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ORIGINAL ARTICLE

Investigation on side-product formation during the synthesis of a lactoferrin-derived lactam-bridged cyclic peptide

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Abstract
Bovine lactoferrin C-lobe is able to prevent both influenza virus hemagglutination and cell infection. In particular, it was demonstrated that the fragment ^{418}SKHSSLDCVLRP^{429} is a potent antiviral peptide. Therefore, we tried to increase the stability of this fragment through side-chain lactam cyclization of the peptide, S[KHSSLD]CVLRP (**1**). However, classic strategy involving solid-supported cyclization of the linear precursor, containing orthogonal allyl/alloc-based protection for the key amino and carboxyl residues, did not provide the desired cyclic peptide. Here, we report the identification of problematic stretches during the sequence assembly process and the optimization of the different parameters involved in the construction of **1**. Results indicated a significant influence of β-protecting group of both aspartic acid and adjacent cysteine residues on the formation of side products. Therefore, the identification of suitable β-protecting groups of these residues allowed us to optimize the synthesis of designed lactam-bridged cyclic peptide.

Keywords Bovine lactoferrin · Solid-phase synthesis · Cyclic peptide · Aspartimide · Allyl ester · Side reactions

Introduction

Bovine lactoferrin (bLf) has a broad anti-influenza activity (Lönnerdal 1995; Van der Strate 2001). In particular, it was demonstrated that bLf C-lobe (418–429) fragment, ^{1}SKHSSLDCVLRP^{12}, is able to inhibit virus hemagglutination and infection of all major virus subtypes, including H1N1 and H3N2, at pico- to femto-molar concentration range (Ammendolia 2012). NMR conformational analysis of this fragment has highlighted global turn conformation, encompassing four β-turns (type IV) and a γ-turn structure (Scala 2017).

Accordingly, the stabilization of the peptide conformation by, for example, the cyclization of linear compounds can be considered a valid approach to the identification of more stable and selective compounds (Mosberg 1999; Camarero 2001; Davies 2003).

As evident from Fig. 1 (Scala 2017), the three β-turns formed by residues 2–5, 4–7, and 6–9, in the reference peptide, could be further stabilized through the formation of a side-chain lactam bridge between Lys^{2} and Asp^{7} residues (Ser-[Lys-His-Ser-Ser-Leu-Asp]-Cys-Val-Leu-Arg-Pro, **1**).

Side-chain lactam bridges linking separated amino acids that are spaced 4–7 residues apart in the linear sequence offer a convenient and flexible method for introducing conformational constraints or for improving the metabolic stability of a peptide (Houston 1995; Taylor 2002).

Solid-phase peptide synthesis (SPPS) is a convenient approach to prepare this type of constrained cyclic peptide (White 2011). In general, its preparation by Fmoc solid-phase methods requires a pair of selectively cleavable protecting groups on the amine and carboxylic acid side chains to be linked (Taylor 2002). Among these protecting groups, the Allyl ester (OAll) for carboxylic acid side chain along with its urethane-based partner for amine side chains, the Alloc group, are the most used. After linear assembly of

