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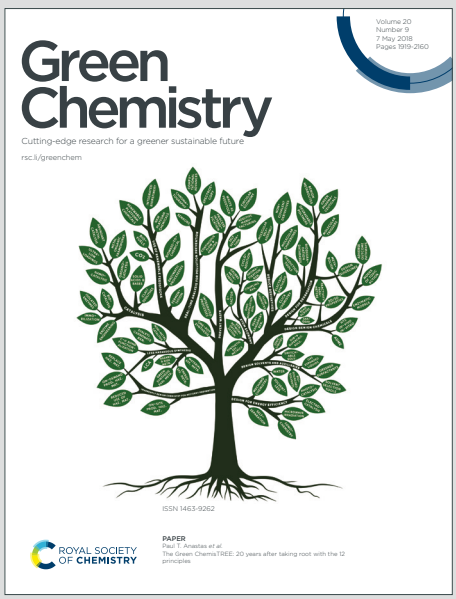

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# A Strategy for The Assembly of Glypromate® and Structure-Related Analogues by Tandem Sequential Peptide Coupling

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## Abstract

This work describes an improved and greener methodology in solution-phase synthesis for the preparation of Glypromate® (glycyl-L-prolyl-L-glutamic acid, GPE), a potent neuropeptide with applications in neurodegenerative conditions such as Huntington's, Parkinson's and Alzheimer's diseases. This protocol comprises the assembly of the perbenzylated form of Glypromate® [Cbz-Gly-Pro-Glu(OBn)-OBn (**5**)] from L-proline. Following a tandem sequential peptide coupling strategy, two chemoselective peptide bonds are formed without the need of purifying the intermediates, in a one-pot fashion synthesis. EcoScale score and E-factor were selected as the green metrics to assess the environmental impact for the preparation of tripeptide **5** under this protocol. After optimization and application of greener conditions, intermediate **5** was obtained with 95% global yield and 99% purity (NMR, HRMS, rp-HPLC), with excellent final EcoScale score of 75 out of 100 and global E-factor of 1.8. Glypromate® is achieved by removal of N- and C-protecting groups by hydrogenolysis using Pd/C as a catalyst in 98% yield, avoiding chromatographic techniques. Moreover, the protocol ensures stereochemical integrity (determined by VT-NMR and rp-HPLC) and was also successfully applied for the preparation of structure-related Glypromate® analogues with higher degree of molecular complexity compatible with functionalized amino acids and with different side chains. For the first time it is reported a one-pot protocol for the assembly of tripeptides with the removal of protecting groups in the same reaction vessel.

**Keywords:** Glypromate®, GPE, Neurodegenerative Diseases, Neuroprotection, One-pot Synthesis.

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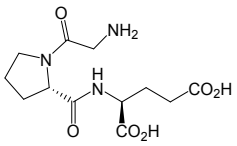
## Introduction

Insulin-like growth factor type 1 (IGF-1) is a potent neurotrophic factor,[1],[2] widely distributed in the mammalian central nervous system,[3] which in brain tissues undergoes metabolization mediated by acid-proteases producing two peptide fragments: the truncated peptide des-(1-3)-IGF-1 and the *N*-terminal sequence of glycyl-L-prolyl-L-glutamic acid (GPE, also known as Glypromate®, as depicted in **Figure 1**).




**Glypromate® (GPE)**

**Figure 1.** Structure of Glypromate® (GPE).

While des-(1-3)-IGF-1 is a potent neurotrophic factor by interaction with IGF-1 receptor, GPE does not bind to this receptor nor has any growth promoting effect.[4] However, it has been shown that GPE is able to displace the binding of [$^3$H]-glutamate to membranes of the anterior brain and hypothalamic synaptosomes,[5],[6] suggesting a possible interaction with the NMDA receptors. Nevertheless, GPE displays several regulatory actions and neuroprotection activity within the brain.[7] For example, it stimulates the release of potassium-induced acetylcholine in the brain cerebral cortex,[4] and dopamine in the striatum.[8] Exogenous administration of GPE prevents neuronal death induced by *N*-methyl-D-aspartate (NMDA), quinolinic acid or 6-hydroxydopamine in different regions of the brain.[9],[5] To date, the neuroprotection mechanism behind GPE's bioactivity is not fully understood.[10]

In a study of 2009,[11] Burgos-Ramos and co-workers show that GPE exerts a neuroprotective effect *in vivo* through the activation of protein kinases B (AKt), which play an important role in multiple cellular processes such as apoptosis, cell proliferation, among others.[11] Moreover, GPE is known to prevent the degeneration of three main phenotypes of striatum neurons (GABA-ergic neurons, and also cholinergic and NADPHd interneurons) in an animal model of Huntington's disease. Other studies in different experimental models showed that the administration of GPE exerts neuroprotective effects after ischemic processes,[12] reducing apoptosis and inhibiting microglial proliferation, thus preventing the loss of neurons after hypoxic ischemia.[4],[5] GPE's neuroprotective effects are well documented in animal models of neurodegenerative processes, such as Huntington's, Parkinson's, and Alzheimer's diseases.[5, 9, 13]

Intravenous administration of GPE in patients who undergo coronary artery bypass grafting (CABG) surgery significantly reduces brain damage,[14] and prevent the decline in cognitive function which is commonly seen during the early postoperative period.[15] Since there are no currently drugs approved to reduce cognitive impairment after CARB surgery, the activity of GPE is of utmost interest as a possible and valuable therapeutic candidate.[16]

Along with the therapeutically potential of GPE in several neurological conditions, Brimble's [17] and García-López's[18] research groups have reported several neuroprotective GPE analogues. Many of these peptidomimetics are based on chemical modifications at one or more GPE's residues.[16] For example, Brimble and co-workers described a GPE analogue in which *N,N*-dimethylation at the glycine residue in GPE structure afforded a bioactive compound (*N*,*N*-dimethyl-Glu-Pro-Glu-OH) with neuroprotective activity at 1 mM with a recovery value of 30-35% comparable to the parent peptide GPE (recovery value from 25% to 40%) at the same concentration.[17b] By the other hand, chemical modifications at the C-terminal, namely the conversion of the α-carboxylic acid in glutamate residue to *N*,*N*-dimethylamide group retains neuroprotective activity with recovery value of 58% in survival assay using striatal cells injured by okadaic acid in respect to the control (GPE) with recovery value of 30.5% at 1 mM concentration.[17c] In another study, this group show a neuroprotective compound in which the side chain of glutamate residue is absent (replaced by glycine).[17a] This analogue showed 20% recovery after striatal cell survival post-apoptosis-induced injury.[17a]

Besides these analogues, Cacciatore and co-workers described the conjugation of (*R*)-α-lipoic acid at the glycine residue of GPE affording a multifunctional codrug with antioxidant and neuroprotective activities.[19]

Considering the potential of GPE and structure-related analogues in neurological conditions[16] along with the intense research seeking for novel neuroprotective agents with enhanced neuroprotection and stability profiles,[20] gram-scale preparation of GPE and structural-related peptides becomes mandatory either to perform chemical modifications or study new formulations and applications.

Peptides, such as GPE can be synthesized by solid-phase peptide synthesis (SPPS) or by conventional solution-phase synthesis (SPS). Although SPPS is an effective strategy for the development of lengthy peptides, this methodology commonly makes use of great excess of reagents (*ca* 5 equiv. per amino acid) [21] in the smaller academic scale and during early drug discovery,[22] moreover it requires the use of highly polar solvents such as *N*,*N*'-dimethylformamide (DMF) to ensure complete dissolution of all the reagents and promote the peptide coupling.[23] Besides DMF, *N*-methyl-2-pyrrolidone and dichloromethane (DCM) are occasionally used.[24] However, all these solvents are classified as hazardous solvents by CHEM21,[25] and its use by the pharmaceutical

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industry is probable to be restricted by REACH (Registration, Evaluation, Authorization and Restriction of Chemicals).[26] Therefore, greener alternatives to the use of DMF in peptide synthesis is mandatory.[24] In addition, SPPS requires specific equipment and resins, which constitute an additional expense for the standard organic chemistry labs.[27]

In contrast, SPS is more adequate for medium to large-scale preparation of small peptides, allowing the use of equimolar quantities of reagents, and thus making this approach suitable for the development of eco-friendly and optimized protocols for the preparation of peptides of high interest.[28]

Trotter and co-workers have developed a straightforward methodology in SPS for the synthesis of GPE, but this methodology involves multiple-step reactions and require the purification of all intermediates.[17c] Sequential reactions in the same reaction vessel (tandem processes) might beneficiate from environment-friendly strategies, avoiding the use and production of toxic substances, and the possibility to build complex molecules with high stereoselectivity.[29]

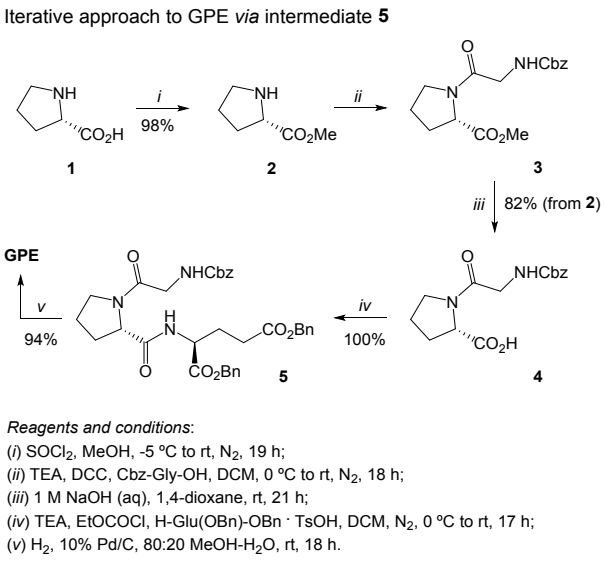
In this work, we sought to contribute with the design of an alternative methodology to the synthesis of GPE and structure-related analogues in SPS, hampered on the green chemistry principles.





