Branched Poly(proline) Peptides: An Efficient New Approach to the Synthesis of Repetitive Branched Peptides

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Proline is unique among the twenty genetically coded amino acids in that it contains both a cyclic backbone and a secondary α-amino group. These structural features impart unique stereochemical properties on proline. Poly(proline) oligomers exist in two distinct conformations in solution. In organic solvents they tend to adopt a conformation known as poly(proline) I (PPI), whereas in aqueous solvents they tend to adopt a different conformation known as poly(proline) II (PPII). We report here a new family of branched poly(proline) peptides. After a careful study of the different approaches, an efficient protocol for the synthesis of this kind of peptide has been established. *cis*-4-Amino-L-proline was mainly used as a branching unit in order to minimize its effects on the conformation of the rest of the molecule. The synthetic strategy employed was based on a convergent solid-phase peptide synthesis methodology that gave crude peptides that were easy to purify. This approach provides a new method for the synthesis of other repetitive branched peptides, minimizing not only synthetic difficulties but also purification problems.

Introduction

The synthesis of branched peptides is an area that has attracted a great deal of interest. These compounds have been used in a wide variety of important applications ranging from multiple antigenic peptide systems[1] to gene carriers.[2]

Proline is unique among the 20 common amino acids in that it bears a side-chain that is cyclized onto the backbone nitrogen atom. The presence of this structure means that the conformation of proline itself is limited. An important feature of poly(proline) (PP) sequences is that they can adopt two clearly different structures in solution. Thus, PP sequences tend to form either the PP II helix in water, which is an extended structure with three proline residues per turn, or the PP I helix in *n*-propanol, which is a less extended structure with 3.3 proline residues per turn.[3,4]

Repetitive sequences based on a single amino acid represent a challenge to peptide chemists due to the inherent synthetic difficulties related to the packing of the growing chain.[5] Furthermore, purification is also a complicated task because all peptides lacking one or more amino acids (deletion peptides) are closely related structurally to the target peptide, and therefore their presence in the final crude peptide mixture can make the purification process very difficult.[6]

One of the major areas of our research program is the study of peptide dendrimers based on PP helices in order to obtain biocompatible dendrimers for drug-delivery applications and, in this respect, we report here the synthesis of branched PP peptides.

Bearing in mind the fact that at least four proline residues are required to obtain a PP II helix,[7] the length of the different peptide segments was fixed to six amino acids. *cis*-4-Amino-L-proline (Amp) (Figure 1) was mainly used as a branching unit in order to maintain structural coherence with the rest of the sequence. Furthermore, three other non-DNA encoded amino acids were used: diaminoacetic acid (Daaa), L-diaminopropionic acid (Dapa) and L-diaminobutyric acid (Daba) (Figure 1). In order to obtain an efficient, neat and rapid synthesis, a solid-phase strategy based on either Pam or Merrifield resin was chosen in conjunction with the two orthogonal protecting groups Boc and Fmoc for the amino functions of the branching unit.

Results and Discussion

Synthesis and Purification of Branched Poly(proline) Peptides

First Approach

(For a list of the abbreviations used in this and the following sections see the beginning of the Exp. Sect.)

The first approach used in the preparation of branched PP peptides (Figure 2) was based on a combination of stepwise and convergent solid-phase peptide synthesis.[8] The

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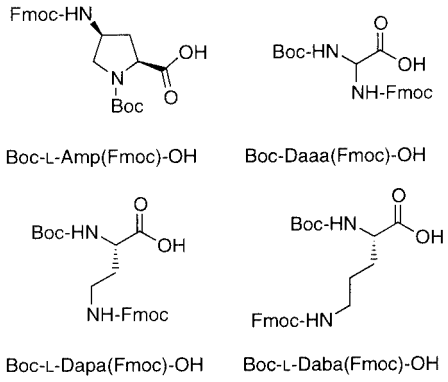

Figure 1. Structure of branching units

first step in this route involved the preparation of the protected segment Fmoc-Pro_5-OH using a hydroxymethyl resin. The first amino acid, Boc-Pro-OH, was incorporated with DIPCDI and catalytic amounts of DMAP in DCM, double coupling for 1 h each time. The incorporation of the third amino acid in poly(proline) peptides is problematic because the sequence Pro-Pro is prone to give diketopiperazines (DKPs).[9] In order to avoid the intramolecular cyclization the third Boc-Pro-OH was introduced by an in situ neutralization approach with DIEA, with BOP/HOBt or PyBOP/HOBt as coupling reagents in DMF.[10] The remaining proline residues were assembled by standard Boc couplings with DCC in DCM, with the exception of the last residue, which was introduced as Fmoc-Pro-OH using DIPCDI/HOBt in DMF. The chloranil test[11] was used to monitor the progress of the couplings. Cleavage of the protected peptide from the resin was carried out by HF acidolysis with 10% anisole. The crude peptide was purified by reversed-phase MPLC and its purity was confirmed by RP-HPLC and MALDI-TOF.