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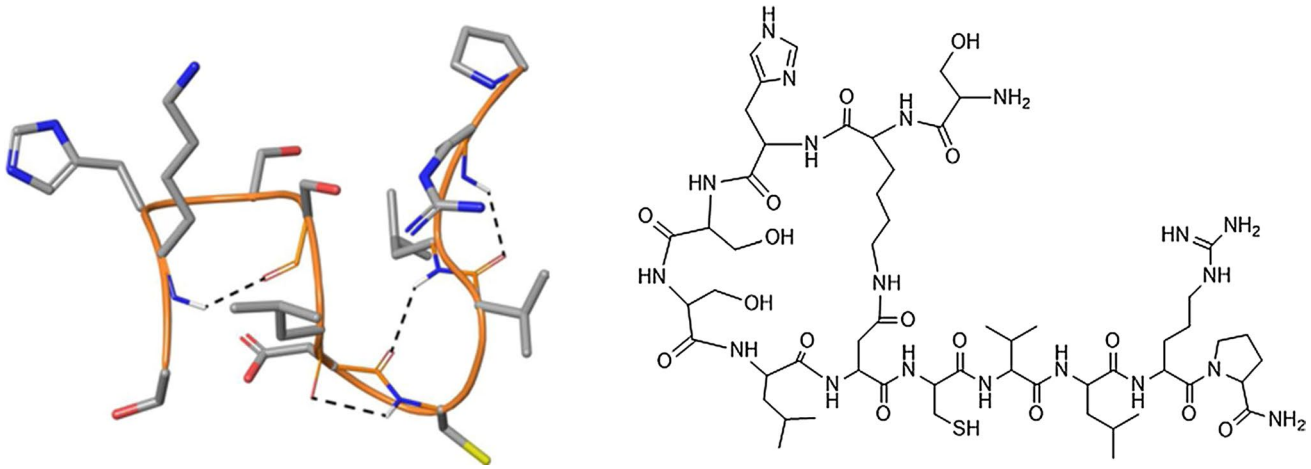

Fig. 1 NMR-derived structures of C-lobe (418–429) fragment (left) and the designed lactam peptide structure (**1**, right)

the full sequence, the OAll and Alloc groups are removed according to well-known protocols and the cyclization is carried out (Grieco 2001). However, this well-accepted protocol to the formation of side-chain lactam led to a major product with a mass difference of + 27 with respect to expected peptide, corresponding to piperidinyl adducts. This paper details the identification and quantification of the side products and their formation mechanisms, as well as the analysis of reaction conditions and optimization of a new protocol for the synthesis of the desired product.

Materials and methods

Chemicals

N^{α}-Fmoc-protected amino acids (Fmoc, fluorenylmethoxycarbonyl), Fmoc-Rink-amide-AM resin, 2-chlorotrityl-chloride resin, *N*-hydroxy-benzotriazole (HOBt), 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluoro-phosphate (HBTU), *N*,*N*-diisopropylethyl-amine (DIEA), piperidine, morpholine, piperazine, triisopropylsilane (TIS), and trifluoroacetic acid (TFA) were purchased from Iris Biotech (Germany). Fmoc-PAL-PEG-PS resin (Applied Biosystems) was purchased from Thermo Fischer Scientific (Italy). Peptide synthesis solvents, as well as CH_3CN for high-performance liquid chromatography (HPLC) were reagent grade and were acquired from commercial sources and used without further purification unless otherwise noted.

Peptide synthesis

Method A: (peptide **2**, peptides **7**–**14**). The synthesis of peptides was performed according to the solid-phase approach using standard Fmoc methodology in a manual reaction vessel (Stewart 1984; Atherton 1989).

The first amino acid was linked on Fmoc-Rink-amide resin (100–200 mesh, 1% DVB, 0.59 mmol/g) or Fmoc-PAL-PEG-PS resin (100–200 mesh, 1% DVB, 0.22 mmol/g) previously deprotected by a 25% piperidine solution in *N*, *N*-dimethylformamide (DMF, 5 min and 30 min).

The following protected amino acids were then added stepwise: N^{α}-Fmoc-Pro-OH, N^{α}-Fmoc-Arg(Pbf)-OH, N^{α}-Fmoc-Leu-OH, N^{α}-Fmoc-Val-OH, N^{α}-Fmoc-Cys(Trt)-OH or N^{α}-Fmoc-Cys(tBu)-OH, N^{α}-Fmoc-Asp(OAll)-OH or N^{α}-Fmoc-Asp(ODmab)-OH or N^{α}-Fmoc-Asp(OtBu)-OH or N^{α}-Fmoc-Asp(2-PhiPr)-OH, N^{α}-Fmoc-Ala-OH, N^{α}-Fmoc-Leu-OH, N^{α}-Fmoc-Ser(tBu)-OH, N^{α}-Fmoc-His(Trt)-OH, N^{α}-Fmoc-Lys(All)-OH or N^{α}-Fmoc-Lys(Mmt)-OH.

