Incorporation of *N*-amidino-pyroglutamic acid into peptides using intramolecular cyclization of α-guanidinoglutaric acid

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N-terminal modification of peptides by unnatural amino acids significantly affects their enzymatic stability, conformational properties and biological activity. Application of *N*-amidino-amino acids, positively charged under physiological conditions, can change peptide conformation and its affinity to the corresponding receptor. In this article, we describe synthesis of short peptides, containing a new building block – *N*-amidino-pyroglutamic acid. Although direct guanidinylation of pyroglutamic acid and oxidation of *N*-amidino-proline using RuO₄ did not produce positive results, *N*-amidino-Glp-Phe-OH was synthesized on Wang polymer by cyclization of α-guanidinoglutaric acid residue. In the course of synthesis, it was found that literature procedure of selective Boc deprotection using TMSOTf/TEA reagent is accompanied by concomitant side reaction of triethylamine alkylation by polymer linker fragment. It should be mentioned that independently from cyclization time and coupling agent (DIC or HCTU), the lactam formation was incomplete. Separation of the cyclic product from the linear precursor was achieved by HPLC in ammonium formate buffer at pH 6. HPLC analysis showed *N*-amidino-Glp-Phe-OH stability at acidic and physiological pH and fast ring opening in water solution at pH 9. The suggested method of *N*-amidino-Glp residue formation can be applied in the case of short peptide chains, whereas synthesis of longer ones will require fragment condensation approach. Copyright © 2009 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: *N*-amidino-pyroglutamic acid; intramolecular cyclization; TMSOTf/TEA deprotection; Wang resin

Introduction

Unnatural amino acids are widely applied in the synthesis of peptides and peptidomimetics, possessing improved biological properties and enzymatic stability [1]. There are numerous indications that *N*-terminal modification has significant influence on biological activity and duration of peptide action [2–5]. Application of *N*-amidino-amino acids seems especially promising in this regard, due to their ability to retain positive charge under physiological conditions, to potential stabilization of quasi-cyclic peptide conformation and enhancement of its interaction with specific receptor [6]. Moreover, it was shown that substitution of amino group by the strongly basic guanidino group sometimes significantly enhances the activity of natural compounds as well as selectivity of their action [7]. Pyroglutamic acid being a five-member lactam of glutamic acid is an *N*-terminal residue in a number of native proteins and peptides. Thus, modification of pyroglutamic acid by introducing the guanidino or *N*-amidino functional groups might represent a useful tool for the structure–activity relationship study.

In this article, we investigated several possibilities of *N*-amidino-pyroglutamic acid incorporation into peptide sequence. Direct synthesis of this unnatural amino acid seems complicated by the low reactivity of lactam nitrogen. On the other hand, attempts of α-guanidinoglutaric acid cyclization, described by Natelson [8,9], resulted in 2-imido-4-oxo-5-(3′-propanoic acid) imidazolidine formation instead of the expected *N*-amidino-pyroglutamic acid (Figure 1). The structure of reaction product is similar to that of creatinine (2-amino-3-methyl-4H-imidazol-5-one). These data

prompted us to investigate the utility of solid-phase technique for the synthesis of peptides, containing *N*-amidino-pyroglutamic acid residue.

Materials and Methods

General

p-Benzyloxybenzyl alcohol resin (Wang resin) (0.68 mmol/g, 200–400 mesh) and Merrifield resin preloaded with Boc-Leu-OH (0.8 mmol/g, 100–200 mesh) were purchased from NovaBiochem (Läufelfingen, Switzerland). All the amino acids and reagents for peptide synthesis were obtained from Sigma Chemical Co. (St Louis, MO, USA), Fisher Scientific (Fair Lawn, NJ, USA), Bachem (Bubendorf, Switzerland), Ferak Berlin (Berlin, Germany), Aldrich (Steinheim, Germany) and IRIS Biotech (Marktredwitz, Germany). *N,N′*-Di-*tert*-butoxycarbonyl-1*H*-benzotriazole-1-carboxamidine (Boc₂CABt) and *N,N′*-Di-*tert*-butoxycarbonyl-1-methyl-2-thiourea were synthesized as described previously [10,11]. Thin layer chromatography (TLC) was performed on Merck F 254 and Silufol plates using specified solvent system. Analytical RP-HPLC was performed using Beckman System Gold with a Phenomenex Luna C₁₈ column (4.6 × 150 mm, 5 µm) using