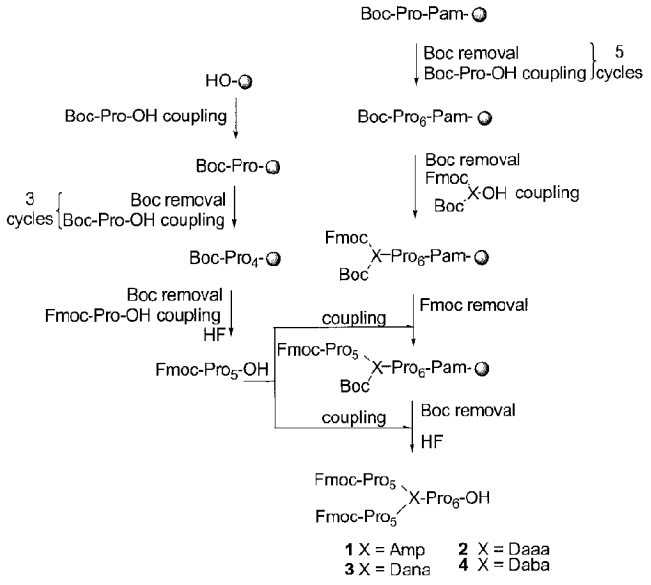

Figure 2. First approach to the synthesis of branched PP peptides

For the second step in the preparation of the PP branched peptide, a Boc-hexaproline resin was synthesized using a Boc-Pro-Pam solid support and an in situ neutralization approach for the third proline residue.[10] In order to achieve greater control of the branched peptide synthesis, the branching unit was introduced as an orthogonally protected Boc-L-Amp(Fmoc)-OH by means of a DIPCDI/HOBt-mediated coupling.

Fmoc-Pro_5-OH was incorporated in both branches using 1.5 equivalents of reagents. This process was carried out by first removing the Fmoc group of the Amp residue, coupling of the first fragment and capping of the possible remaining amino groups with Ac_2O. This stage was followed by removal of the Boc group, coupling of the second fragment and capping with benzoyl chloride. Success proved elusive after several attempts at coupling using DIPCDI/HOAt in DCM at 25 °C, and therefore a systematic study was carried out with the aim of obtaining the optimal conditions for coupling the fragments. Selective capping, as mentioned above, with Ac_2O and benzoyl chloride allowed us to quantify the extent of the condensation reactions at the two different amino positions by HPLC. Synthesis of standards for the side products was carried out using chemistry similar to that described above. Fragment couplings were explored using different coupling mixtures (Table 1) [e.g. DIPCDI/HOAt, DIPCDI/HOOBt, DIPCDI/HOBt and PyAOP/DIEA (single and double additions)], different solvents [DCM, DMF, $CHCl_3$/TFE (3:1), DMSO/toluene (1:3)] and several temperatures (−20 °C, 25 °C and 60 °C). It was found that DMF was a better solvent than DCM, mixtures of $CHCl_3$/TFE (3:1)[12] or DMSO/toluene (1:3).[13] Although the degrees of condensation at 60 °C were similar to their analogues at 25 °C, larger amounts of side products were observed at the higher temperature. The best results were obtained by using DIPCDI/HOAt in DMF at 25 °C for the first fragment coupling, followed by the phosphonium reagent PyAOP[14] with DIEA in DMF at 25 °C for the second fragment coupling. Although a 34% yield of the desired compound was obtained the crude peptide proved to be difficult to purify by chromatographic techniques (Figure 3).

Taking into account the rigidity of the pyrrolidine ring in the Amp residue, the other branching units, which are more flexible, were also tested: Boc-Daaa(Fmoc)-OH, Boc-L-Dapa(Fmoc)-OH, Boc-L-Daba(Fmoc)-OH. Similar results were obtained in these studies, showing that the difficulty of the couplings is not directly related to the presence of the Amp pyrrolidine ring.

Second Approach

The second approach to the synthesis of the PP branched peptide (Figure 4) involved the preparation of a Boc-L-Amp(Fmoc)-hexaproline peptide resin using the methodology described for the first approach. In order to decrease the steric hindrance at the α-position of the Amp residue, the Boc group was removed and a Boc-Pro-OH unit was incorporated. Removal of the Fmoc group and incorpora-

Table 1. Reaction conditions explored and extents of the segment condensation reactions quantified by RP-HPLC using the first synthetic approach; SP 1: Benzoyl-L-Amp(Ac)-Pro_6-OH; SP 2: Fmoc-Pro_5-L-Amp(Ac)-Pro_6-OH; SP 3: Benzoyl-L-Amp(Fmoc-Pro_5)-Pro_6-OH