Each coupling reaction was accomplished using a threefold excess of amino acid with HBTU and HOBt in the presence of DIEA (6 equiv). The peptide resin was washed with dichloromethane (DCM, 3×, DMF (3×) and DCM (3×) and the Fmoc deprotection protocol, described above, was repeated after each coupling step. The Fmoc removal was accomplished by treating the protected peptide resin with a 25% solution of piperidine in DMF (5 min and 25 min) or 25% piperazine/DMF, 25% morpholine/DMF, and 25% piperidine/DMF + 0.1 M HOBt (as shown in Table 1). In addition, after each step of deprotection and after each coupling step, Kaiser test was performed to confirm the complete removal of the Fmoc protecting group and to verify that complete coupling has occurred on all the free amines on the resin. After peptide assembling, the N-terminal Fmoc group was removed. After peptide assembling, the N-terminal Fmoc group was removed and peptides were released from the resin using a cleavage mixture containing 90% TFA, 5% TIS, and 5% H_2O for 3 h. The resin was removed

Table 1 Influence of the solid support and base on the amount of by-products formed during the synthesis of Asp(OAll)-Cys-Val-Leu-Arg-Pro

Entries	Type of resin	Fmoc deprotection (for 5 + 25 min)	By-product[a]
a	Rink amide	25% piperidine/DMF	4
b	Fmoc-PAL-PEG-PS	25% piperidine/DMF	4
c	2-Chlorotrityl-chloride	25% piperidine/DMF	4
d	Rink amide	25% Piperazine/DMF	5
e	Rink amide	25% Morpholine/DMF	6
f	Rink amide	25% piperidine/DMF + HOBt 0.1 M	4

[a]All by-products were formed in quantitative yield after 3 h base treatment

by filtration, and the crude peptide was recovered by precipitation with cold anhydrous ethyl ether to give a white powder and then lyophilized.

Method B: (peptide **2** on 2-Chlorotrityl-chloride resin). Peptide was synthesized using a 2-chlorotrityl-chloride resin (100–200 mesh, 1.8 mmol/g). The first amino acid, N^{α}-Fmoc-Pro-OH (3 equiv relative to resin capacity) was dissolved in dry DCM (approximately 10 mL per gram of resin). A small amount of DMF may be needed to achieve complete dissolution. This solution was added to the resin followed by 12 equiv (relative to resin capacity) of DIEA. The mixture was agitated for 60 min, and then, the resin was washed with (17/2/1) DCM/Methanol/DIEA (3×), DCM (×3), DMF (2×), and DCM (2×). Successively, Fmoc protective group was removed and synthesis was accomplished as previously described.

Synthesis of lactam peptide **1**: the corresponding linear peptide was synthesized as described above and the amino acids N^{α}-Fmoc-Asp(2-PhiPr)-OH and N^{α}-Fmoc-Lys(Mmt)-OH were used as lactam precursors. After linear assembly, the 2-PhiPr and Mmt groups were removed according to the following procedure: 200 mg of peptide resin was washed with dichloromethane (DCM) and a solution of 1% TFA/DCM was added. The reaction was allowed to proceed for 30 min. The peptide resin was washed with DCM (3×), DMF (3×), and DCM (4×). The macrocyclic lactam ring formation was mediated by the addition of HBTU (6 equiv), HOBt (6 equiv), and DIEA (12 equiv) for 2 h. The reaction was carried out under an inert atmosphere to avoid the oxidation of thiol group of cysteine. The process was repeated if necessary (Kaiser test used to monitor completion).

The N-terminal Fmoc group was removed as described above. Finally, the peptides were released from the resin with TFA/TIS/H_2O (90:5:5) for 3 h. The resin was removed by filtration, and the crude peptide was recovered by precipitation with cold anhydrous ethyl ether to give a white powder and then lyophilized.