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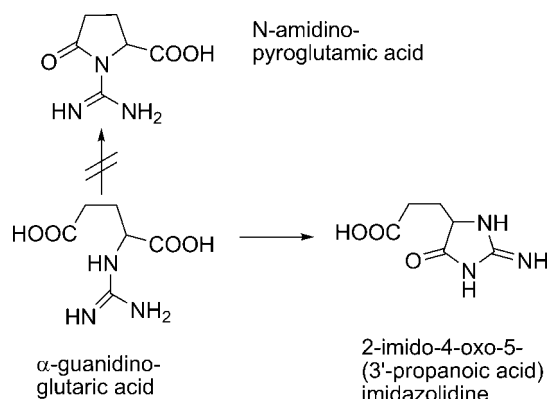Institute of Macromolecular Compounds RAS, 199004, St Petersburg, Russia

Figure 1. Synthesis of *N*-amidino-pyroglutamic acid according to [8,9].

UV detection at 229 nm, buffer A: 0.1% H_3PO_4 in H_2O, buffer B: 0.1% H_3PO_4 in 50% MeCN–H_2O at gradient rate of 1% per min and flow rate of 1 ml/min. Preparative RP-HPLC was performed with a reverse phase Nucleosil C_{18} column (10 × 250 mm, 5 μm), buffer A: 0.1% TFA in H_2O, buffer B: 0.1% TFA in 50% MeCN–H_2O with nonlinear gradient and 5 ml/min flow rate. Mass spectra were recorded on Finnigan LCQ ion-trap mass spectrometer with an electrospray ionization (ESI) source in the positive mode. Analytical RP-HPLC and electrospray MS confirmed the purity and molecular mass of the synthesized peptides. ^{1}H-NMR and ^{13}C-NMR spectra were recorded at 400 and 125 MHz, respectively, on a Bruker Avance 400 spectrometer or at 200 and 50 MHz on a Bruker AC-200. Calibration was done with solvent residual signals as internal standards. Chemical assignments were carried out on the basis of heteronuclear correlated spectroscopy experiments.

N-amidino-proline

N-amidino-L-proline was synthesized as reported elsewhere [12]. Briefly, L-proline was guanidinylated with cyanamide in water–methanol solution and purified by precipitation in isopropyl alcohol and recrystallization from water. ESI-MS [obzd: m/z (M+H)$^+$ 158.12; calcd for $C_6H_{11}N_3O_2$: 157.20]. Elemental analysis: calcd for $C_6H_{11}N_3O_2$: C, 41.13%; H, 7.43%; N, 23.99%; found: C, 41.21%; H, 7.43%; N, 23.94%. ^{1}H-NMR (400 MHz, D_2O) δ 4.26-4.23 (dd, 1H, CHα), 3.56-3.51 (td, 1H, CHδ^1), 3.48-3.41 (q, 1H, CHδ^2), 2.33-2.16 (m, 1H, CHβ^1), 2.16-2.1 (m, 1H, CHβ^2), 2.05-1.88 (m, 2H, CHγ). ^{13}C-NMR (125MHz, D_2O) δ 177.78 (COOH), 154.86 (C$_{amidino}$), 62.43 (Cα), 47.84 (Cδ), 31.04 (Cβ), 23.33 (Cγ).

Solid-phase Peptide Synthesis

Peptides were assembled manually using polypropylene syringe (10 ml) fitted with a polyethylene porous disc. The first residues were attached according to the standard methods [13].

α-guanidino-glutaryl-leucine diethylamide

Solid-phase peptide synthesis was carried out using Merrifield resin preloaded with Boc-Leu-OH. Removal of Boc group was accomplished with 65% TFA in DCM (1 and 15 min) and followed by washings with DCM (3 × 1 min), DMF (1 min), DIEA/DMF (5 : 95, v/v) (1 and 2 min) and DMF (3 × 1 min). A threefold molar excess of Boc-Glu(OBu^t)-OH and DIC/*Cl*-HOBt was used with a standard coupling time of 2 h, and the resin was washed with DMF (2×1 min)