Coupling Method	Sub Product 1 (SP 1)	Sub Product 2 (SP 2)	Sub Product 3 (SP 3)	Product (P)
DIPCDI/HOAt, DCM, 25 °C	0%	46%	43%	11%
DIPCDI/HOAt, DMF, 25 °C	3%	23%	55%	19%
PyAOP/DIEA, DCM, 25 °C	7%	37%	50%	6%
PyAOP/DIEA, DMF, 25 °C	0%	17%	74%	11%
DIPCDI/HOOBt, $CHCl_3$/TFE (3:1), 25 °C	23%	16%	58%	3%
PyAOP/DIEA (double addition), DCM, 25 °C	9%	26%	59%	6%
PyAOP/DIEA (double addition), DMF, 25 °C	4%	38%	47%	11%
DIPCDI/HOAt, DMF, 25 °C + PyAOP/DIEA, DMF, 25 °C	0%	8%	58%	34%
DIPCDI/HOAt, DMF, 25 °C + PyAOP/DIEA, DMF, −20 °C	3%	40%	43%	14%
DIPCDI/HOAt, DMF, 25 °C + PyAOP/DIEA, DMF, 60 °C	0%	61%	9%	30%
DIPCDI/HOBt, DMSO/toluene (1:3), 25 °C	4%	30%	59%	7%
DIPCDI/HOBt, DMSO/toluene (1:3), 60 °C	0%	46%	43%	11%

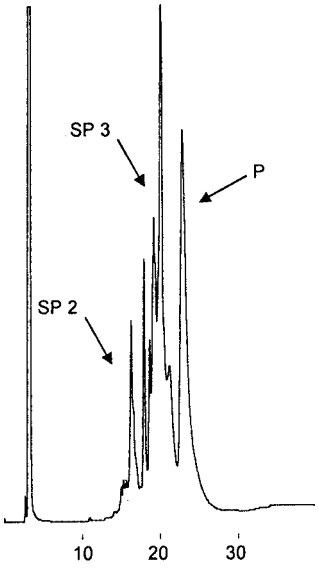

Figure 3. Example of an analytical RP-HPLC, on a C_4 column, of crude branched PP peptide using the first approach, Amp as branching unit, DIPCDI/HOAt and PyAOP/DIEA as coupling mixtures for first and second fragment couplings, respectively, in DMF at 25 °C; elution conditions: A: 0.045% TFA in H_2O; B: 0.036% TFA in CH_3CN; linear gradient from 0% to 100% in 30 min; flow rate, 1 mL/min λ = 220 nm
Note: In other syntheses of the same series (PyAOP/DIEA, DCM, 25 °C), SP 1 appeared at around 9 minutes.

tion of the Fmoc-Pro_5-OH peptide, followed by removal of the Boc group and incorporation of the Fmoc-Pro_4-OH peptide, obtained in a similar way to Fmoc-Pro_5-OH, should have led to the target compound. Despite the fact that fragment couplings were carried out using experimental conditions optimized in the previously described synthetic approach (PyAOP/DIEA in DMF at 25 °C), satisfactory results were not obtained in this case.

Third Approach

The first and second approaches were based on the nucleophilic character of Amp amino functions in the fragment coupling reactions. In the third approach (Figure 5) the carbonyl group of the Amp residue was used as an electrophilic component in the key coupling. Thus, the synthesis of Fmoc-Pro_5-L-Amp(Fmoc-Pro_5)-OH (**5**) was started by incorporation of Boc-L-Amp(Fmoc)-OH onto a Merrifield resin, with DIPCDI and catalytic amounts of DMAP in DCM. Elongation of the chains was carried out simultaneously, apart from the first proline residue, using Boc-Pro-OH for all residues bar the last one, which employed Fmoc-