## Results and Discussion

The synthesis of GPE by SPS is well documented in literature by Trotter and co-workers.[17c] This synthetic route relies on the preparation of perbenzylated intermediate **5** as the key intermediate (**Scheme 1**). This precursor (**5**) is then readily converted into GPE by concomitant removal of both N and C-terminal protecting groups by hydrogenolysis catalyzed by palladium on carbon (Pd/C).




*Reagents and conditions*:
(*i*) $SOCl_2$, MeOH, -5 ºC to rt, $N_2$, 19 h;
(*ii*) TEA, DCC, Cbz-Gly-OH, DCM, 0 ºC to rt, $N_2$, 18 h;
(*iii*) 1 M NaOH (aq), 1,4-dioxane, rt, 21 h;
(*iv*) TEA, EtOCOCl, H-Glu(OBn)-OBn · TsOH, DCM, $N_2$, 0 ºC to rt, 17 h;
(*v*) $H_2$, 10% Pd/C, 80:20 MeOH-$H_2O$, rt, 18 h.

**Scheme 1.** Iterative synthesis of GPE in solution-phase *via* intermediate **5**.[17c]

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This iterative approach starts with readily available L-proline (**1**), which is converted to methyl ester derivative **2** upon activation with thionyl chloride in methanol. The amino ester **2** is then coupled with Cbz-glycine using 1,3-dicyclohexylcarbodiimide (DCC) and triethylamine (TEA) in DCM to afford dipeptide **3** (**Scheme 1**). Treatment of **3** with 1 M NaOH using 1,4-dioxane as solvent leads to the obtention of dipeptide **4** with a global yield of 82% (from **2**). Dipeptide **4** is then activated *in situ* using ethyl chloroformate to give the corresponding mixed anhydride (under basic conditions), and subsequently treated with dibenzyl L-glutamate to provide perbenzylated tripeptide **5** as a single optical isomer in 100% yield. The global yield reported from **1→5** is 80%, yet the process requires the changing of different (hazardous) solvents, three chromatographic purifications, harsh conditions, and an extensive overall time of the process (75 h, from **1→5**). The final step in the sequence (hydrogenolysis of tripeptide **5**) is very effective, affording GPE in 94% yield after trituration in diethyl ether ($Et_2O$).

After the successful proof-of-concept of this one-pot process,[30] we hypothesize the possibility to run these isolated reactions in the same reaction vessel hampered on the fundamentals of green chemistry,[31] in order to develop a tandem process based on a one-pot synthesis approach.

In some cases, workup and isolation of the intermediates are not always necessary. When multiple reactions are conducted in a single reactor, without the need to isolate or purify the intermediates, it is possible to achieve the so-called "pot-economy".[32] This concept is more than a practical and cheaper way to synthesize a target molecule – it represents a greener alternative to do it. The advantages are clear: reduction of the amount of solvents, less waste production, and lower time associated to the overall process. Furthermore, this approach obviates the necessity to isolate intermediates, alleviating the labour required for the synthesis of a specific molecular target and reducing the intrinsic costs.

Therefore, a great advance in the synthetic route from **1** to **5** proposed by Trotter and co-workers,[17c] would be achieved by preparation of dipeptide **4** without the need of protecting L-proline (**1**), avoiding useless steps. Additionally, acylation of **4** with dibenzyl L-glutamate to afford perbenzylated tripeptide **5** would benefit from *in situ* activation of **4** without isolation of this intermediate, ensuing a one-pot protocol.

By examination of the retrosynthetic map for the preparation of intermediate **5** depicted in **Scheme 2**, dipeptide **4** can be synthesized from fully deprotected L-proline (**1**) and the commercially available preactivated glycine in the form of succinimidyl ester (Cbz-Gly-OSu**)** in presence of a tertiary amine. At this point, there is no necessity to isolate intermediate **4** before coupling with dibenzyl L-glutamate, **H**-Glu(OBn)-OBn, since a tertiary amine is also required in the next peptide coupling (*in situ* activation) upon the addition of a coupling reagent to afford perbenzylated tripeptide **5**.

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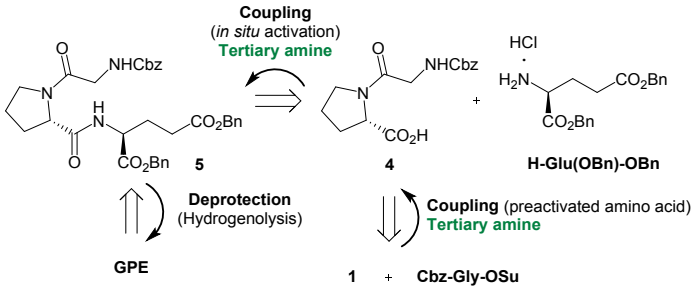
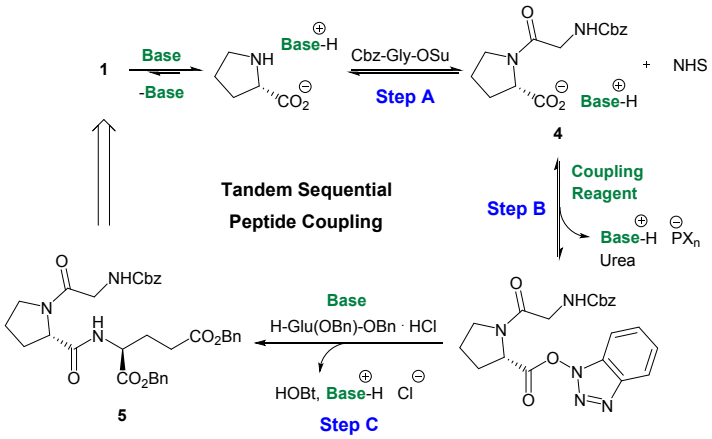



**Scheme 2.** Retrosynthetic map for the preparation of GPE *via* intermediate **5** highlighting a one-pot strategy.

Considering a tertiary amine is required in both strategies (*in situ* activation and peptide coupling of preactivated amino acids), there is a synthetic advantage to carry out the these highly effective single reactions in the same reactor,[29] ensuing a one-pot synthesis without the need to isolate intermediate **4**, as highlighted in **Scheme 3**.




**Scheme 3.** Tandem sequential peptide coupling process for the assembly of tripeptide **5**.

To test the viability of this process for the preparation of tripeptide **5**,[33] it was selected standard conditions in SPS: DCM as solvent, TEA as tertiary amine and 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate (TBTU),[34] as coupling reagent (entries 1-3, **Table 1**). All the reactions were performed at room temperature.

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**Table 1.** Optimization of the one-pot protocol for preparation of tripeptide **5**.

| Entry | Solvent[a] | Tertiary amine | Coupling Reagent | Time / h Step | | | Purification | Yield (5) / % | EcoScale[b] |
|---|---|---|---|---|---|---|---|---|---|
| | | | | A | B | C | | | |
| **1** | DCM | TEA | TBTU | 6 | 1 | 2 | Ex + CC | 86 | 47 |
| **2** | DCM | TEA | TBTU | 6 | 0.5 | 2 | Ex + CC | 95 | 52 |
| **3** | DCM | TEA | TBTU | 6 | 0.5 | 1 | Ex + CC | 93 | 51 |
| **4** | EtOAc | TEA | TBTU | 6 | 0.5 | 1 | Ex + CC | 72 | 35 |
| **5** | EtOAc | TEA | TBTU | 12 | 0.5 | 1 | Ex + CC | 93 | 46 |
| **6** | EtOAc | DIPEA | TBTU | 12 | 0.5 | 1 | Ex + CC | 91 | 50 |
| **7** | EtOAc | DIPEA | BOP | 12 | 0.5 | 1 | Ex + CC | 91 | 48 |
| **8** | EtOAc | DIPEA | PyBOP | 12 | 0.5 | 1 | Ex + CC | 90 | 59 |
| **9** | EtOAc | DIPEA | PyBOP | 12 | 0.5 | 1 | PP | 95 | 75 |

[a] 50 mL solvent for 4 mmol of L-proline

CC: Column chromatography; Ex: Liquid-liquid extraction; PP: Precipitation

*N*-Hydroxysuccinimide (NHS) esters are widely used in peptide science due to their high reactivity towards amines, relative stability to hydrolysis, and low toxicity.[35] Following the one-pot cascade reactions depicted in **Scheme 3**, it was found that the coupling between L-proline and Cbz-Gly-OSu (Step A, entry 1 of **Table 1**) proceeds smoothly at rt, with complete consumption of Cbz-Gly-OSu after 6 h (TLC). The following steps B and C are critical, since the activation of the dipeptide intermediate **4** (step B) and the aminolysis (step C) are intended to occur without removal of the byproducts.

After *in situ* activation of **4** with TBTU and addition of H-Glu(OBn)-OBn it was detected a new spot in TLC. Due to its high-water solubility, NHS, a byproduct formed in step A, can be easily removed from the reaction media by simple aqueous extraction.[35] Therefore, after the workup protocol (liquid-liquid extractions), the oil obtained was chromatographed. Gratifyingly, tripeptide **5** was isolated in 86% yield as a white solid (entry 1, **Table 1**). Changing the time reaction of steps B and C (0.5 and 1 h, respectively, in entry 3 of **Table 1**), had no significative impact on the yield when compared with the conditions used in entries 1 and 2 of **Table 1**.