and DCM (1 min). In the course of guanidinylation, H_2N-Glu-Leu-O-resin was treated by means of 3 eq of Boc_2CABt and 3 eq of DIEA in DMF or NMP. The mixture was shaken for 2 h and the reaction was monitored using Kaiser test. After the deprotection, peptidyl resin was shaken in DMF for 18 h with 6 eq of DIC and 6 eq of *Cl*-HOBt followed by washings with DMF (3 × 1 min), DCM (3 × 1 min) and diethyl ether (2 × 1 min). The peptidyl resin obtained was cleaved (4 h, 0 °C) by anhydrous ethylamine. Ethylamine was evaporated under reduced pressure, and peptide/resin mixture was washed three times with AcOH followed by filtration. The filtrate was analyzed by RP-HPLC and ESI MS without subsequent purification steps.

N-amidino-pyroglutamyl-phenylalanine

Solid-phase peptide synthesis was carried out using Wang resin and DIC/*Cl*-HOBt as coupling reagent. A threefold molar excess of protected amino acid (Fmoc-Phe-OH and Fmoc-Glu(OBu^t)-OH correspondingly) was used with a standard coupling time of 2 h followed by washings with DMF (2 × 1 min) and DCM (1 min). Removal of the Fmoc group was carried out with piperidine : DMF (2 : 8, v/v) (2 and 8 min) and followed by washings with DMF (3 × 1 min), iPrOH (2 × 1 min) and DMF (3 × 1 min). In the course of guanidinylation, H_2N-Glu(OBu^t)-Phe-O-resin was treated by means of 3 eq of Boc_2CABt and 3 eq of DIEA in DMF or NMP. The mixture was shaken for 2 h and the reaction was monitored using Kaiser test. Boc and OBu^t protecting groups were removed by trimethylsilyl triflate (TMSOTf) according to Lejeune *et al*. [14]. Briefly, peptidyl-polymer was treated by 0.2 M TMSOTf/0.4 M TEA/DCM (15 min), washed by DCM; TEA/DCM (10 : 90, v/v); after DCM washing, the procedure was repeated. The resulted peptidyl resin with free γ-carboxyl group of guanidinoglutaric acid was shaken in DMF for 18 h with 6 eq of DIC and 6 eq of *Cl*-HOBt. After completion of the cyclization, the resin was washed with DMF (3 × 1 min) and DCM (3 × 1 min). The peptidyl resin obtained was cleaved (2–4 h, rt) by TFA/TIS/H_2O (95 : 2.5 : 2.5, v/v) and dry diethyl ether was added to precipitate the cleaved peptide. The precipitate was filtrated and purified by preparative HPLC (purity ≥97%): Nucleosil C_{18} column (10 × 250 mm, 5 μm), 229 nm UV detection, the acetonitrile gradient in ammonium formate buffer (0.1% HCOOH and NH_4OH until pH 6) at flow rate of 5 ml/min. Fractions were desalted manually on C_{18} syringe (buffer A: 0.1% TFA in H_2O, buffer B: 0.1% TFA in 25% MeCN–H_2O) and lyophilized. Yield: 20%. ESI-MS [obzd: m/z (M+H)$^+$ 319.13; calcd for $C_{15}H_{18}N_4O_4$: 318.13]. ^{1}H-NMR (400 MHz, DMSO-d$_6$) δ 12.9 (COOH), 8.60-8.50 (m, 4H, NH), 7.26 (m, 5H, CH$_{Ar}$), 5.0 (dt, 1H, CHα$_{Glp}$), 4.45 (m, 1H, CHα$_{Phe}$), 3.11-3.08 (dd, 1H, CHβ$^1_{Phe}$), 2.96-2.92 (m, 1H, CHβ$^2_{Phe}$), 2.56 (m, 2H, CHγ$_{Glp}$), 2.4 (m, 1H, CHβ$^1_{Glp}$), 2.03 (t, 1H, CHβ$^2_{Glp}$). ^{13}C-NMR (100 MHz, DMSO-d$_6$) δ 129.0 (C$_{aromatic}$), 128.07 (C$_{aromatic}$), 126.31 (C$_{aromatic}$), 58.81 (Cα$_{Glp}$), 36.26 (Cβ$_{Phe}$), 30.39 (Cγ$_{Glp}$), 22.76 (Cβ$_{Glp}$).

