivatives were obtained in moderate to good yield using conventional solution synthesis. Now, to avoid the tedious purification steps of intermediates, we decided to explore a convenient solid-phase procedure for the generation of GPE analogues. New modifications at Gly and Pro residues were selected for this purpose. Thus, a set of representative aromatic (Phe), acid (Asp), basic (Lys), and aliphatic (Nle, Ile) amino acids were proposed as replacements for Gly. The already described N-terminal Ala and D-Ala derivatives were also included in the study to compare solution versus solid-phase approaches. Based on the promising biological results obtained by modification of the Pro residue,[14] and to better understand the role of this central amino acid, a series of GPE analogues, incorporating substituted prolines (Amp, Hyp), conformationally restricted analogues (Oic, b_7Pro), and Pro homologues (Aze, Pip), was also synthesized. The b_7Pro residue combines alkylation at 2 and 5 positions of Pro residue, two substitutions that when made independently both resulted in highly significant neuroprotective GPE analogues.[14] We also investigated the incorporation at the central residue of two 1-aminocycloalkanecarboxylic acids, Acp and Ach, which unlike Pro, favor all *trans* conformation around the Gly–Xaa amide bond, but similar to Pro serve to constrain the peptide backbone.[15] In the present study, we report the solid-phase synthesis, binding affinities at glutamate receptors, and the neuroprotective effects in cultured hippocampal neurons of the two indicated small libraries of tripeptide derivatives.

GPE analogues were synthesized manually in parallel on a 2-chlorotrityl polystyrene resin.[16] This solid support does not favor the on-resin formation of diketopiperazines, due to the steric hindrance of the bulky 2-chlorotrityl, and this minimizes losses of C-terminal dipeptides.[17,18] The following protected amino acids were used: Fmoc-Pro-OH, Fmoc-Amp(Boc)-OH, Fmoc-Hyp(^{t}Bu)-OH, Fmoc-Oic-OH, Alloc-b_7Pro-OH, Fmoc-Aze-OH, Fmoc-Pip-OH, Fmoc-Acp-OH, Fmoc-Ach-OH, Fmoc-Ala-OH, Fmoc-D-Ala-OH, Fmoc-Phe-OH, Fmoc-Asp(O^{t}Bu)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Nle-OH, and Fmoc-Ile-OH. The conformationally restricted Pro analogue H-b_7Pro-OH was prepared from H-Ser-OMe following a described procedure,[19] and converted into the Alloc-b_7Pro-OH derivative by N-protection with Alloc-Cl in the presence of tetramethylamonium hydroxide (Scheme 1).[20] Resin **1**, loaded with the first amino acid [H-Glu(O^{t}Bu)], was coupled to the central amino acid residue PG-Xaa-OH, as preformed active ester by using HOBt/DIC (Scheme 1, compounds **2**–**10**). The N-terminal residue was then similarly incorporated, after deprotection of the Fmoc (20% piperidine/DMF) or Alloc [$PhSiH_3$/$Pd(PPh_3)_3$] N-protecting groups, into intermediates **11a**–**11o**. Final tripeptides **12a**–**12o** were obtained by cleavage from the resin



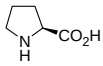
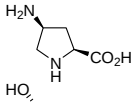
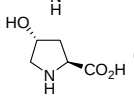
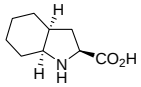
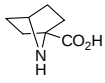
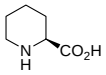
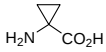
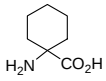
Scheme 1.

with AcOH/TFE, followed by treatment with TFA, to accomplish the removal of Boc and ^{t}Bu side-chain protecting groups. As shown in Table 1, all GPE analogues were recovered in good to excellent yield (68–98%) and high purity (>95%).[21] This solid-phase strategy afforded compounds **12a** and **12b** in 78% and 93% overall yield, respectively, which represents 38% and 47% of enhanced efficacy with respect to their preparation via solution methodologies.[10]

Displacement of L-[^{3}H]glutamate from rat brain synaptic membranes by all these GPE analogues was determined and the results were compared to that obtained for the endogenous model tripeptide GPE (Table 1). N-Terminal Ala, D-Ala, and Phe derivatives **12a**–**12c** displayed one order of magnitude higher affinity for the glutamate receptors than GPE, while Asp, Lys, and Nle analogues **12d**–**12f** were equipotent. No displacement was found for derivative **12g**, having a branched aliphatic residue at the N-terminal position.