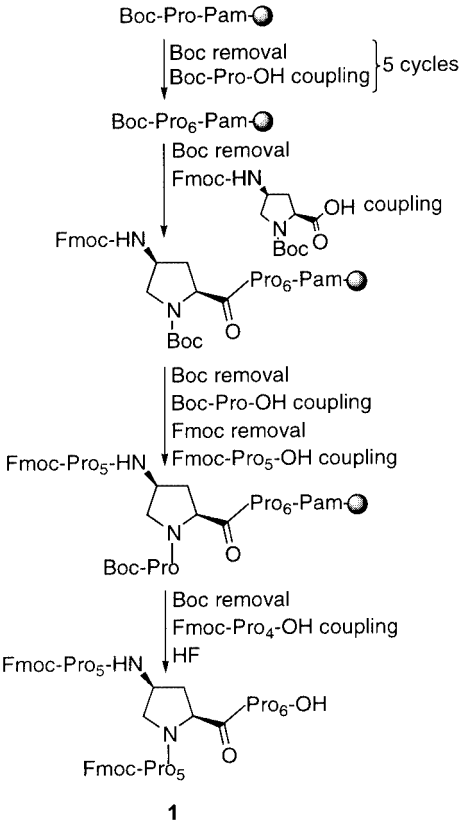

Figure 4. Second approach to the synthesis of branched PP peptide

Pro-OH. An in situ neutralization approach for the third position (second proline residue) of the peptide resin was used to avoid DKP formation.[10] The peptide was purified by semi-preparative RP-HPLC and purities were confirmed by RP-HPLC and MALDI-TOF mass spectrometry. A systematic study into the condensation of peptide **5** (Table 2), as described for fragment condensations in the first approach, was carried out by exploring different coupling reagents, reaction temperatures and using DMF as solvent. The extent of the condensation reaction was quantified by RP-HPLC. The best result was obtained in the condensation of peptide **5** with the hexaproline resin where the coupling conditions involved double addition of one of the most reactive phosphonium reagents, PyAOP,[14] and DIEA in DMF at 25 °C. Although the reaction extents were similar to those obtained for the first approach, in this case only two compounds — with remarkably different affinities to the chromatographic column — could be formed, a situation leading to crude peptides that were easy to purify (Figure 6).

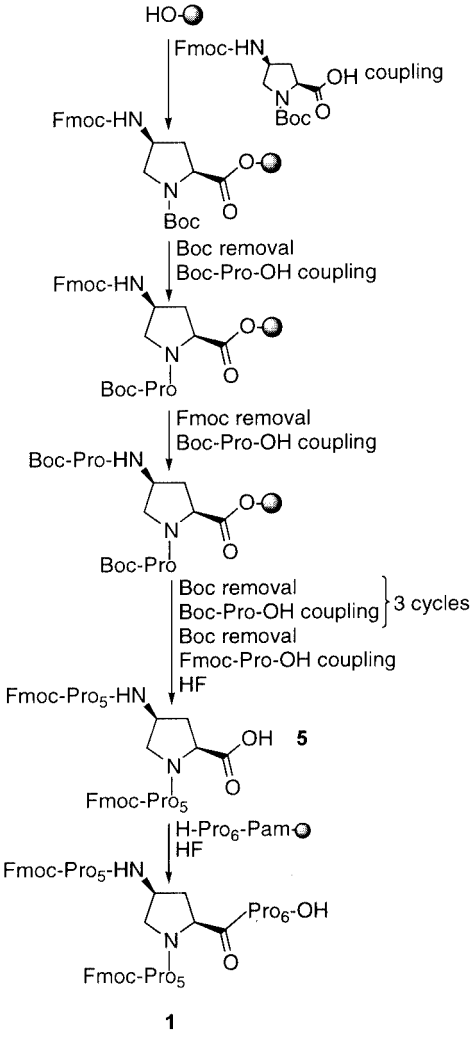

Figure 5. Third approach to the synthesis of branched PP peptide

In summary, the preparation of branched poly(proline) peptides was found to be very challenging. However, after

Table 2. Reaction conditions explored and extent of the segment condensation reaction quantified by RP-HPLC using the third synthetic approach. SP 4: Ac-Pro_6-OH

Coupling Method	Sub Product 4 (SP 4)	Product (P)
PyAOP/DIEA, DMF, 25 °C	89%	11%
PyAOP/DIEA (double addition), DMF, 25 °C	66%	34%
PyAOP/DIEA, DMF, 60 °C	93%	7%
DIPCDI/HOAt, DMF, 25 °C	72%	28%

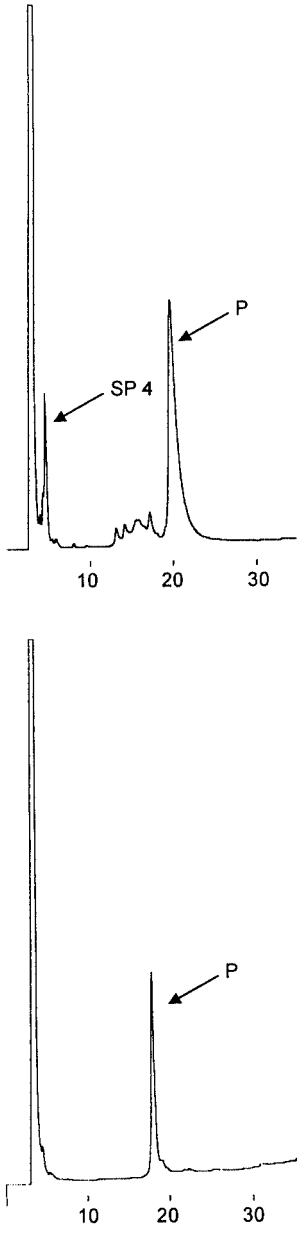

Figure 6. Analytical RP-HPLC, on a C_4 column, of crude (top) and pure (bottom) branched PP peptide, using the third approach and PyAOP/DIEA as coupling mixture in DMF at 25 °C elution conditions: A: 0.045% TFA in H_2O; B: 0.036% TFA in CH_3CN; linear gradient from 20% to 100% in 30 min; flow rate, 1 mL/min λ = 220 nm