[(4-Hydroxypenyl)Methyl]Triethylazanium Trifluoroacetate

This compound was obtained as a result of side reaction during the synthesis of *N*-amidino-pyroglutamyl-phenylalanine. Yellow liquid oil, ESI-MS [obzd: m/z (M)$^+$ 208.18; calcd for $C_{13}H_{22}NO$: 208.17]. ^{1}H-NMR (200 MHz, D_2O) δ 7.32-7.30 (d, 2H, CH$_{Ar}$), 6.90-6.88 (d, 2H, CH$_{Ar}$), 4.24 (s, 2H, CH$_2$-Ph), 3.14-3.07 (q, 6H, CH$_2^{Et}$), 1.31-1.26 (t, 9H, CH$_3^{Et}$). ^{13}C-NMR (50MHz, D_2O) δ 163.60, 163.12 (COOH$_{TFA}$), 134.49 (C$_{aromatic}$), 119.21 (C$_{aromatic}$), 116.31 (C$_{aromatic}$), 59.83 (CH$_2$-Ph), 52.21 (CH$_2^{Et}$), 7.34 (CH$_3^{Et}$).

Results and Discussion

Our initial experiments were directed toward the development of reliable methods for solution phase synthesis of *N*-amidino-pyroglutamic acid and its derivatives. Apart from intramolecular cyclization of α-guanidinoglutaric acid, the principal possibilities comprise guanidinylation of pyroglutamic acid and *N*-amidino-proline oxidation.

Guanidinylation of Pyroglutamic Acid

The reduced nucleophilic reactivity of amide nitrogen in pyrrolidone ring due to electron pair delocalization significantly complicates synthesis of *N*-substituted derivatives [15]. An efficient procedure for synthesis of protected pyroglutamic acid is based on Boc/Z-glutamic acid anhydride formation and subsequent dicyclohexylamine treatment [16,17]. In order to improve the re-activity of amide nitrogen in the acylation process, several authors [15,18–20] applied a variety of bases, such as DMAP, DIEA or NaH. Thus, Tomasini and Villa [15] described acylation of pyroglutamic acid ester by alanine pentafluorophenyl ester in the presence of sodium hydride. It was shown that interaction between pyroglutamic acid ring and NaH or LiHMDS with formation of an anion afforded the desired dipeptide in good to high yield. Considering these data, one can expect that NaH may facilitate the process of pyroglutamic acid esters guanidinylation that would result in the synthesis of *N*-amidino-pyroglutamic acid derivatives.

There is a wide range of efficient guanidinylating agents used for primary and secondary amino group modification [21]. For our studies, we have chosen *N*,*N*′-Di-*tert*-butoxycarbonyl-1*H*-benzotriazole-1-carboxamidine (Boc$_2$CABt), a powerful reagent, suggested by Musiol and Moroder [11] (Figure 2). However, application of reaction conditions, described in the literature [15], and addition of different bases, including NaH, did not result in the desired product. Thus, although acylation of pyroglutamic acid can be achieved, use of strong bases seems insufficient for its guanidinylation or requires optimization of the reaction procedure.
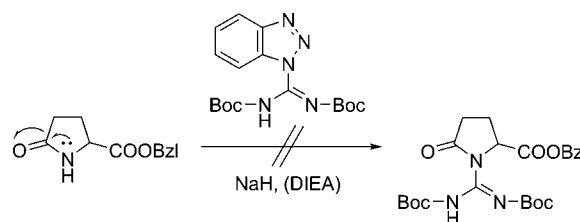

Figure 2. Unsuccessful attempt of pyroglutamic acid guanidinylation.

N-amidino-proline Oxidation

Due to the structure similarity, proline represents an attractive template for the synthesis of pyroglutamic acid derivatives. Muramatsu *et al*. [22] showed that *N*-acyl-L-proline can be readily oxidized by KMnO$_4$ in the presence of strong acids producing pyroglutamyl residue. Other examples of this approach comprise synthesis of protected pyroglutamic acid and peptides, containing its *N*-acyl derivatives using ruthenium tetroxide as an efficient oxidizing agent [23].