Proven the feasibility of this tandem regioselective process, alternative approaches were considered to upgrade this protocol under the twelve pillars of green chemistry and design a competitive synthetic route for the preparation of GPE in SPS. To accomplish this goal, green substrates, eco-friendly reagents and solvents as well as mild reaction conditions are compulsory.[36]

Regarding its importance for a laboratory scale study, the EcoScale score[37] was selected as the green metric of choice to assess the environmental impact of this tandem process. EcoScale scores a chemical process based on several aspects: yield, cost, safety, technical set up, the temperature and time of reaction, workup and purification procedures. An ideal chemical process is characterized by

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high selective reactions which require as little steps as possible, and that make use of inexpensive and easily commercial available reagents.[38] The score obtained for a chemical process are considered excellent if the EcoScale score is >75, acceptable if >50 and inadequate if <50.[39] The EcoScale score ranges from 0 (totally failed reaction) to 100 (ideal reaction) and attributes penalty points to each of the parameters that are subtracted from the ideal score of 100. EcoScale is a powerful tool to study a chemical process and offers several advantages: easy score calculation, easy comparison of chemical procedures and accounts with many aspects of environmental impact in its procedure assessment.[36]

All the EcoScale scores for the conditions tested were obtained from the parameters previously described in literature,[37] using the EcoScale calculator (http://ecoscale.cheminfo.org/calculator). The scores listed in entries 1-3 in **Table 1** ranged from 47-52, which are in the borderline of an acceptable synthesis. To improve the overall EcoScale score eco-compatible conditions and reagents were explored for the synthesis of tripeptide **5**.

Starting with the solvent, it was checked the possibility to change DCM, which is classified as hazardous,[25] seeking for greener alternatives. Among the list of recommended solvents,[25] ethyl acetate (EtOAc) was selected due to its low toxicity (EtOAc has been approved by the U.S. Food & Drug Administration as a food additive),[40] and higher vapor pressure. However, when we attempted replacing the solvent to EtOAc under the same conditions previously listed in entry 3 of **Table 1**, the global yield dropped from 93 to 72% (entry 4 of **Table 1**). It was found that due to lower solubility of **1** in EtOAc, step A required more time for completion.

In fact, in the beginning of the reaction it is observed the formation of a suspension that progresses into a clear solution after 12 h, delivering **5** in 93% yield (entry 5, **Table 1**). Steps B and C did not require further optimization. However, despite the replacement of DCM by EtOAc and the excellent overall yield obtained, the final EcoScale score was 46 (entry 5, **Table 1**), which is considered an inadequate synthesis.[37]

To reduce the environmental impact of this process, it was envisioned the replacement of TEA by a more convenient tertiary amine such as *N*,*N*-diisopropylethylamine (DIPEA), also known as Hünig's base.[34] This simple operation had no significant impact on the yield of **5**, nevertheless the EcoScale score was improved from 46 to 50 (entry 6, **Table 1**), resulting in an acceptable synthetic process.[37]

Despite TBTU is very effective as a coupling reagent with low epimerization, it is classified as explosive,[41] displaying a great impact in the final Ecoscale score, and therefore it should be replaced. Recently, other uronium coupling reagents based on HOBt and HABt such as HBTU, HCTU and HATU were reported to cause anaphylaxis,[42] and therefore should be avoided. Combination of benzotriazole derivatives with carbodiimides are also used for peptide coupling,[34, 43] however the use of carbodiimides is incompatible with this one-pot protocol since they are known to react with the

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NHS (generated in the reaction) to form succinimidoxycarbonyl-β-alanine-hydroxysuccinimide ester, resulting in β-alanine insertions.[44] Likewise, ethyl cyano(hydroxyimino)acetate also known as "oxyma pure",[45] which is considered as a green additive for peptide synthesis to replace HOBt/HOAt requires the use of carbodiimides and therefore cannot be employed in this protocol.

Phosphonium salts such (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP, also known as Castro's reagent),[34] are widely used as coupling reagents, providing high coupling efficiency and low amino acid racemization. BOP has been used in the preparation of GPE analogues.[18a] Despite the coupling efficiency demonstrated by BOP was comparable with TBTU based on the yields obtained (entry 7, **Table 1**), the EcoScale score was decreased to 48. BOP reagent is known to originate hexamethylphosphoramide (HMPA), a carcinogenic byproduct, which may account for the lower score.[46]

To overcome this issue, benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate (PyBOP), a derivative of BOP that does not produce HMPA,[47] was used as coupling reagent. PyBOP has been successfully applied in difficult peptide couplings without noticeable racemization,[48] in contrast to other phosphonium reagents such as PyBrOP which is less effective in suppressing epimerization.[48] Despite PyBOP is listed in the group of "least preferred" reagents for peptide production (along with COMU) based on differential scanning calorimetry (DSC) studies, the average major left limit onset is 121 °C, being therefore problematic only at high temperatures and larger scales. Nevertheless, PyBOP display excellent properties that make it an appropriated candidate as coupling reagent to be used in the early phase of drug development: high coupling efficiency, low epimerization and safety/toxicity profile.[22]

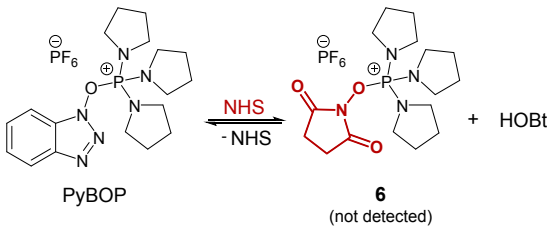
By employing PyBOP the EcoScale score has considerably increased (59 out of 100) with no significant effect on the global yield (entry 8, **Table 1**), which represents a satisfactory process. Although DIPEA and PyBOP can also be employed in SPPS, the one-pot process uses practically stoichiometric amounts, minimizing the waste of these reagents.

Chromatography techniques have also a dramatic impact on the final EcoScale score,[37] therefore efforts were made to avoid chromatography without compromising the purity of the final product. Bearing in mind that tripeptide **5** is a solid and that after the liquid-liquid extractions the TLC is very clean, it was envisioned the precipitation of the desired peptide from the crude. Several solvents were tested and $Et_2O$ found the best solvent to induce precipitation. Crystallization was also easily attained in a mixture of EtOAc/$Et_2O$ 1:9, affording colourless needle-like crystals upon filtration and washings with cold $Et_2O$. The perbenzylated tripeptide **5** was obtained in excellent yield (95%) and purity (99%, NMR and HPLC (see ESI† for details). With this workup the global EcoScale score was significantly improved, achieving 75 out of 100 (**Table 1,** entry 9), which indicate an excellent synthetic process.

Environmental factor (E-factor) is a useful green metric to assess the environmental impact of a chemical process.[49] A higher E-factor means more waste production and, consequently, a pronounced negative environmental impact.[49] The ideal E-factor is zero. The synthesis of tripeptides by SPPS display, in average, an E-factor score of 81.0,[50] which compares unfavourably with other competitive available methodologies such as classical SPS or mechanochemical processes (ball-milling). These two strategies display, in average, E-factor scores of 7.1 and 5.0, respectively.[50] This data agrees with the global E-factor calculated for the classical synthesis of tripeptide **5** developed by Trotter (E-factor = 6.3, see ESI† for details).[17c] The global E-factor for the assembly of **5** using this one-pot methodology proves to be superior to all the other methodologies, scoring only 1.8.

Interestingly, tripeptide **5** exhibit *cis-trans* conformers (72:27) in DMSO-$d_6$ at room temperature, as demonstrated by variable-temperature NMR experiments (VT-NMR, **Figure S29** of ESI†). The experimental energy barrier for *cis-trans* isomerization was found to be of 18.8 kcal mol$^{-1}$, being the *cis* conformers the most populated at room temperature. The *cis-trans* isomerization seems to be associated to the prolyl residue as demonstrated by ROESY and further supported by *in silico* studies (see **Figures S30** and **S31** of ESI† for more details).

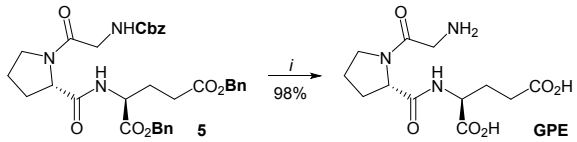
In a previous work, our research group has found that TBTU interact with NHS to generate *in situ* another coupling reagent, *O*-[*N*-Succinimidyl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TSTU).[30] Regarding that NHS is formed as byproduct during step A, the possibility of a putative NHS-PyBOP interaction upon the addition of PyBOP in step B was checked to study the stability of the coupling reagent in presence of NHS. Therefore, a $^1$H-NMR experiment was performed using equimolar quantities of these reagents in DMSO-$d_6$ for 12 h (**Scheme 4**).




**Scheme 4.** $^1$H-NMR study for the PyBOP-NHS interaction.

The data retrieved from $^1$H-NMR spectrum in this study excludes the formation of the hypothesized intermediate **6** under this protocol (**Figures S26** and **S27** of ESI†), ensuring the stability of PyBOP in the presence of NHS. It is worth mentioning that due to the presence of residual water associated with DMSO-$d_6$ it is possible to detect the hydrolysis of PyBOP (approx. 27%, note the lack of water typical signal at 3.30 ppm in **Figures S26** and **S27** of ESI†).

After optimization of the synthetic protocol for perbenzylated tripeptide **5**, GPE was easily obtained by hydrogenolysis using Pd/C in methanol/water in practically quantitative yield (**Scheme 5**),[17c] with no need of chromatographic purification after filtration and precipitation in $Et_2O$ (see experimental section).




*Reagents and conditions*:
*i*) Pd/C, $H_2$ (50 psi), MeOH/$H_2O$ (1:1, v/v).

**Scheme 5.** Synthesis of GPE from perbenzylated tripeptide **5**.

HRESIMS and HPLC experiments were performed to complement the spectroscopic data (NMR) obtained for tripeptide **5**. The results are shown in **Figure 2**. HRESIMS reveals the presence of two peaks with $m/z$ = 616.26893 and 1231.52941 a.m.u. ($[M + H]^+$ and $[2M + H]^+$, respectively), which is in agreement with the mass expected for tripeptide **5**. The HPLC chromatogram (insert, **Figure 2**) clearly advocate the success of the purification process from the crude mixture, showing a single peak at $t_R$ = 22.2 min (99.0% purity).