a careful study of different approaches, an efficient protocol for the synthesis of this kind of peptide has been established. This strategy is based on a convergent solid-phase peptide synthesis methodology involving coupling of pure Fmoc-Pro_5-L-Amp(Fmoc-Pro_5)-OH to a hexaproline Pam resin with PyAOP/DIEA in DMF at 25 °C. This procedure gives rise to reasonable reaction extents and, more remarkably, to only two compounds, which have very different chromatographic properties. These chromatographic differences lead to a straightforward purification process that yields branched peptides of 98% purity.

Finally, this approach provides a new method for the synthesis of other repetitive branched peptides and demonstrates the suitability of the convergent solid-phase synthesis for the preparation of such peptides.

Experimental Section

Abbreviations: ACN: acetonitrile; Ac_2O: acetic anhydride; Amp:*cis*-4-amino-L-proline; Boc: *tert*-butoxycarbonyl; BOP: benzotriazol-1-yl-oxytris(dimethylamino)phosphonium hexafluorophosphate; Daaa: diaminoacetic acid; Daba: L-diaminobutyric acid; Dapa: L-diaminopropionic acid; DCC: *N,N′*-dicyclohexylcarbodiimide; DCM: dichloromethane; DIEA: *N,N*-diisopropylethylamine; DIPCDI: *N,N′*-diisopropylcarbodiimide; DKP: 2,5-diketopiperazine; DMAP: 4-dimethylaminopyridine; DMF: *N,N*-dimethylformamide; DMSO, dimethyl sulfoxide; Fmoc: 9-fluorenylmethoxycarbonyl; HOAc: acetic acid; HOAt: 1-hydroxy-7-azabenzotriazole; HOBt: 1-hydroxybenzotriazole; HOOBt: 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine; MALDI-TOF: matrix assisted laser desorption ionization, time of flight; MeOH: methanol; MPLC: medium pressure liquid chromatography; Pam: 4-hydroxymethylphenylacetic acid; PP: poly(proline); PyAOP: 7-azabenzotriazol-1-yl-oxytris(pyrrolidino)phosphonium hexafluorophosphate; PyBOP: benzotriazol oxytris(pyrrolidino)phosphonium hexafluorophosphate; RP-HPLC: reversed-phase high performance liquid chromatography; TFA: trifluoroacetic acid; TFE: 2,2,2-trifluoroethanol.

Materials and Equipment: Protected amino acids were obtained from Calbiochem-NovaBiochem AG (Läufelfingen, Switzerland) and Neosystem (Strasbourg, France). DIPCDI, DMAP, HOBt, HOOBt were obtained from Fluka Chemika (Buchs, Switzerland). BOP, PyBOP and solid supports for peptide synthesis were supplied by Calbiochem-NovaBiochem AG. HOAt was purchased from Applied Biosystems (Framingham, MA). These reagents were used without further purification. Solvents for peptide synthesis and RP-HPLC were obtained from Scharlau (Barcelona, Spain). Trifluoroacetic acid was supplied by KaliChemie (Bad Wimpfen, FRG). Other chemicals used were obtained from Aldrich (Milwaukee, WI, U.S.A.) and were of the highest purity commercially available. Peptide syntheses were performed manually in a polypropylene syringe fitted with a polyethylene disc. HF was obtained from Air Products and Chemicals, Inc. (Allentown, Canada) and the equipment from Peptide Institute Inc., Minoh, Osaka, Japan. Analytical RP-HPLC was performed using a Waters (Milford, MA, U.S.A.) or Shimadzu (Kyoto, Japan) chromatography system with a reversed-phase Nucleosil C_4 (0.4 × 25 cm, 10 μm) column. Semi-preparative RP-HPLC was performed using a Waters (Milford, MA, U.S.A.) chromatography system with a Vydac C_8 (1 × 25 cm, 10 μm) column. Compounds were detected by UV absorption at 220 nm. Reversed-phase MPLC was performed on a Lichroprep RP-8 column (44 × 3.7 cm; 40−60 μm) using a LDC/Milton Roy pump, a variable wavelength detector and an automatic fraction collector. Mass spectra were recorded on a MALDI Voyager DE RP time of-flight (TOF) spectrometer (Applied Biosystems, Framingham). α-Cyano-4-hydroxycinnamic acid was used as matrix and was purchased from Aldrich.