Previously we described synthesis of *N*-amidino-proline and its derivatives for the application in classical and solid-phase peptide synthesis [12]. These data prompted us to investigate the practical utility of *N*-amidino-proline oxidation leading to the formation of *N*-amidino-pyroglutamic acid. The typical oxidation protocol is based on *in situ* ruthenium tetroxide generation from RuO$_2$ under the action of 10% NaIO$_4$ in the mixture of EtOAc and water [24]. Considering the solubility in organic medium, as a starting compound we have chosen N^G,N^G-*bis*-Boc-*N*-amidino-proline benzyl ester. RP-HPLC monitoring of the reaction process indicates formation of compounds eluting earlier than the starting material. In order to eliminate possible oxidative degradation of protecting groups, further experiments were carried out using free *N*-amidino-proline and stoichiometric amount of oxidizing agent (Figure 3). Although RP-HPLC analysis does not permit to identify the desired product, ESI-MS data revealed the
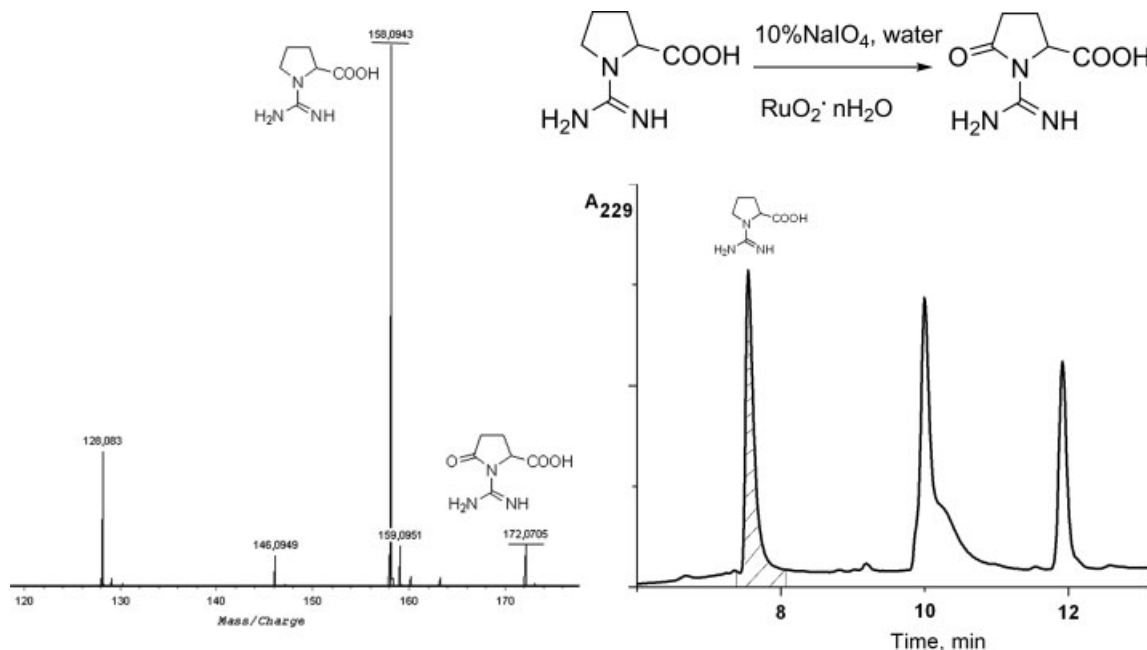

Figure 3. Oxidation of *N*-amidino-proline. ESI-MS (left) and RP-HPLC (right) analysis of the crude product.

minor amount of *N*-amidino-pyroglutamic acid in the reaction mixture. Thus, although these experiments confirm the principal possibility of *N*-amidino-pyroglutamic acid formation, oxidation of *N*-amidino-proline cannot be considered a reliable method of synthesis.

Cyclization of α-Guanidinoglutaric Acid

It is well known that both glutamic acid and glutamine residues are prone to cyclization when located at the *N*-terminus of peptide sequence [25,26]. Moreover, glutamic acid can be easily transformed to pyroglutamic acid in water solution at elevated temperature [27]. There are also examples showing that in strong acidic conditions this reaction can take place even in the middle of peptide sequence, resulting in *N*-acylpyroglutamic acid formation [26]. These data suggest that intramolecular cyclization of α-guanidinoglutaric acid might serve as an alternative route for the synthesis of *N*-amidino-pyroglutamic acid.