Concerning modifications at the central residue, the incorporation of 4-amino- and 4-hydroxy-substituted prolines into derivatives **12h** and **12i**, respectively, led to a slight increase in the binding affinity, while no significant change was observed after the inclusion of the restricted Pro analogue Oic into compound **12j**. In contrast, excellent affinities for glutamate receptors were observed for tripeptides **12k** (60-fold increase) and **12m** (13-fold enhancement), with a highly restricted Pro ring and the superior Pro homologue, pipecolic acid. No

Table 1. List of synthesized compounds, synthetic yield, and binding to glutamate receptors

Dipeptide intermediate	Tripeptide intermediate	Final compound	Xaa	R^1	R^2	Yield[a] (%)	K_i[b] (μM)
		GPE	 (Pro)	H	H	—	31.24 ± 15.65
2	**11a**	**12a**	Pro	CH_3 (Ala)	H	78	2.66 ± 0.31
2	**11b**	**12b**	Pro	H	CH_3 (D-Ala)	93	5.40 ± 0.75
2	**11c**	**12c**	Pro	CH_2Ph (Phe)	H	98	4.85 ± 1.02
2	**11d**	**12d**	Pro	CH_2CO_2H (Asp)	H	88	41.30 ± 6.48
2	**11e**	**12e**	Pro	$(CH_2)_4NH_2$ (Lys)	H	93	22.03 ± 5.48
2	**11f**	**12f**	Pro	$(CH_2)_3CH_3$ (Nle)	H	86	19.50 ± 7.64
2	**11g**	**12g**	Pro	$CH(CH_3)CH_2$ CH_3 (Ile)	H	85	>100
3	**11h**	**12h**	 (Amp)	H	H	80	15.54 ± 4.78
4	**11i**	**12i**	 (Hyp)	H	H	68	9.24 ± 1.74
5	**11j**	**12j**	 (Oic)	H	H	93	22.37 ± 5.30
6	**11k**	**12k**	 (b_7Pro)	H	H	81	0.48 ± 0.09
7	**11l**	**12l**	 (Aze)	H	H	89	>100
8	**11m**	**12m**	 (Pip)	H	H	96	2.39 ± 0.17
9	**11n**	**12n**	 (Acp)	H	H	71	>100
10	**11o**	**12o**	 (Ach)	H	H	76	>100

[a] Yield of isolated compounds.
[b] Displacement of L-[^{3}H]glutamate from rat brain synaptic membranes.

appreciable affinity, at concentrations up to 100 μM, was found either for the low Pro homologue, Aze-containing derivative **12l**, or for compounds **12n** and **12o**, incorporating the nonproline-like residues Acp and Ach.

All synthesized compounds, regardless of their effectiveness in displacing L-[^{3}H]glutamate from rat brain membranes, were investigated for their neuroprotective effects on cultured hippocampal neurons exposed to NMDA (100 μM).[22] As shown in Figure 1, the measured neuroprotective effects were in all cases of lower potency than that of GPE in this in vitro assay. However, the neuronal death caused by NMDA administration was still significantly prevented on pretreatment with the tripeptide analogues **12c**, **12l**, **12n**, and **12o**, added at a 100 μM concentration. These outcomes seem to indicate that the neuroprotective activity of the GPE analogues in this assay is not directly related to the binding potency at glutamate receptors, since the best neuroprotection was obtained for GPE analogues that do not displace tritiated glutamate from these receptors.