General Procedures

Coupling of the First Amino Acid onto a Hydroxymethyl Resin: (1) Amino acid (3 equiv.), DIPCDI (3 equiv.) and DMAP (0.3 equiv.) in DMF (2 × 1 h); (2) DMF (4 × 1 min); (3) DCM (4 × 1 min). The possible remaining hydroxyl groups on the resin were blocked by acetylation with HOAc (5 equiv.), DIPCDI (5 equiv.) and DMAP (0.5 equiv.) in DCM (1 × 20 min).

Boc Group Removal (Except for the Second Proline Residue): (1) DCM (4 × 1 min); (2) 40% TFA/DCM (1 × 1 min + 2 × 15 min); (3) DCM (4 × 1 min); (4) 5% DIEA/DCM (4 × 3 min); (5) DCM (4 × 1 min).

Incorporation of Boc-Pro-OH (Except for the Third Proline Residue): (1) Boc-Pro-OH (5 equiv.) and DCC (5 equiv.) in DCM (1 × 2 h); (2) DCM (4 × 1 min); (3) DMF (4 × 1 min).

In Situ Neutralization Coupling of Boc-Pro-OH: (1) Boc-Pro-OH (5 equiv.), DIEA (10 equiv.), BOP (5 equiv.) or PyBOP (5 equiv.) and HOBt (5 equiv.) in DMF (1 × 2 h); (2) DMF (4 × 1 min); (3) DCM (4 × 1 min).

Incorporation of Fmoc-Pro-OH: (1) Fmoc-Pro-OH (5 equiv.), DIPCDI (5 equiv.) and HOBt (5 equiv.) in DMF (1 × 2 h); (2) DMF (4 × 1 min); (3) DCM (4 × 1 min).

Fmoc Group Removal: (1) DMF (4 × 1 min); (2) 20% piperidine/DMF (1 × 1 min + 2 × 10 min); (3) DMF (4 × 1 min).

Incorporation of Branching Units (First and Second Approach): (1) Boc-Xxx(Fmoc)-OH (2.5 equiv.) and DCC (2.5 equiv.) in DCM (1 × 2 h); (2) DCM (4 × 1 min); (3) DMF (4 × 1 min).

Coupling Efficiencies: Monitored using the chloranil test for secondary amines and the ninhydrin test for primary amines.

Capping with Acetic Anhydride: Ac_2O (240 μL, 5 mmol) and DIEA (425 μL, 5 mmol) in DCM (1 × 15 min).

Capping with Benzoyl Chloride: Benzoyl chloride (230 μL, 2 mmol) and DIEA (33 μL, 0.2 mmol) in DMF (1 × 15 min).

Acidolytic Cleavage with HF: The peptide resin was washed with methanol (3 × 1 min + 1 × 15 min), dried and treated with HF in the presence of 10% anisole for 1 h at 0 °C. The crude peptide was precipitated with anhydrous diethyl ether, dissolved in HOAc and lyophilized.

Synthesis and Purification of Fmoc-Pro_5-OH and Fmoc-Pro_4-OH: This synthesis was carried out on a hydroxymethyl resin (2.0 g, 0.9 mmol/g) following the methodology described in the general procedure section. The peptide resin (250 mg) was cleaved with HF. The crude peptide Fmoc-Pro_5-OH was purified by MPLC using 20% ACN in H_2O (containing 1% TFA) and 70% ACN in H_2O (containing 1% TFA). The purity of the fractions was verified by analytical RP-HPLC and indicated a peptide purity of 98%. MALDI [M + H]: calcd. 726.7; found 726.6. Purification was not required for Fmoc-Pro_4-OH; the crude peptide was 99% pure by analytical RP-HPLC. MALDI [M + H]: calcd. 629.6; found 629.5.

Synthesis of Boc-Pro$_6$-Resin: Amino acids were incorporated on a Boc-Pro-Pam resin (1.0 g, 0.5 mmol/g) following the methodology described in the general procedure section. In order to assess the purity of the peptide a small amount of the peptide resin was cleaved with HF, providing a crude peptide that was 95% pure by analytical RP-HPLC. MALDI [M + H]: calcd. 601.6; found 601.5.