The problem of imidazolidine formation (Figure 1), encountered by Natelson can be overcome by using appropriate protecting groups or solid-phase synthesis technique. Apart from high efficiency of SPPS relative to solution phase chemistry, its well-known advantages comprise simplicity, rapidity and versatility in the synthesis of various peptide sequences. Therefore, we used solid-phase approach as the method of choice for the synthesis of peptides, containing *N*-amidino-pyroglutamic acid residue.

For the optimization of reaction conditions, we selected the model compound α-guanidino-glutaryl-leucine, synthesized on Merrifield resin. Among different cyclization reagents, described in the literature [28–30], DIC/HOBt seems appropriate for this case. The guanidinylation was performed using Boc$_2$CABt/DIEA in accordance with procedure for synthesis of peptides containing *N*-amidino-proline [12]. However, we encountered multiple problems at the stage of peptide cleavage. Application of TFMSA significantly complicates purification of the final product. On the other hand, cleavage of peptide by ethylamine (Figure 4) resulted in by-products formation. RP-HPLC and ESI-MS analysis of reaction mixture evidenced both incomplete deprotection of the guanidine group in the course of TFA treatment [31] and lactam ring opening leading to corresponding ethylamide with mass difference of 144 a.m.u. as compared with the desired peptide.

In order to achieve peptide cleavage in mild acidic conditions and to simplify purification of the final product, further experiments were performed using Wang polymer. The problem of selective Boc deprotection of peptidyl resin can be

solved applying recently suggested protocols [14,32]. In particular, TMSOTf/TEA [14] ensures efficient Boc removal with less than 10% loss of resin loading. Synthesis of a model peptide *N*-amidino-pyroglutamyl-phenylalanine was carried out as shown in Figure 5. The peptidyl-polymer was deprotected by stirring for 15 min in TMSOTf/TEA/DCM followed by repetitive treatment. However, it should be mentioned that the control of protecting groups' removal is complicated and its completeness remains unclear. The on-resin cyclization of α-guanidinoglutaric acid was carried out using sixfold excess of DIC/HOBt in DMF during 18 h. The resin-bound peptide was cleaved off using standard protocol (TFA/TIS/H$_2$O, 95:2.5:2.5, v/v) and analyzed by RP-HPLC and ESI-MS.

The desired product, *N*-amidino-pyroglutamyl-phenylalanine, was obtained with 20% yield. The principal impurities comprise its linear precursor containing α-guanidinoglutaric acid and an unknown by-product with an intensive peak in mass-spectrum at M/z 208 and high absorption at λ_{229}. Interestingly enough, this molecular mass is exactly the same as that of guanidinophenylalanine. The variation of TMSOTf content and treatment duration has no significant influence on by-product formation. Identification of compounds containing free guanidino group was performed using qualitative Sakaguchi reaction [33]. Opposite to expectations, peptide with molecular mass corresponding to *N*-amidino-pyroglutamyl-phenylalanine exhibited a positive Sakaguchi reaction, unlike by-product with 208 a.m.u. mass. These data exclude the possibility of guanidinophenylalanine formation in the course of solid-phase synthesis or subsequent treatment.

Taking into account pH sensitivity of Wang resin [34], we suggested that TMSOTf/TEA treatment is the key stage of side product formation. Giraud *et al.* described the process of Trp alkylation by the linker, in the course of peptide cleavage from Wang resin [35]. One can expect similar triethylamine alkylation as a side reaction during TMSOTf deprotection. Indeed, NMR analysis revealed [(4-hydroxypenyl)methyl]triethylazanium trifluoroacetate formation (Figure 5). However, it is not clear whether it results from the on-resin process with subsequent cleavage of product by TFA, or it simply dissociates from the polymer at the final stage.