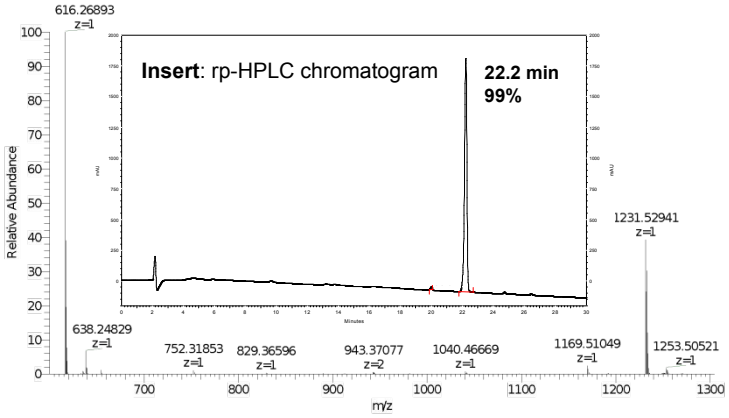



**Figure 2**. HRESIMS of tripeptide **5** ($[M + H]^+$ = 616.26893 and $[2M + H]^+$ = 1231.52941 a.m.u.). Insert: rp-HPLC chromatogram. Purity of 99% ($t_R$ = 22.2 min).

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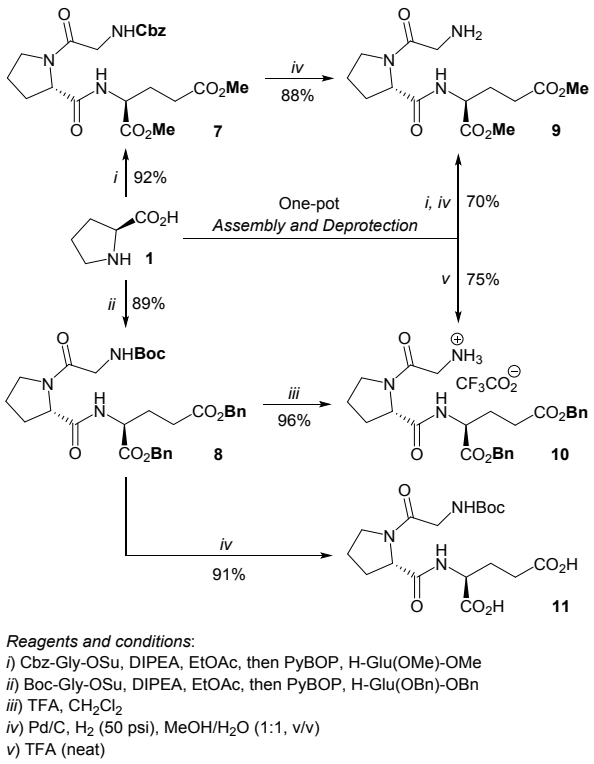
Considering the importance of GPE and its structural-related analogues, the synthetic potential of this protocol was also explored for the assembly of valuable GPE intermediates which may allow subsequent functionalization either at the amino or carboxy termini sites. To accomplish this task, two orthogonally protected GPE intermediates were designed: **Cbz**-Gly-Pro-Glu(O**Me**)-O**Me** and **Boc**-Gly-Pro-Glu(O**Bn**)-O**Bn** (tripeptides **7** and **8**, respectively, **Scheme 6**).




**Scheme 6.** One-pot synthesis of peptides **7-10** and preparation of GPE derivatives **9-11** in two-pots.

In the case of tripeptide **7** (**Scheme 6**), dimethyl L-glutamate was used instead of the benzylic ester counterpart employed for derivative **5**, delivering tripeptide **7** in 92% yield under the same one-pot methodology. For the assembly of tripeptide **8** (**Scheme 6**), the appropriated Boc-protected glycine preactivated as *N*-hydroxysuccinimidyl ester (Boc-Gly-OSu) was used instead of the Cbz amino ester, delivering **8** in high yields (89%). Considering the lability of *N*-Boc protecting group under acidic conditions,[30] the workup for tripeptide **8** was performed using $NaHCO_3$ (aq).

With peptides **7** and **8** in hands it was performed the selective deprotection either at N or C-terminal (**Scheme 6**). For tripeptide **7**, selective removal of Cbz group was performed by hydrogenolysis catalyzed by Pd/C, enabling the preparation of *N*-deprotected tripeptide **9** in 88% yield. Brimble and co-workers have previously described that peptide **9** displays neuroprotective activity in striatal cell survival post-apoptosis-induced injury.[17a]

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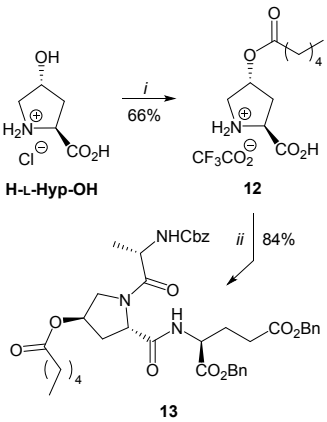
In contrast, under the same conditions, selective hydrogenolysis of tripeptide **8** afforded the C-deprotected tripeptide **11** in high yield (91%). In both cases, peptides **9** and **11** were induced to precipitate in $Et_2O$, avoiding the need of chromatographic purifications.

Acidolysis of the carbamate **8** using trifluoroacetic acid (TFA), delivered *N*-deprotected tripeptide **10** as a trifluoroacetate ammonium salt in practically quantitative yield (96%) and with high purity after the workup by precipitation in $Et_2O$.

In order to improve the potential of this protocol, the assembly and orthogonal deprotection were carried out in the same reaction vessel. This unprecedented synthetic approach lead to the preparation of peptides **9** and **11** directly from L-Pro. In the case of peptide **9**, after the assembly of the intermediate **7**, the solvent was removed *in vacuo* and the *N*-protecting group (Cbz) was removed by hydrogenolysis catalyzed by Pd/C without isolation of **7**. The reaction proved to be feasible with 70% yield for the global process, with the need of chromatographic isolation of tripeptide **9**. In a similar way, for the preparation of peptide **10**, after the one-pot synthesis of intermediate **8**, the solvent was removed in *vacuo*, followed by the removal of Boc group by acidolysis upon the addition of TFA (neat). The reaction delivered tripeptide **10** after chromatography with 75% global yield.

Modification on peptide side chains is commonly employed as a chemical strategy to tune bioactivity. Moreover, many neuroprotective GPE analogues are composed by other amino acids with more functionalities. In this sense, to ensure the compatibility of this one-pot methodology with the preparation of GPE peptidomimetics it was designed and synthesized tripeptide **13** (**Scheme 7**). This peptide contains a functionalized *trans*-4-hydroxy-L-proline (Hyp) and L-alanine residue as proline and glycine surrogates, respectively (**Scheme 7**).




*Reagents and conditions*:
*i*) Caproyl chloride, TFA
*ii*) Cbz-L-Ala-OSu, DIPEA, EtOAc, then PyBOP, H-Glu(OBn)-OBn

**Scheme 7.** One-pot synthesis of peptidomimetic **13**.

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Functionalized amino acid **12** was prepared by direct *O*-acylation of H-Hyp-OH with captoyl chloride in TFA as previously described in the literature.[51] The one-pot protocol was then applied to amino acid **12** using Cbz-Ala-OSu and H-Glu(OBn)-OBn, affording tripeptide **13** in 84% yield. This result shows that this one-pot methodology can be used for the preparation of GPE analogues with higher degree of molecular complexity proving to be compatible with other amino acids.

**Determination of the Stereochemical Purity using this Protocol**

A paramount aspect when describing new methodologies employing chiral compounds such as amino acids is the possible loss of optical activity. In peptide synthesis the activation step is critical, and the most significant epimerization mechanism involves the transitory formation of azlactones.[30],[34] Coupling reagents such as PyBOP, which contains the racemization suppressant HOBt, are known to be very effective with minimal epimerization.[34] Azlactone formation is the major mechanism that leads to loss of optical purity and generally occurs during the activation of carboxyl groups of amino acids (except for glycine or proline).[30] Thus, this protocol is not expected to show significative epimerization phenomena since activation takes place at proline and hence, is not prone to undergo azlactone formation.[52] Furthermore, the mild conditions employed in this one-pot process, the short overall time and the use of reagents in stochiometric amounts decreases the likelihood of loss of optical activity.

Although the NMR data for the peptide products synthesized using this protocol (**5**, **7**, **8** and **13**) show that these peptides are diastereomerically pure, further experiments were envisioned to corroborate the spectroscopic data and unambiguously determine the diastereomeric ratio (*dr*). Regarding that diastereoisomers may be resolved by simple chromatographic techniques such as rp-HPLC, we prepared the mixtures of epimers for these peptides synthesized under the one-pot methodology using D,L-proline instead of the optically active L-proline. These pairs of epimers were then screened by rp-HPLC in order to access which pair suited better to access the diastereomeric ratio (*dr*). Among the mixture of epimers tested, the pair **7**/*epi*-**7** were easily resolved by rp-HPLC. For that purpose, both diastereopure peptides **7** and *epi*-**7** (using L-proline or D-proline, respectively) were prepared (**Scheme 8**).

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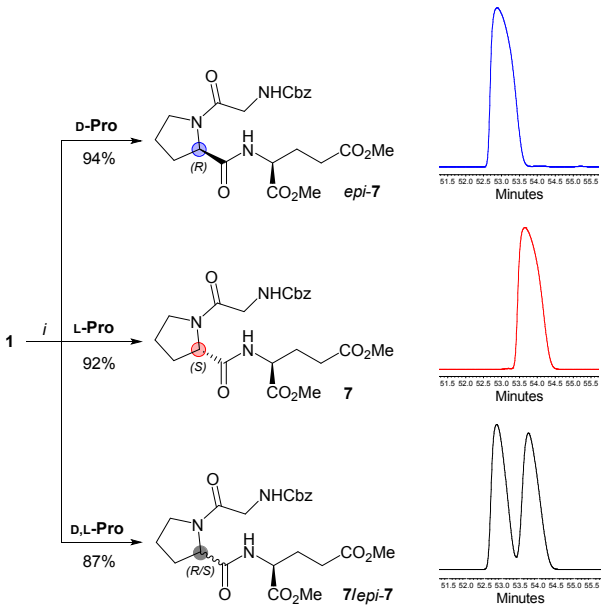


*Reagents and conditions*:
*i*) Cbz-Gly-OSu, DIPEA, EtOAc, then PyBOP, H-Glu(OMe)-OMe.
Elution in gradient 1-40% acetonitrile in water, 80 min, flow: 1 mL $min^{-1}$;
$t_R$ for **7**: 53.7 min; $t_R$ for *epi*-**7**: 52.8 min.