Purification and characterization

All crude peptides were purified by RP-HPLC on a preparative C18-bonded silica column (Phenomenex Kinetex AXIA 100 Å, 100 × 21.2 mm, 5 µm) using a Shimadzu SPD 20A UV/VIS detector, with detection at 220 and 254 nm. The column was perfused at a flow rate of 15 mL/min with a linear gradients of CH_3CN (0.1% TFA) in water (0.1% TFA), from 5 to 90% over 20 min. Analytical purity and retention time (tr) of each peptide were determined using analytical HPLC performed on a Supelco, Ascentis express peptide C18 column (50 × 3.00 mm, 2.7 µm) with a flow rate of 0.800 mL/min using linear gradients of CH_3CN (0.1% TFA) in water (0.1% TFA), from 5 to 90% over 9 min.

All analogues showed > 97% purity when monitored at 220 nm. Homogeneous fractions, as established using analytical HPLC, were pooled and lyophilized. Peptides molecular weights were determined by ESI mass spectrometry and LC–MS in an LC–MS 2010 instrument fitted with Phenomenex, Ascentis express peptide C18 column (50 × 3.00 mm, 2.7 µm), eluted with a linear gradient from 5 to 90% B over 9 min, at a flow rate of 0.600 mL/min. Electro-spray ionization mass spectrometry (ESI–MS) analysis in positive ion mode was made using a Finnigan LCQ Deca ion trap instrument, manufactured by Thermo Finnigan (San Jose, CA, USA), equipped with the Excalibur software for processing the data acquired. The sample was dissolved in a mixture of water and methanol (50/50) and injected directly into the electro-spray source, using a syringe pump, which maintains constant flow at 5 mL/min. The temperature of the capillary was set at 220 °C.

Results and discussion

Sequence assembly of the lactam peptide Ser-c[Lys-His-Ser-Ser-Leu-Asp]-Cys-Val-Leu-Arg-Pro, **1** was performed with standard Fmoc peptide synthesis using α-allyl-protected aspartic acid residue on a rink-amide resin. Unfortunately, this approach failed completely. Therefore, we studied the stepwise elongation of the peptide and we observed the formation of side products for the first time after the Aspartic acid Fmoc deprotection with piperidine (25% v/v in DMF, 30 min). In fact, LC–MS analysis of the sequence H-Asp(OAll)-Cys-Val-Leu-Arg-Pro-NH_2 (**2**) revealed the formation of three compounds: desired compound (**2**), aspartimide (**3**), and piperidinyl derivative(s) (**4**) (Fig. 2).