The second question is the origin of the linear peptide with α-guanidinoglutaric acid, found in the reaction mixture as a side product with 10–15% yield. There are two possibilities: incomplete intramolecular cyclization and lactam ring opening during peptide cleavage. RP-HPLC analysis showed complete stability of *N*-amidino-pyroglutamyl-phenylalanine at acidic and
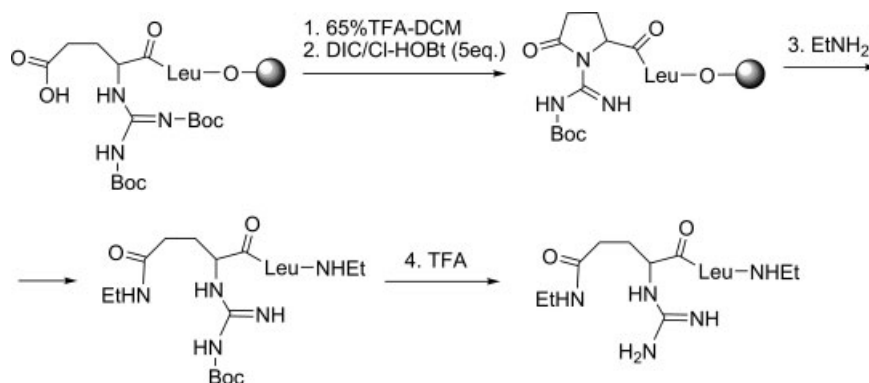

Figure 4. Synthesis of α-guanidinoglutaryl-leucine diethylamide on Merrifield resin.
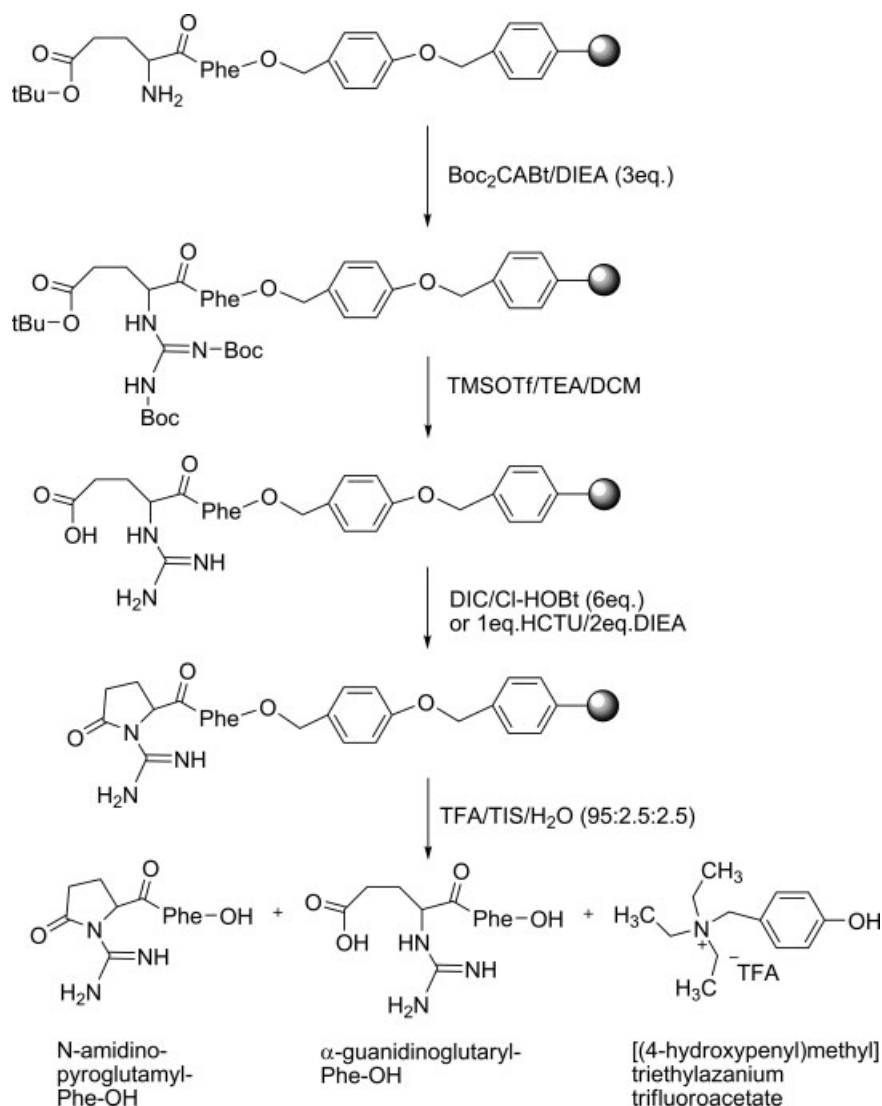
Figure 5. Synthesis of *N*-amidino-pyroglutamyl-phenylalanine on Wang polymer.