**Scheme 8.** Epimerization assessment under the one-pot conditions for derivative **7** by rp-HPLC.

Using the one-pot protocol, tripeptides *epi*-**7** and **7**/*epi*-**7** were obtained in 94% and 87% yields, respectively, starting from the appropriate proline. The chromatograms obtained for each peptide along with the NMR data for **7** and *epi*-**7** (available in ESI) unarguably show that these peptides are virtually free of epimerization (*dr* > 99%) by comparison with the NMR data and the chromatogram obtained for the mixture of diastereoisomers **7**/*epi*-**7**.

## Conclusion

In this work is described a short, greener and effective strategy for the preparation of GPE *via* an optimized tandem sequential peptide coupling reactions to assemble tripeptide **5** as the key intermediate. This peptide is prepared in 13-14 h in contrast with classical procedures (75 h), with 90-95% global yield and 99% purity (NMR, HRESIMS and rp-HPLC). Using VT-NMR experiments it was found a high energy barrier for *cis-trans* isomerization for tripeptide **5** (18.8 kcal $mol^{-1}$) in DMSO-$d_6$ which seems to be associated to the prolyl residue as demonstrated by ROESY and further corroborated by *in silico* studies.

GPE is obtained from tripeptide **5** in gram-scale by simple hydrogenolysis using Pd/C as catalyst with 98% yield, avoiding the need of chromatographic purifications. Additionally, it has been

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demonstrated the broad applicability of this methodology through the preparation of GPE analogues using orthogonal protecting groups, enabling its N and C-functionalization. The assembly of tripeptides and the removal of *N*-protecting groups in the same reaction vessel is unprecedented, as demonstrated for preparation of peptides **9** and **10**. Furthermore, this protocol allows the assembly of peptides virtually free of epimerization (*dr* > 99%, rp-HPLC, NMR).

Although this methodology requires pre-activated amino acids (HOSu esters) for the success of the tandem sequential peptide coupling process these amino acids are commercially available and/or are easily activated using the NHS/DCC chemistry.

This highly efficient and competitive one-pot protocol offers a greener alternative for the gram-scale preparation of GPE and structure-relates analogues with different side chains and functional groups, boosting the development of novel neuroprotective peptides and the assembly of small peptides in solution-phase.





## Experimental Section

### Chemistry. General

All chemicals were obtained from Bachem, Sigma-Aldrich and Fluorochem (reagent grade) and were used without further purification. All reactions were carried out under argon atmosphere. Analytical TLC was carried out on pre-coated silica gel plates (Merck 60 $F_{254}$, 0.25 mm) using UV light and an ethanolic solution of phosphomolybdic acid (followed by gentle heating) for visualization. HPLC analyses were performed on a Merck-Hitachi Lachrom Elite instrument equipped with a diode array detector (DAD) and thermostated (Peltier effect) autosampler, using a Purospher STAR RP-18e column (150 mm × 4.6 mm; particle size, 5 μm) and using the following conditions: 1–100% gradient of B in A (A = $H_2O$ with 0.05% of trifluoroacetic acid; B = acetonitrile) during 30 min with a flow rate of 1 mL $min^{-1}$ and detection at λ = 220 nm (tripeptide **5**); 1-40% gradient of B in A during 80 min with a flow rate of 1 mL $min^{-1}$ and detection at λ = 220 nm (tripeptides **7,** *epi*-**7** and **7**/*epi*-**7**); Mass spectra were recorded with a LTQ OrbitrapTM XL hybridmass spectrometer (Thermo Fischer Scientific, Bremen, Germany) controlled by LTQ Tune Plus 2.5.5 and Xcalibur 2.1.0 (Centro de Materiais da Universidade do Porto, CEMUP). The capillary voltage of the electrospray ionization source (ESI) was set to 3.1 kV. The capillary temperature was set at 275 °C. The sheath gas was set at 6 (arbitrary unit as provided by the software settings). The capillary voltage was set at 35 V and the tube lens voltage set at 110 V. $^{1}$H- and $^{13}$C-NMR spectra were recorded at CEMUP with a Bruker Avance III 400 at 400 MHz and 100 MHz, respectively. The NMR spectra were calibrated using residual protic solvents ($D_2O$: $\delta_H$ = 4.79; DMSO-$d_6$: $\delta_H$ = 2.50; 3.30, $\delta_C$ = 39.52) and are reported in ppm.[53] The nomenclature

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used for the assignment of protons and/or carbons for each α-amino acid residue in the peptide chains followed the three-letter system in subscript for amino acid residue (Glu: L-Glutamic acid; Gly: Glycine; Hyp: *trans*-4-hydroxy-L-proline; Pro: L-Proline). The numeration of proton(s) and/or carbons in the structures was performed starting with the carbonyl carbon of the main chain of each α-amino acid residue as follows: $H_{AA}$-X (for proton or magnetically equivalent protons) or $C_{AA}$-X (for carbon); AA: amino acid residue using three-letter system; X: proton(s) or carbon numeration starting from carbonyl carbon of each residue of amino acid. In the case of presence of rotamers, the chemical shift of the *minor* rotamer is assigned with "*".



**Dibenzyl *N*-(benzyloxycarbonyl)-glycyl-L-prolyl-L-glutamate (5)**

In a round bottom flask previously flushed with Ar was charged H-L-Pro-OH (0.46 g, 4.0 mmol) in EtOAc (50 mL), followed by the addition of DIPEA (1.39 mL, 7.98 mmol) and Cbz-Gly-OSu (1.23 g, 4.02 mmol). The resulting suspension was stirred overnight after which a clear solution was achieved. To this solution was added PyBOP (2.18 g, 4.19 mmol) with further stirring for 25-30 min until complete dissolution, followed by the addition of H-Glu(OBn)-OBn hydrochloride (1.60 g, 4.40 mmol) and the solution was left stirring for 1 h. The suspension was transferred to a separatory funnel and was washed with HCl 1M (2 x 50 mL) and $NaHCO_3$ (2 x 50 mL). The organic extract was dried over anhydrous $Na_2SO_4$, filtered and the solvent was removed *in vacuo*. The crude oil was either a) chromatographed using EtOAc (2.22 g, 90% yield) or b) induced to precipitate by addition of cold $Et_2O$/EtOAc (9:1) and filtrated (2.34 g, 95% yield), affording tripeptide **5** as white solid. Mp = 118-120 ºC [lit.[17c] 116-118 ºC]. $[\alpha]_D^{23}$: $-98.8 \pm 0.1$ (*c*1.06, $CHCl_3$) [lit.[17c] −55.9 (*c*0.37, $CH_2Cl_2$)]. $R_f$: 0.61 in EtOAc. HPLC: > 99% purity ($t_R$ = 22.3 min). **$^1$H-RMN (DMSO-$d_6$, 400 MHz, 110 ºC) δ ppm**: 7.96 (br s, 1H, $NH_{Glu}$), 7.60 – 7.07 (m, 15H, ArH), 6.62 (br s, 1H, $NH_{Gly}$), 5.13 (d, *J* = 4.0 Hz, 2H, $CH_2Ph$), 5.10 (s, 2H, $CH_2Ph$), 5.05 (s, 2H, $CH_2Ph$), 4.50 – 4.35 (m, 2H, $H_{Pro}$-2 + $H_{Glu}$-2), 3.84 (d, *J* = 4.0 Hz, 1H, $H_{Gly}$-2), 3.80 (d, *J* = 3.5 Hz, 1H, $H_{Gly}$-2), 3.49 (t, *J* = 6.7 Hz, 2H, $H_{Pro}$-5), 2.46 (t, *J* = 7.6 Hz, 2H, $H_{Glu}$-4), 2.12 (tt, *J* = 15.9, 7.9 Hz, 1H), 2.70 – 1.70 (m, 5H). **$^{13}$C-RMN (DMSO-$d_6$, 100 MHz, 110 ºC) δ ppm**: [171.3 (C), 171.1 (C), 170.4 (C), 166.8 (C), $C_{Gly}$-1 + $C_{Pro}$-1 + $C_{Glu}$-1 + $CO_2Bn$], 155.5 (C, $NCO_2Bn$), [136.6 (C), 135.7 (C), 135.4 (C), 3 x ArC], [127.7 (CH), 127.7 (CH), 127.6 (CH), 127.3 (CH), 127.2 (CH), 127.1 (CH), 127.1 (CH), 127.0 (CH), 126.8 (CH), 3 x ArC], [65.5 ($CH_2$), 65.0 ($CH_2$), 65.0 ($CH_2$), 3 x $CH_2Ph$], [58.7 (CH), 51.0 (CH), $C_{Pro}$-2 + $C_{Glu}$-2], 45.6 ($CH_2$), 42.4 ($CH_2$), 29.5 ($CH_2$), 25.7 ($CH_2$). **HRMS (ESI-TOF) *m*/*z*:** $[M + H]^+$ calcd for $C_{34}H_{38}N_3O_8^+$ 616.2653; found: 616.2689.