Synthesis of Fmoc-Pro$_5$-L-Amp(Fmoc-Pro$_5$)-Pro$_6$-OH (First Approach): Boc-L-Amp(Fmoc)-Pro$_6$-Pam resin (1 g, 0.5 mmol/g) was synthesized following the protocol described in the general procedure section. The optimal conditions for the peptide fragment condensations were obtained by studying 40 mg aliquots of the orthogonally protected peptide resin. After Fmoc removal, the first Fmoc-Pro$_5$-OH (26 mg, 1.5 equiv.) was coupled. An acetylation reaction was then carried out with Ac_2O. The Boc group was removed and, after washing, the second Fmoc-Pro$_5$-OH (26 mg, 1.5 equiv.) was coupled and the peptide resin was capped with benzoyl chloride. Conditions explored: (1) DIPCDI (4 μL, 1.5 equiv.), HOAt (4 mg, 1.5 equiv.) in DCM (1 × 3 h) at 25 °C. (2) DIPCDI (4 μL, 1.5 equiv.), HOAt (4 mg, 1.5 equiv.) in DMF (1 × 3 h) at 25 °C. (3) PyAOP (14 mg, 1.5 equiv.), DIEA (5 μL, 1.5 equiv.) in DCM (1 × 3 h) at 25 °C. (4) PyAOP (14 mg, 1.5 equiv.), DIEA (5 μL, 1.5 equiv.) in DMF (1 × 3 h) at 25 °C. (5) DIPCDI (4 μL, 1.5 equiv.), HOOBt (4 mg, 1.5 equiv.) in $CHCl_3$/TFE (3:1) (1 × 3 h) at 25 °C. (6) PyAOP (14 mg, 1.5 equiv.), DIEA (5 μL, 1.5 equiv.) (double addition) in DCM (1 × 3 h) at 25 °C. (7) PyAOP (14 mg, 1.5 equiv.), DIEA (5 μL, 1.5 equiv.) (double addition) in DMF (1 × 3 h) at 25 °C. (8) DIPCDI (4 μL, 1.5 equiv.), HOAt (4 mg, 1.5 equiv.) in DMF (1 × 3 h) at 25 °C for the first fragment and PyAOP (14 mg, 1.5 equiv.), DIEA (5 μL, 1.5 equiv.) in DMF (1 × 3 h) at 25 °C for the second fragment. (9) DIPCDI (4 μL, 1.5 equiv.), HOAt (4 mg, 1.5 equiv.) in DMF (1 × 3 h) at 25 °C for the first fragment and PyAOP (14 mg, 1.5 equiv.), DIEA (5 μL, 1.5 equiv.) in DMF (1 × 3 h) at −20 °C for the second fragment. (10) DIPCDI (4 μL, 1.5 equiv.), HOAt (4 mg, 1.5 equiv.) in DMF (1 × 3 h) at 25 °C for the first fragment and PyAOP (14 mg, 1.5 equiv.), DIEA (5 μL, 1.5 equiv.) DMF (1 × 3 h) at 60 °C for the second fragment. (11) DIPCDI (4 μL, 1.5 equiv.), HOBt (4 mg, 1.5 equiv.) in DMSO/toluene (1:3) (1 × 3 h) at 25 °C. (12) DIPCDI (4 μL, 1.5 equiv.), HOBt (4 mg, 1.5 equiv.) in DMSO/toluene (1:3) (1 × 3 h) at 60 °C. Peptide resins were cleaved with HF. The reaction extents were quantified by analytical RP-HPLC and are summarized in Table 1.

Synthesis of Fmoc-Pro$_5$-L-Xxx(Fmoc-Pro$_5$)-Pro$_6$-OH (Xxx = Daaa, Dapa, Daba): Fmoc-L-Xxx(Boc)-Pro$_6$-Pam resin (100 mg, 0.5 mmol/g) was synthesized using the methodology described in the general procedure section. Coupling of the two Fmoc-Pro$_5$-OH peptides (68 mg, 1.5 equiv.) was carried out using PyAOP (39 mg, 1.5 equiv.), DIEA (15 μL, 1.5 equiv.) in DCM (1 × 3 h) at 25 °C following the protocol described for the previous synthesis. Acidolytic cleavages were carried out with HF. Crude peptides were analyzed by analytical RP-HPLC and MALDI and the compounds had purities no higher than 30% by analytical RP-HPLC.

Synthesis of Fmoc-Pro$_5$-L-Amp(Fmoc-Pro$_5$)-Pro$_6$-OH (Second Approach): Boc-L-Amp(Fmoc)-Pro$_6$-Pam resin (55 mg, 0.5 mmol/g) was synthesized following the protocol described in the general procedure section. After Boc removal, Boc-Pro-OH was coupled, the Fmoc group was removed and Fmoc-Pro$_5$-OH (33 mg, 1.5 equiv.) was coupled with PyAOP (20 mg, 1.5 equiv.), DIEA (7 μL, 1.5 equiv.) in DCM (1 × 3 h) at 25 °C. An acetylation reaction was then carried out. The Boc group was removed, Fmoc-Pro$_4$-OH (29 mg, 1.5 equiv.) was coupled with PyAOP (20 mg, 1.5 equiv.), DIEA (7 μL, 1.5 equiv.) in DCM (1 × 3 h) at 25 °C and the peptide resin was capped with benzoyl chloride. Acidolytic cleavage with HF was carried out. The crude peptide was analyzed by analytical RP-HPLC and MALDI and the compound had a purity no higher than 40% by analytical RP-HPLC.