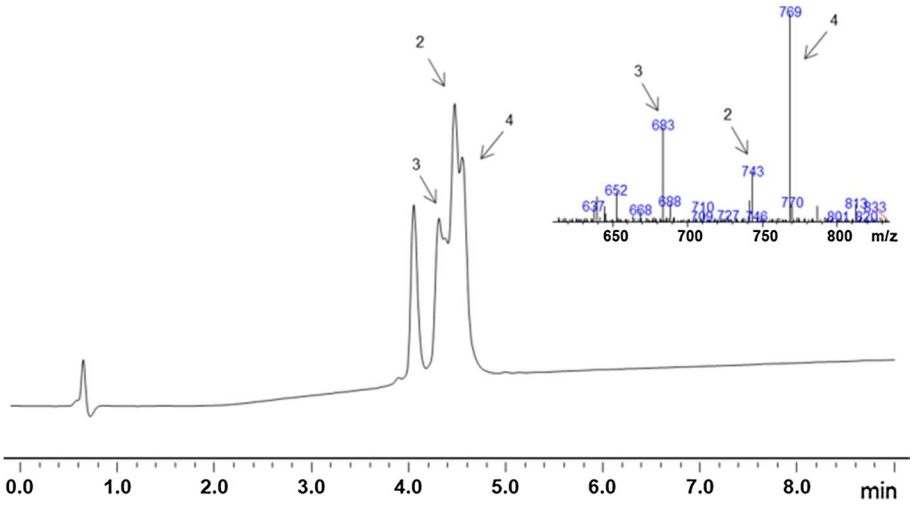

Fig. 2 LC-MS analysis of crude peptide DCVLRP, after cleavage with TFA/TES/H_2O (90:5:5 v/v, rt. 1.5 h), revealed the formation of three compounds: (**2**) desired product (calc. *m/z* = 743); (**3**) aspartimide-containing product (calc. *m/z* = 683); (**4**) piperidide-containing product (calc. *m/z* = 769). HPLC conditions: RP-C18, 5–90% CH_3CN in 0.1% TFA over 9 min, 0.6 ml min^{-1}

According to the literature (Dölling 1994; Yang 1994; Mergler 2003), our results could be interpreted as a spontaneous removal of allyl group from aspartic acid side chain, assuming an aspartimide formation followed by aminolysis with piperidine (Fig. 3). The reaction involves the nucleophilic attack of the nitrogen atom attached to the α-carboxylic group to the side-chain carboxyl group of aspartic acid. Subsequent nucleophilic attack of piperidine to the imide ring results in the formation of α- and β-piperidides. NMR analysis in $DMSO_{d6}$ solution clearly demonstrated the presence of two peptides which differed for the presence of an allyl or a piperidinyl (pip) group on the Asp residue (see supporting info). Considering the two possible α- or β-piperidides, relatively intense NOE correlations peaks between the signals of Hβ protons of Asp and Hα proton of Cys, and between the Hα proton of Asp and the