physiological pH and fast α-guanidinoglutaryl peptide formation in water at pH 9. These data are in accord with the earlier mentioned lactam ring opening during ethylamine cleavage of *N*-amidino-pyroglutamyl-leucine from the Merrifield polymer and *N*-amidino-pyroglutamyl-phenylalanine visualization by Sakaguchi reaction. On the other hand, additional experiments showed that both cyclization time and the type of coupling agent (DIC or HCTU) have no visible influence on lactam formation efficiency.

Taking into account similar chromatographic behavior of the desired peptide and its linear precursor, for the preparative purification of the final product it was necessary to optimize separation conditions. Application of TFA buffer is efficient only for [(4-hydroxypenyl)methyl]triethylazanium removal, whereas final purification was achieved using ammonium formate buffer, pH 6. The presence of additional carboxylic group due to its ionization significantly increases the retention time of α-guanidino-glutaryl-phenylalanine and consequently simplifies isolation of desired product (Figure 6). The structure of *N*-amidino-pyroglutamyl-phenylalanine was confirmed by ESI-MS and high resolution NMR spectroscopy.

Our attempts of α-guanidinoglutaric acid intramolecular cyclization in the case of longer peptide sequences (GnRH analogue containing 10 amino acid residues) failed. Both RP-HPLC and ESI-MS analyses showed that crude peptide contains more than 70% of α-guanidinoglutaryl-*D*-Phe-Pro-Ser-Tyr-*D*-Lys-Leu-Arg-Pro-Gly-NH$_2$. Considering obvious problems at the stage of peptide purification, it seems that synthesis of oligopeptides containing *N*-amidino-pyroglutamic acid will require fragment condensation approach.

Conclusions

The intramolecular cyclization of α-guanidinoglutaric acid represents an efficient approach to synthesis of short peptides containing a new building block – *N*-amidino-pyroglutamic acid. RP-HPLC study showed that such peptides undergo fast ring opening in water solution at pH 9 but are completely stable in acidic conditions and at physiological pH. These data show that *N*-amidino-pyroglutamic acid may represent a valuable tool for peptide modification and structure–activity relationships study.
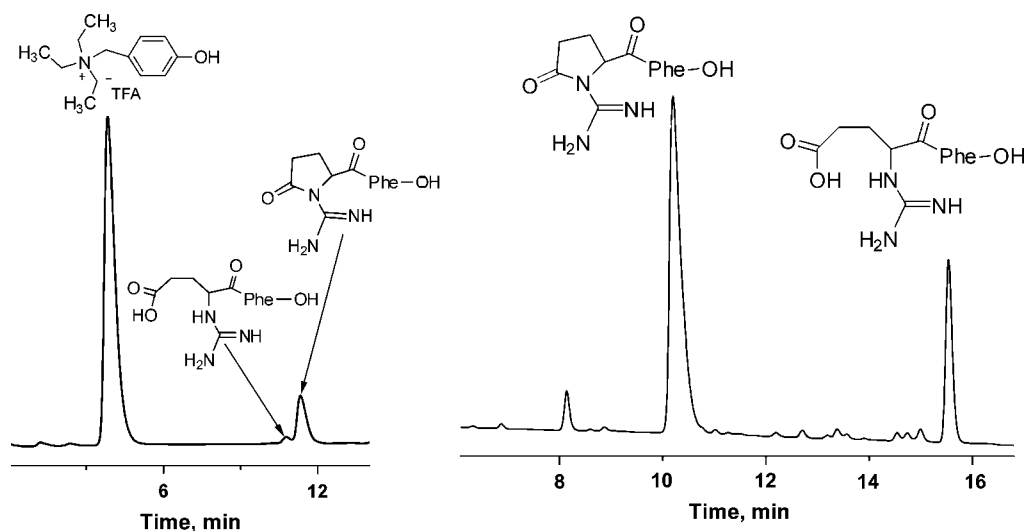
Figure 6. RP-HPLC separation of *N*-amidino-pyroglutamyl-phenylalanine and its precursor in TFA buffer (left) and ammonium formate buffer, pH 6 (right).

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