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**Glycyl-L-prolyl-L-glutamic acid (Glypromate®, GPE)**[17c]

A mixture of tripeptide **5** (3.00 g, 4.87 mmol) and 10 wt % Pd/C (0.30 g) in 4:1 methanol–water (60 mL; v/v) was prepared in a hydrogenation vessel and transferred into a hydrogenation apparatus, flushed with $H_2$ (60 psi) and mechanically stirred at room temperature, protected from light, for 4 h. The reaction mixture was filtered through a Celite™ pad and the pad washed with 1:1 methanol–water (100 mL; v/v). The filtrate was concentrated to dryness under reduced pressure and the residue triturated with anhydrous $Et_2O$ to afford GPE as a white solid (1.39 g, 98%). mp: 215-216 °C (lit.[17c] 216 °C). $[\alpha]_D^{18}$: $-61.6 \pm 0.1$ (*c*1.04, $H_2O$) [lit.[17c] $[\alpha]_D^{26}$: $-84.8$ (*c*0.034, $H_2O$)]. **$^1$H-RMN ($D_2O$, 400 MHz, 26 ºC) δ ppm**: 4.51 (dd, *J* = 8.5, 4.1 Hz, 1H, $H_{Pro}$-2); 4.26 (dd, *J* = 8.8, 5.0 Hz, 1H, $H_{Glu}$-2), 4.05 (d, *J* = 16.5 Hz, 0.80H, $H_{Gly}$-2), 4.00 (d, *J* = 16.5 Hz, 0.80H, $H_{Gly}$-2), 3.93* (d, *J* = 16.3 Hz, 0.20H, $H_{Gly}$-2), 3.76* (d, *J* = 16.3 Hz, 0.20H, $H_{Gly}$-2), 3.71 – 3.51 (m, 2H, $H_{Pro}$-5), [2.45 (t, *J* = 7.6 Hz), 2.43* (t, *J* = 7.6 Hz), 2H, $H_{Glu}$-4], 2.32 – 2.23 (m, 1H, $H_{Pro}$-3), 2.23 – 2.07 (1H, m, $H_{Glu}$-3), 2.07 – 1.80 (m, 4H, $H_{Pro}$-3 + $H_{Glu}$-3 + $H_{Pro}$-4). **$^{13}$C-RMN ($D_2O$, 100 MHz, 26 ºC) δ ppm**: [179.4* (C), 179.3 (C), $C_{Glu}$-5], [178.1 (C), 178.1* (C), $C_{Glu}$-1], [174.4 (C), 174.0* (C), $C_{Pro}$-1], [167.0* (C), 166.6 (C), $C_{Gly}$-1], [61.5 (CH), 61.1* (CH), $C_{Pro}$-2], [55.4* (CH), 55.1 (CH), $C_{Glu}$-2], [48.5* ($CH_2$), 47.8 ($CH_2$), $C_{Pro}$-5], [41.3 ($CH_2$), 41.1* ($CH_2$), $C_{Gly}$-2], [32.6* ($CH_2$), 32.4* ($CH_2$), 31.9 ($CH_2$), 30.3 ($CH_2$), $C_{Glu}$-4 + $C_{Pro}$-3], [27.9 ($CH_2$), 27.4* ($CH_2$), $C_{Glu}$-3], [25.1 ($CH_2$), 22.9* ($CH_2$), $C_{Pro}$-4]. **HRMS (ESI-TOF) *m/z*:** $[M + H]^+$ calcd for $C_{12}H_{20}N_3O_6^+$ 302.1347; found: 302.1348.

**Dimethyl *N*-(benzyloxycarbonyl)glycyl-L-prolyl-L-glutamate (7)**

Following the same one-pot protocol described for preparation of tripeptide **5**, to a suspension of H-L-Pro-OH (0.46 g, 4.0 mmol) in EtOAc (50 mL), was added DIPEA (1.39 mL, 7.98 mmol) and Cbz-Gly-OSu (1.23 g, 4.02 mmol). To the resulting solution was added PyBOP (2.18 g, 4.19 mmol) and H-Glu(OMe)-OMe hydrochloride (0.93 g, 4.4 mmol). After the typical workup the crude oil was chromatographed using EtOAc as eluent affording the title compound as a colourless oil (1.71 g, 92%). $[\alpha]_D^{26}$: $+3.4 \pm 0.2$ (*c*1.31, $CHCl_3$) [lit.[17a] $[\alpha]_D$: $-62.3$ (*c*0.23, $CH_2Cl_2$)]. **$^1$H-RMN (DMSO-$d_6$, 400 MHz, 110 ºC) δ ppm**: 7.89 (br s, 1H, $NH_{Glu}$), 7.40 – 7.34 (m, 4H, ArH), 7.34 – 7.27 (m, 1H, ArH), 6.65 (br s, 1H, $NH_{Gly}$), 5.07 (s, 2H, $CH_2Ph$), 4.43 (dd, *J* = 8.4, 2.7 Hz, 1H, $H_{Pro}$-2), 4.34 (dt, *J* = 8.1, 5.7 Hz, 1H, $H_{Glu}$-2), 3.96 – 3.71 (m, 2H, $H_{Gly}$-2), 3.65 (s, 3H, $CO_2CH_3$), 3.61 (s, 3H, $CO_2CH_3$), 3.51 (t, *J* = 7.0 Hz, 2H, $H_{Pro}$-5), 2.39 (t, *J* = 7.5 Hz, 2H, $H_{Glu}$-4), 2.15 – 2.01 (m, 2H), 2.01 – 1.83 (m, 4H). **$^{13}$C-RMN (DMSO-$d_6$, 100 MHz, 110 ºC) δ ppm**: [171.9 (C), 171.0 (C), 166.9 (C), $C_{Gly}$-1 + $C_{Pro}$-1 + $C_{Glu}$-

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1 + $CO_2Me$], 155.6 (C, $NCO_2Bn$), 136.6 (C, ArC), 127.6 (2 x CH, ArC), 127.0 (CH, ArC), 126.9 (2 x CH, ArC), 65.1 ($CH_2$, $CH_2Ph$), [58.9 (CH), 51.1 (CH), $C_{Pro}$-2 + $C_{Glu}$-2], [50.9 ($CH_3$), 50.5 ($CH_3$), 2 x $CO_2CH_3$], 45.7 ($CH_2$), 42.4 ($CH_2$), 29.3 ($CH_2$), 25.7 ($CH_2$). **HRMS (ESI-TOF) *m/z*:** $[M + H]^+$ calcd for $C_{22}H_{30}N_3O_8^+$ 464.2027; found: 464.2030.

**Dimethyl *N*-(benzyloxycarbonyl)glycyl-D-prolyl-L-glutamate (*epi*-7)**

Following the same one-pot protocol described for preparation of tripeptide **5**, to a suspension of H-D-Pro-OH (0.46 g, 4.0 mmol) in EtOAc (50 mL), was added DIPEA (1.39 mL, 7.98 mmol) and Cbz-Gly-OSu (1.23 g, 4.02 mmol). To the resulting solution was added PyBOP (2.18 g, 4.19 mmol) and H-Glu(OMe)-OMe hydrochloride (0.93 g, 4.4 mmol). After the typical workup the crude oil was chromatographed using EtOAc as eluent affording the title compound as a colourless oil (1.74 g, 94%). $[\alpha]_D^{23}$: + 148.9 ± 0.2 (*c*1.065, $CHCl_3$). **$^1$H-RMN (DMSO-$d_6$, 400 MHz, 110 ºC) δ ppm**: 7.71 (br s, 1H, $NH_{Glu}$), 7.39 – 7.33 (m, 4H, ArH), 7.33 – 7.27 (m, 1H, ArH), 6.67 (br s, 1H, $NH_{Gly}$), 5.07 (s, 2H, $CH_2Ph$), 4.45 – 4.39 (m, 1H, $H_{Pro}$-2), 4.35 (dt, *J* = 8.3, 5.6 Hz, 1H, $H_{Glu}$-2), 3.88 (d, *J* = 15.7 Hz, 1H, $H_{Gly}$-2), 3.86 (d, *J* = 15.6 Hz, 1H, $H_{Gly}$-2), 3.65 (s, 3H, $CO_2CH_3$), 3.61 (s, 3H, $CO_2CH_3$), 3.51 (t, *J* = 7.0 Hz, 2H, $H_{Pro}$-5), 2.37 (t, *J* = 7.5 Hz, 2H, $H_{Glu}$-4), 2.14 – 2.02 (m, 2H), 1.98 – 1.82 (m, 4H). **$^{13}$C-RMN (DMSO-$d_6$, 100 MHz, 110 ºC) δ ppm**: [171.9 (C), 171.0 (C), 167.1 (C), $C_{Gly}$-1 + $C_{Pro}$-1 + $C_{Glu}$-1 + $CO_2Me$], 155.6 (C, $NCO_2Bn$), 136.6 (C, ArC), 127.6 (2 x CH, ArC), 127.0 (CH, ArC), 126.9 (2 x CH, ArC), 65.1 ($CH_2$, $CH_2Ph$), [59.1 (CH), 51.1 (CH), $C_{Pro}$-2 + $C_{Glu}$-2], [50.9 ($CH_3$), 50.5 ($CH_3$), 2 x $CO_2CH_3$], 45.7 ($CH_2$), 42.5 ($CH_2$), 29.3 ($CH_2$), 25.8 ($CH_2$). **HRMS (ESI-TOF) *m/z*:** $[M + H]^+$ calcd for $C_{22}H_{30}N_3O_8^+$ 464.2027; found: 464.2029.