In general, modifications at the N-terminal Gly residue led to GPE analogues with diminished potency. However, while Ala, D-Ala, and Phe derivatives (**12a**–**12c**) still showed significant prevention of neuronal cell death, the incorporation of acidic, basic of high aliphatic residues at this position was detrimental for the neuroprotective activity (compounds **12d**–**12g**).

Minute changes at the Pro residue, by incorporation of 4-NH_2 and 4-OH groups, led to GPE analogues **12h** and **12i** with 2- and 5-fold lower neuroprotective values, respectively, than the model tripeptide. Approximately half of the GPE activity was also retained by the conformationally restricted Oic and b_7Pro derivatives **12j** and **12k**, and by the pipecolic analogue **12m**. Nevertheless, compounds **12l**, **12n**, and **12o** preserved considerable neuroprotective activity at 100 μM, with recovery values ranging from 27% to 34%.

In summary, we have developed a fast and efficient solid-phase approach for the preparation of new GPE analogues. The biological evaluation of the synthesized tripeptide library has demonstrated that most of the GPE analogues maintain or improve the glutamate receptor binding affinity showed by GPE, but their neuroprotective activity was of lower potency than that

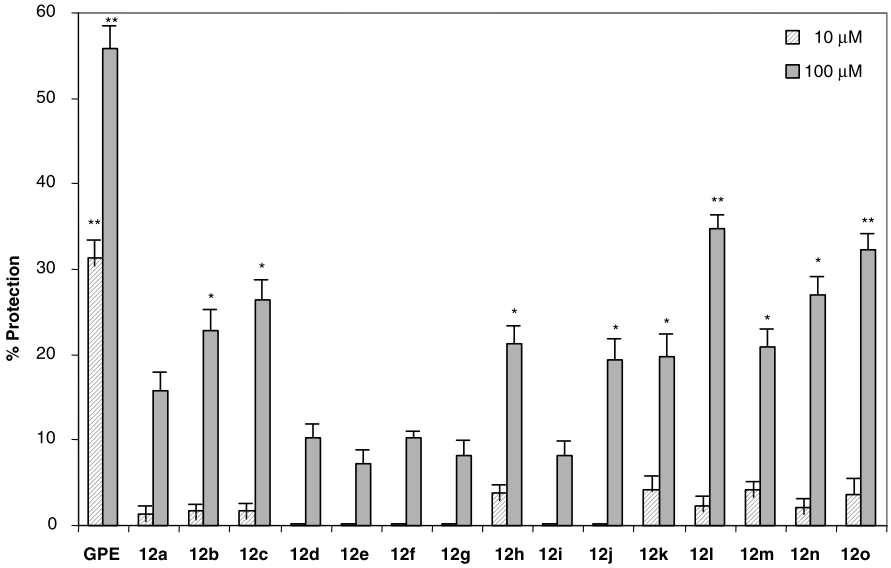


Figure 1. Percentage of protection of rat hippocampal neurons from NMDA (100 μM) excitotoxicity. Cell survival was estimated by measuring the activity of mitochondrial dehydrogenase on the tetrazolium derivative MTT. Values are means of 8–13 experiments. Asterisks indicate difference from control (*$P < 0.05$, **$P < 0.01$, Student's t test).

of this endogenous tripeptide. A general inspection of the biological results obtained in the binding and neuroprotection assays indicates that the prevention of neuronal death by these GPE analogues after NMDA injury is not directly linked to their affinity for glutamate receptors.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2005.11.040.

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20. Alloc-b_7Pro-OH: mp 76–77 °C (hexane/EtOAc). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.45–1.51 (m, 2H), 1.80–1.90 (m, 4H), 2.09–2.18 (m, 2H), 4.41 (dd, 1H), 4.52–4.55 (m, 2H), 5.18 (m, 1H), 5.26 (m, 1H), 5.84 (m, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 29.32, 33.59, 59.72, 66.58, 69.14, 118.20, 132.08, 156.88, 175.67.
21. Solid-phase experimental procedures, and significant analytical and spectroscopic data of all tripeptide derivatives are recorded in the supplementary data.
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