Synthesis and Purification of Fmoc-Pro$_5$-L-Amp(Fmoc-Pro$_5$)-OH: The first Pro residue was incorporated on a hydroxymethyl resin (2.0 g, 0.9 mmol/g) as Boc-L-Amp(Fmoc)-OH using the methodology described in the general procedure section. After Boc removal, Boc-Pro-OH was coupled, the Fmoc group was removed, and another Boc-Pro-OH was coupled. The desired branched peptide was obtained by simultaneous introduction of two Boc-Pro-OH units at every step by following the program sequence described in the general procedure section. The final amino acid for both chains was introduced as an Fmoc-Pro-OH. The peptide was cleaved from the resin with HF and then lyophilized. The crude peptide was purified by semi-preparative RP-HPLC using a linear gradient of ACN (containing 1% TFA) and H_2O (containing 1% TFA). The purity of each of the fractions was verified by analytical RP-HPLC and the final peptide was 98% pure. MALDI: calcd. [M + H] 1546.5; found [M + Na] 1567.9.

Synthesis of Fmoc-Pro$_5$-L-Amp(Fmoc-Pro$_5$)-Pro$_6$-OH (Third Approach): Fmoc-Pro$_5$-L-Amp(Fmoc-Pro$_5$)-OH (72 mg, 1.5 equiv.) was incorporated on a Boc-Pro$_6$-resin (50 mg, 0.5 mmol/g), in which the Boc group had previously been removed. The peptide resin was then capped with Ac_2O. Several sets of conditions were explored: (1) PyAOP (20 mg, 1.5 equiv.), DIEA (7 μL, 1.5 equiv.) in DMF (1 × 3 h) at 25 °C. (2) PyAOP (20 mg, 1.5 equiv.), DIEA (7 μL, 1.5 equiv.) (double addition) in DMF (1 × 3 h) at 25 °C. (3) PyAOP (20 mg, 1.5 equiv.), DIEA (7 μL, 1.5 equiv.) in DMF (1 × 3 h) at 60 °C. (4) DIPCDI (5 μL, 1.5 equiv.), HOAt (5 mg, 1.5 equiv.) in DMF (1 × 3 h) at 25 °C. Acidolytic cleavages were carried out with HF. Reaction extents were quantified by analytical RP-HPLC and are summarized in Table 2. The crude peptide for experiment (2) was purified by semi-preparative RP-HPLC using a linear gradient of ACN (containing 1% TFA) and H_2O (containing 1% TFA). The purity of each of the fractions was verified by analytical RP-HPLC and the final peptide was 98% pure. MALDI: calcd. [M + H] 2129.1; found [M + Na] 2151.3.

Synthesis of RP-HPLC Standards: Boc-L-Amp(Fmoc)-Pro$_6$-Pam resin (100 mg, 0.5 mmol/g) was split into three aliquots. SUB PRODUCT 1: the first aliquot was treated with 20% piperidine in DMF, acetylated, the Boc group removed, and capped with benzoyl chloride. SUB PRODUCT 2: the second aliquot was treated with 20% piperidine in DMF, acetylated, the Boc group removed and five Fmoc-Pro-OH units were attached in a step-wise manner. SUB PRODUCT 3: the third aliquot was treated with 40% TFA in DCM and capped with benzoyl chloride, the Fmoc group removed and five Fmoc-Pro-OH units were attached in a step-wise manner. SUB PRODUCT 4: this compound was synthesized by acetylation of an aliquot of Boc-Pro$_6$-resin (25 mg, 0.5 mmol/g), from which the Boc group had previously been removed. PRODUCT: the RP-HPLC standard Fmoc-Pro$_5$-L-Amp(Fmoc-Pro$_5$)-Pro$_6$-OH was obtained by purifying a small aliquot from one of the syntheses described in the previous section. Acidolytic cleavages with HF were carried out and crude peptides were purified by semi-preparative RP-HPLC to give standards of 96−98% purity. MALDI for Benzoyl-L-Amp-(Ac)-Pro$_6$-OH (SP 1): calcd. [M + H] 859.7; found [M + H] 859.2. MALDI for Fmoc-Pro$_5$-L-Amp(Ac)-Pro$_6$-OH (SP 2): calcd. [M + H] 1463.4; found [M + H] 1463.1. MALDI for Benzoyl-L-Amp(Fmoc-Pro$_5$)-Pro$_6$-OH (SP 3): calcd. [M + H] 1525.4; found [M + H] 1525.4. MALDI for Ac-Pro$_6$-OH (SP 4): calcd. [M + H] 643.6;

found [M + Na] 665.5. MALDI for Fmoc-Pro_5-L-Amp(Fmoc-Pro_5)-Pro_6-OH (P): calcd. [M + H] 2129.1; found [M + Na] 2151.1.

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