**Dimethyl *N*-(benzyloxycarbonyl)glycyl-D,L-prolyl-L-glutamate (7/*epi*-7)**

Following the same one-pot protocol described for preparation of tripeptide **5**, to a suspension of H-D,L-Pro-OH (0.46 g, 4.0 mmol) in EtOAc (50 mL), was added DIPEA (1.39 mL, 7.98 mmol) and Cbz-Gly-OSu (1.23 g, 4.02 mmol). To the resulting solution was added PyBOP (2.18 g, 4.19 mmol) and H-Glu(OMe)-OMe hydrochloride (0.93 g, 4.4 mmol). After the typical workup the crude oil was chromatographed using EtOAc as eluent affording the title compound as a colourless oil (1.61 g, 87%). **$^1$H-RMN (DMSO-$d_6$, 400 MHz, 110 ºC) δ ppm (*mixture of epimers*)**: 7.89 (br s, 2H, $NH_{Glu}$ + $NH_{Glu'}$), 7.46 – 7.34 (m, 8H, ArH), 7.33 – 7.26 (m, 2H, ArH), 6.66 (br s, 2H, $NH_{Gly}$ + $NH_{Gly'}$), 5.07 (s, 4H, 2 x $CH_2Ph$), 4.47 – 4.39 (m, 2H, $H_{Pro}$-2 + $H_{Pro}$-2'), 4.39 – 4.30 (m, 2H, $H_{Glu}$-2 + $H_{Glu}$-2'), 3.97 – 3.71 (m, 4H, $H_{Gly}$-2 + $H_{Gly}$-2'), 3.65 (s, 6H, 2 x $CO_2CH_3$), 3.61 (s, 6H, 2 x $CO_2CH_3$), 3.55 – 3.47 (m, 4H, $H_{Pro}$-5 + $H_{Pro}$-5'), 2.41 – 2.34 (m, 4H, $H_{Glu}$-4 + $H_{Glu}$-4'), 2.18 – 2.02 (m, 4H), 2.01 – 1.83 (m, 8H). **$^{13}$C-RMN (DMSO-$d_6$, 100 MHz, 110 ºC) δ ppm (*mixture of epimers*)**: [171.9 (C), 171.8 (C), 171.1

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(C), 171.0 (C), 171.0 (C), 167.0 (C), 166.9 (C), $C_{Gly}$-1 + $C_{Gly}$-1' + $C_{Pro}$-1 + $C_{Pro}$-1' + $C_{Glu}$-1 + $C_{Glu}$-1' + 2 x $CO_2Me$], 155.6 (C, 2 x $NCO_2Bn$), 136.6 (2 x C, ArC), 127.6 (4 x CH, ArC), 127.0 (2 x CH, ArC), 126.9 (4 x CH, ArC), 65.1 (2 x $CH_2$, 2 x $CH_2Ph$), [58.9 (CH), 51.1 (CH), 51.1 CH), $C_{Pro}$-2 + $C_{Pro}$-2' + $C_{Glu}$-2 + $C_{Glu}$-2'], [50.9 ($CH_3$), 50.5 ($CH_3$), 4 x $CO_2CH_3$], 45.6 (2 x $CH_2$), 42.4 (2 x $CH_2$), [29.3 ($CH_2$), 29.3 ($CH_2$)], 25.7 (2 x $CH_2$). **HRMS (ESI-TOF) *m/z*:** $[M + H]^+$ calcd for $C_{22}H_{30}N_3O_8^+$ 464.2027; found: 464.2029.



**Dibenzyl *N*-(*tert*-butyloxycarbonyl)glycyl-L-prolyl-L-glutamate (8)**

Following the same one-pot protocol described for preparation of tripeptide **5**, to a suspension of H-L-Pro-OH (0.46 g, 4.0 mmol) in EtOAc (50 mL), was added DIPEA (1.39 mL, 7.98 mmol) and Boc-Gly-OSu (1.09 g, 4.02 mmol). To the resulting solution was added PyBOP (2.18 g, 4.19 mmol) and H-Glu(OBn)-OBn hydrochloride (1.60 g, 4.40 mmol). The suspension was transferred to a separatory funnel and was washed with $NaHCO_3$ (3 x 50 mL). The organic extract was dried over anhydrous $Na_2SO_4$, filtered and the solvent was removed *in vacuo*. The crude oil was chromatographed using EtOAc obtaining the title compound as a colourless oil (2.07 g, 89% yield). $[\alpha]_D^{23}$: $+1.7 \pm 0.1$ (*c*1.07, $CHCl_3$). **$^1$H-RMN (DMSO-$d_6$, 400 MHz, 110 ºC) δ ppm**: 7.96 (br s, 1H, $NH_{Glu}$), 7.43 – 7.27 (m, 10H, ArH), 6.07 (br s, 1H, $NH_{Gly}$), 5.14 (d, *J* = 3.7 Hz, 2H, $CH_2Ph$), 5.11 (s, 2H, $CH_2Ph$), 4.47 – 4.37 (m, 2H, $H_{Pro}$-2 + $H_{Glu}$-2), 3.94 – 3.57 (m, 2H, $H_{Gly}$-2), 3.48 (t, *J* = 6.7 Hz, 2H, $H_{Pro}$-5), 2.46 (t, *J* = 7.6 Hz, 2H, $H_{Glu}$-4), 2.19 – 2.09 (m, 1H), 2.05 – 1.77 (m, 5H), 1.40 (s, 9H, Boc). **$^{13}$C-RMN (DMSO-$d_6$, 100 MHz, 110 ºC) δ ppm**: [171.3 (C), 171.1 (C), 170.4 (C), 167.1 (C), $C_{Gly}$-1 + $C_{Pro}$-1 + $C_{Glu}$-1 + $CO_2Bn$], 154.9 (C, Boc), 135.7 (C, ArC), 135.4 (C, ArC), 127.7 (2 x CH, ArC), 127.7 (2 x CH, ArC), 127.3 (CH, ArC), 127.2 (CH, ArC), 127.1 (2 x CH, ArC), 127.1 (2 x CH, ArC), 77.6 (C, Boc), 65.6 ($CH_2$, $CH_2Ph$), 65.0 ($CH_2$, $CH_2Ph$), [58.8 (CH), 51.1 (CH), $C_{Pro}$-2 + $C_{Glu}$-2], 45.5 ($CH_2$), 42.2 ($CH_2$), 29.7 ($CH_2$), 29.5 ($CH_2$), 27.6 (3 x $CH_3$, Boc), 25.7 ($CH_2$). **HRMS (ESI-TOF) *m/z*:** $[M + H]^+$ calcd for $C_{31}H_{40}N_3O_8^+$ 582.2810; found: 582.2811.

**Dimethyl glycyl-L-prolyl-L-glutamate (9)**

Method A) Following the same protocol described for preparation of GPE, a mixture of tripeptide **7** (0.50 g, 1.1 mmol) and 10 wt % Pd/C (0.10 g) in methanol (40 mL) was prepared in a hydrogenation vessel and transferred into a hydrogenation apparatus, flushed with $H_2$ (60 psi) and mechanically stirred at room temperature, protected from light, for 4 h. After the typical workup the title compound was obtained as light pink solid (0.32 g, 88%). Method B) Following the one-pot protocol described for preparation of **7**, after the addition of H-Glu(OMe)-OMe hydrochloride, the solvent was removed *in vacuo* and the crude mixture was dissolved in methanol (40 mL) followed by the addition of

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10 wt % Pd/C (0.10 g) and the reaction vessel was flushed with $H_2$ (1 atm), at room temperature and protected from light with magnetic stirring, for 4 h. The reaction mixture was filtered through a Celite™ pad and the pad washed with 1:1 methanol–water (100 mL; v/v). The filtrate was concentrated to dryness under reduced pressure and chromatographed with DCM:MeOH 20:1 affording the title compound as a light pink solid (0.25 g, 70%). $[\alpha]_D^{23}$: $-60.7 \pm 0.2$ (*c*1.075, $CH_3OH$) [lit.[17a] $[\alpha]_D$: $-90.7$ (*c*0.20, $H_2O$)]. **$^1$H-RMN (DMSO-$d_6$, 400 MHz, 110 ºC) δ ppm**[54]: 8.13 (br s, 1H, $NH_{Glu}$), 4.48 (br s, 1H, $H_{Pro}$-2), 4.29 (dd, *J* = 13.7, 7.9 Hz, 1H, $H_{Glu}$-2), 3.92 – 3.67 (m, 2H, $H_{Gly}$-2), 3.65 (s, 3H, $CO_2CH_3$), 3.61 (s, 3H, $CO_2CH_3$), 3.53 (t, *J* = 7.2 Hz, 2H, $H_{Pro}$-5), 2.40 (t, *J* = 7.5 Hz, 2H, $H_{Glu}$-4), 2.16 – 2.02 (m, 2H), 2.01 – 1.81 (m, 4H). **$^{13}$C-RMN (DMSO-$d_6$, 100 MHz, 110 ºC) δ ppm**: [171.9 (C), 171.0 (C), 170.7 (C), 164.3 (C), $C_{Gly}$-1 + $C_{Pro}$-1 + $C_{Glu}$-1 + $CO_2$Me], [59.1 (CH), 51.1 (CH), $C_{Pro}$-2 + $C_{Glu}$-2], [51.1 ($CH_3$), 50.6 ($CH_3$), 2 x $CO_2CH_3$], 45.7 ($CH_2$), 39.8 ($CH_2$), 39.4 ($CH_2$), 29.5 ($CH_2$), 25.6 ($CH_2$). **HRMS (ESI-TOF) *m*/*z*:** $[M + H]^+$ calcd for $C_{14}H_{24}N_3O_6^+$ 330.1660; found: 330.1661.

**Dibenzyl glycyl-L-prolyl-L-glutamate trifluoroacetate (10)**

Method A) A solution of tripeptide **8** (0.52 g, 0.89 mmol) in TFA (2.05 mL, 26.8 mmol) was magnetically stirred until consumption of the starting material (TLC). Excess of TFA was removed using a rotavapor affording the title compound as a yellow oil (0.51, 96%). Method B) Following the one-pot protocol described for preparation of **8**, after the addition of H-Glu(OBn)-OBn hydrochloride, the solvent was removed *in vacuo* and the crude mixture was dissolved in TFA (10 mL) and the solution was magnetically stirred until consumption of the starting material (TLC). Excess of TFA was removed using a rotavapor and the mixture was chromatographed with DCM:MeOH 20:1 to 10:1 affording the title compound as a yellow oil (0.40 g, 75%). $[\alpha]_D^{23}$: $-42.9 \pm 0.2$ (*c*1.135, $CH_3OH$). **$^1$H-RMN (DMSO-$d_6$, 400 MHz, 110 ºC) δ ppm**[54]: 8.09 (br s, 1H, $NH_{Glu}$), 7.46 – 7.21 (m, 10H, ArH), 5.14 (d, *J* = 5.1 Hz, 2H, $CH_2$Ph), 5.11 (s, 2H, $CH_2$Ph), 4.47 (d, *J* = 7.3 Hz, $H_{Pro}$-2), 4.41 (dd, *J* = 13.5, 7.5 Hz, $H_{Pro}$-2), 3.82 – 3.62 (m, 2H, $H_{Gly}$-2), 3.50 (t, *J* = 6.3 Hz, 2H, $H_{Pro}$-5), 2.47 (t, *J* = 7.5 Hz, 2H, $H_{Glu}$-4), 2.23 – 1.73 (m, 6H, $H_{Pro}$-4 + $H_{Glu}$-4 + $H_{Glu}$-5). **$^{13}$C-RMN (DMSO-$d_6$, 100 MHz, 110 ºC) δ ppm**: [171.3 (C), 170.7 (C), 170.4 (C), 164.0 (C), $C_{Gly}$-1 + $C_{Pro}$-1 + $C_{Glu}$-1 + $CO_2$Bn], [135.7 (C), 135.4 (C), ArC], [127.8 (CH), 127.7 (CH), 127.4 (CH), 127.3 (CH), 127.2 (CH), 127.2 (CH), ArC], [65.6 ($CH_2$), 65.1 ($CH_2$), 2 x $CH_2$Ph], [58.9 (CH), 51.2 (CH), $C_{Pro}$-2 + $C_{Glu}$-2], 45.6 ($CH_2$), 39.4 ($CH_2$), 29.7 ($CH_2$), 28.4 ($CH_2$), 25.7 ($CH_2$), 23.3 ($CH_2$). **HRMS (ESI-TOF) *m*/*z*:** $[M + H]^+$ calcd for $C_{26}H_{32}N_3O_6^+$ 482.2286; found: 482.2282.

***N*-(*tert*-Butyloxycarbonyl)glycyl-L-prolyl-L-glutamic acid (11)**

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Following the same protocol described for preparation of GPE, a mixture of tripeptide **8** (0.48 g, 0.83 mmol) and 10 wt % Pd/C (0.10 g) in 4:1 methanol–water (40 mL; v/v) was prepared in a hydrogenation vessel and transferred into a hydrogenation apparatus, flushed with $H_2$ (60 psi) and mechanically stirred at room temperature, protected from light, for 4 h. After the typical workup the title compound was obtained as a hygroscopic white solid (0.30 g, 91%). $[\alpha]_D^{23}$: $-24.9 \pm 0.1$ (*c*1.21, $CH_3OH$). **$^1$H-RMN (DMSO-$d_6$, 400 MHz, 110 ºC) δ ppm**[55]: 7.77 (br s, 1H, $NH_{Glu}$), 6.10 (br s, 1H, $NH_{Gly}$), 4.46 – 4.41 (m, 1H, $H_{Pro}$-2), 4.30 – 4.22 (m, 1H, $H_{Glu}$-2), 3.84 – 3.66 (m, 2H, $H_{Gly}$-2), 3.50 (t, *J* = 6.8 Hz, 2H, $H_{Pro}$-5), 2.38 (t, *J* = 7.6 Hz, 2H, $H_{Glu}$-4), 2.20 – 1.83 (m, 6H, $H_{Glu}$-3 + $H_{Pro}$-3 + $H_{Pro}$-4), 1.41 (s, 9H, Boc). **$^{13}$C-RMN (DMSO-$d_6$, 100 MHz, 110 ºC) δ ppm**: [172.9 (C), 172.0 (C), 170.9 (C), 167.1 (C), $C_{Gly}$-1 + $C_{Pro}$-1 + $C_{Glu}$-1 + $CO_2H$], 154.9 (C, Boc), 77.7 (C, Boc), [58.9 (CH), 51.1 (CH), $C_{Pro}$-2 + $C_{Glu}$-2], 45.6 ($CH_2$), 42.2 ($CH_2$), 29.9 ($CH_2$), 29.5 ($CH_2$), 27.7 (3 x $CH_3$, Boc), 26.2 ($CH_2$), 26.1 ($CH_2$). **HRMS (ESI-TOF) *m/z*:** [M - H]$^-$ calcd for $C_{17}H_{26}N_3O_8^-$ 400.1725; found: 400.1729.

***O*-Caproyl-*trans*-4-hydroxy-L-proline (12)**

In a round bottom flask was dissolved H-L-Hyp-OH (1.02 g, 7.78 mmol) in TFA (5 mL) at 0 ºC using an ice bath, followed by caproyl chloride (2.20 mL, 15.7 mmol). The solution was stirred for 16 h and the excess of TFA was removed *in vacuo*. To the resulting mixture was added cold $Et_2O$ (50 mL) to induce the precipitation. The precipitate was filtered and dried affording the title compound as a fine white powder (1.76 g, 66%). The spectroscopic and spectrometric data is in agreement with the data previously reported in the literature.[51]

**Dibenzyl *N*-(benzyloxycarbonyl)-L-alanyl-(*O*-caproyl-*trans*-4-hydroxy)-L-prolyl-L-glutamate (13)**

Following the same one-pot protocol described for preparation of tripeptide **5**, to a suspension of **12** (1.37 g, 4.00 mmol) in EtOAc (50 mL), was added DIPEA (1.39 mL, 7.98 mmol) and Cbz-Ala-OSu (1.29 g, 4.02 mmol). To the resulting solution was added PyBOP (2.18 g, 4.19 mmol) and H-Glu(OBn)-OBn hydrochloride (1.60 g, 4.40 mmol). After the typical workup the crude oil was chromatographed using EtOAc as eluent affording the title compound as a colourless oil (2.50 g, 84%). $[\alpha]_D^{24}$: $-66.0 \pm 0.2$ (*c*1, MeOH). **$^1$H-RMN ($CDCl_3$, 400 MHz, 26 ºC) δ ppm**: 7.43 – 7.27 (m, 15H, ArH), 7.24 (d, *J* = 8.0 Hz, 1H, $NH_{Glu}$), 5.72 (d, *J* = 7.6 Hz, 1H, $NH_{Gly}$), 5.35 (br s, 1H, $H_{Hyp}$-4), 5.15 (s, 2H, $CH_2Ph$), 5.08 (s, 2H, $CH_2Ph$), 5.07 (s, 2H, $CH_2Ph$), 4.67 – 4.53 (m, 2H, $H_{Hyp}$-2 + $H_{Glu}$-2), 4.50 – 4.36 (m, 1H, $H_{Ala}$-2), 3.78 (d, *J* = 2.9 Hz, 2H, $H_{Hyp}$-3), 2.50 – 2.16 (m, 7H), 2.03 – 1.93 (m, 1H), 1.66 – 1.55 (m, 2H, $CH_2(CH2)_2CH_3$), 1.38 – 1.21 [1.25 (d, *J* = 6.7 Hz), 7H, $H_{Ala}$-3 + $(CH_2)_2CH_3$], 0.89 (t, *J* = 6.7 Hz, 3H, $CH_3$). **$^{13}$C-RMN ($CDCl_3$, 100 MHz, 26 ºC) δ ppm**: [174.4 (C), 173.4 (C), 172.7 (C), 172.6 (C), 171.4 (C), 170.5 (C), $C_{Ala}$-1 + $C_{Hyp}$-1 + $C_{Glu}$-1 + $CO_2Bn$], 155.7 (C, $NCO_2Bn$), [136.4 (C),

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135.8 (C), 135.2 (C), 3 x ArC], [128.7 (CH), 128.6 (CH), 128.4 (CH), 128.3 (CH), 128.2 (CH), 128.1 (CH), ArC], 72.7 (CH, $C_{Hyp}$-4), [67.5 ($CH_2$), 66.9 ($CH_2$), 66.6 ($CH_2$), 3 × $CH_2Ph$], 58.8 (CH, $C_{Hyp}$-2), 52.8 ($CH_2$, $C_{Hyp}$-3), [51.9 (CH), 51.8 (CH), $C_{Hyp}$-2 + $C_{Glu}$-2], 48.5 (CH, $C_{Ala}$-2), 34.2 ($CH_2$), 33.6 ($CH_2$), 31.3 ($CH_2$), 30.0 ($CH_2$), 27.3 ($CH_2$), 24.5 ($CH_2$), 22.4 ($CH_2$), 18.1 ($CH_3$, $C_{Ala}$-3), 14.0 ($CH_3$, $CH_2\underline{C}H_3$). **HRMS (ESI-TOF) *m/z*:** $[M + Na]^+$ calcd for $C_{41}H_{49}N_3NaO_{10}^+$ 766.3310; found: 766.3308.

## Author Contributions

Conceived and designed the experiments: IES-D. Performed the experiments: IES-D. *In silico* calculations: LPS. Analyzed the data: IES-D, LPS, SGS, XG-M, JER-B. Wrote the paper: IES-D. All authors have given approval to the final version of the manuscript.

## Supporting Information

1D ($^1H$, $^{13}C$, DEPT-135) and 2D (TOCSY, COSY, HSQC) NMR data for peptide products, free energy calculations for the *cis-trans* isomerization of tripeptide **5**, and the cartesian coordinates for *cis*-**5** and *trans*-**5** conformers.

## Conflicts of interest

The authors declare no competing financial interest.

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[54] Amine protons are too broadened due to rapid proton exchange at 110 ºC.

[55] Carboxylic acids protons are too broadened due to rapid proton exchange at 110 ºC.

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## Graphical Abstract