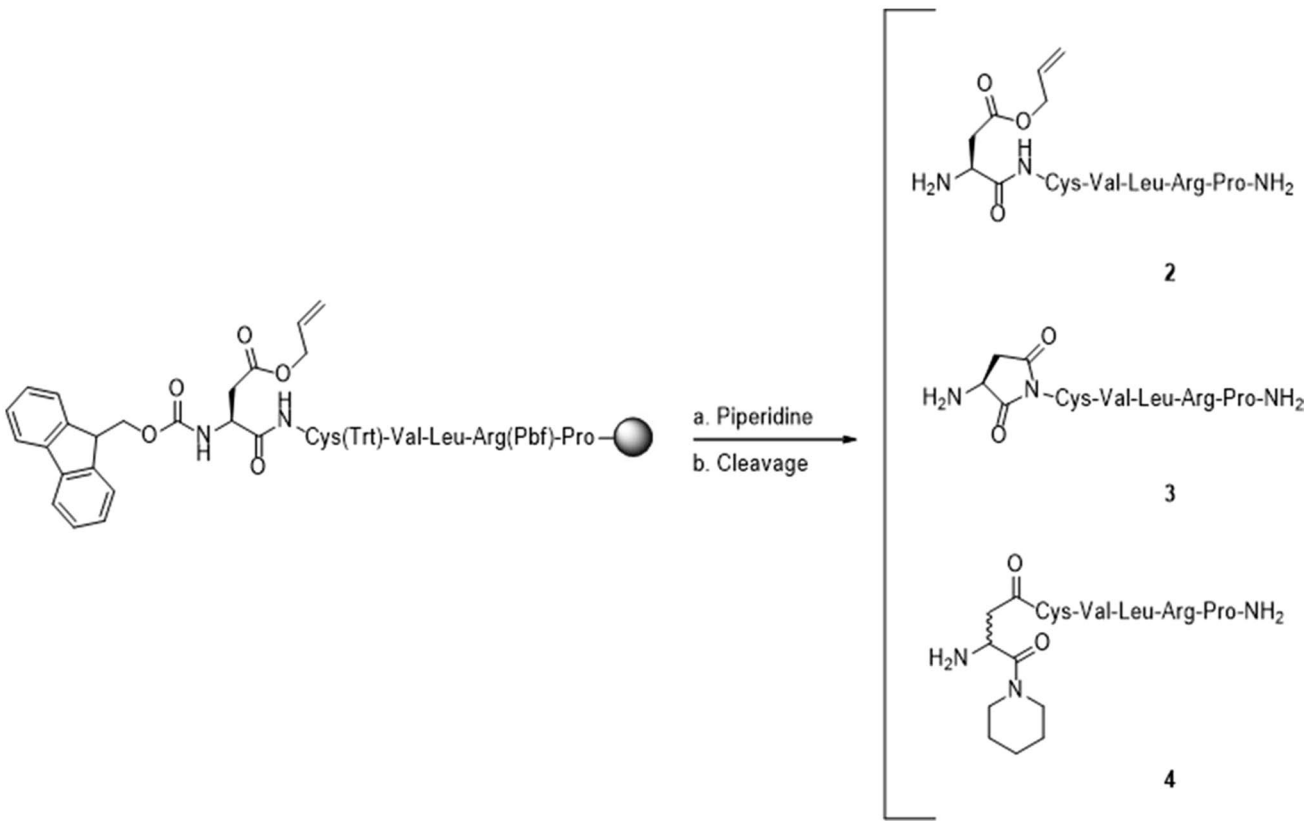

Fig. 3 Formation of side products. **a** Piperidine (25% v/v in DMF). **b** TFA/TES/H_2O (90:5:5 v/v, rt. 1.5 h)

piperidine proton signals (Fig. S1) demonstrate the presence of the α-piperidide by-product, while the β-isomer was not observed. Electron withdrawing effect of the terminal amine group on the α-carbonyl can explain the selective attack of the piperidine nitrogen on this group.

To minimize the aspartimide-related by-product formation, we studied the influence of resins, protecting groups, bases, and amino acid sequence on the side-product formation. To simulate deprotection conditions of prolonged synthesis, all peptides were incubated with piperidine (25%v/v in DMF) for 30 min, 1, 2, 3, 6, 12, and 16 h (Dölling 1994; Lauer 1995).

Factors influencing by-product formation

Solid support

It has been observed that the aspartimide impurity formation is also dependent on the polymeric support (Lukszo 1996; Subirós-Funosas 2011). Use of resin linkers such as Fmoc-PAL-PEG-PS helps to reduce the amount of secondary products. In this resin, chains of polyethylene glycol are grafted with the result that they move away from the functional groups of the resin core; hence, the reaction conditions become more "solution-like" (Forns and Fields 2000). It is also reported that the use of 2-chlorotrityl-chloride resin, which offers the possibility of mild acidolysis of the peptide chain, prevents this undesired cyclization (Ruczynski 2008; Dixon D2009). However, in our case, the uses of both solid supports were not successful (Table 1, entries b and c, respectively).

Base

The type of the secondary amine used in the Fmoc deprotection protocol also affects both the amount and the ratio of by-products (Dölling 1994; Yang 1994). Thus, we replaced the piperidine (pKa = 11.12) with the milder base piperazine (pKa = 9.73) and morpholine (pKa = 8.3), which could reduce aspartimide formation, even at the cost of the reaction rate. As observed in Table 1, the use of these bases was ineffective with the prevailing formation of side products, piperazine (Fig. 4, compound **5**, Table 1, entry d) and morpholine (Fig. 4 compound **6**, Table 1, entry e) derivatives.

The addition of *N*-hydroxylamine-based derivative, as hydroxybenzotriazole (HOBt), contributes to the wide arsenal of approaches to prevent the formation of aspartimide and derived by-products (Flora 2005; Michels 2012). The abstraction of the amide backbone proton has been proposed as the crucial step in the cyclization that leads to aspartimide. Thus, the addition of relative strong acid HOBt results in competition with the Asp-X amide backbone for the base present in the medium (Scheme 1). The effect of the HOBt should be the decrease of the percentage of negatively charged amide backbone nitrogen, which is responsible for initiating aspartimide derivative formation.

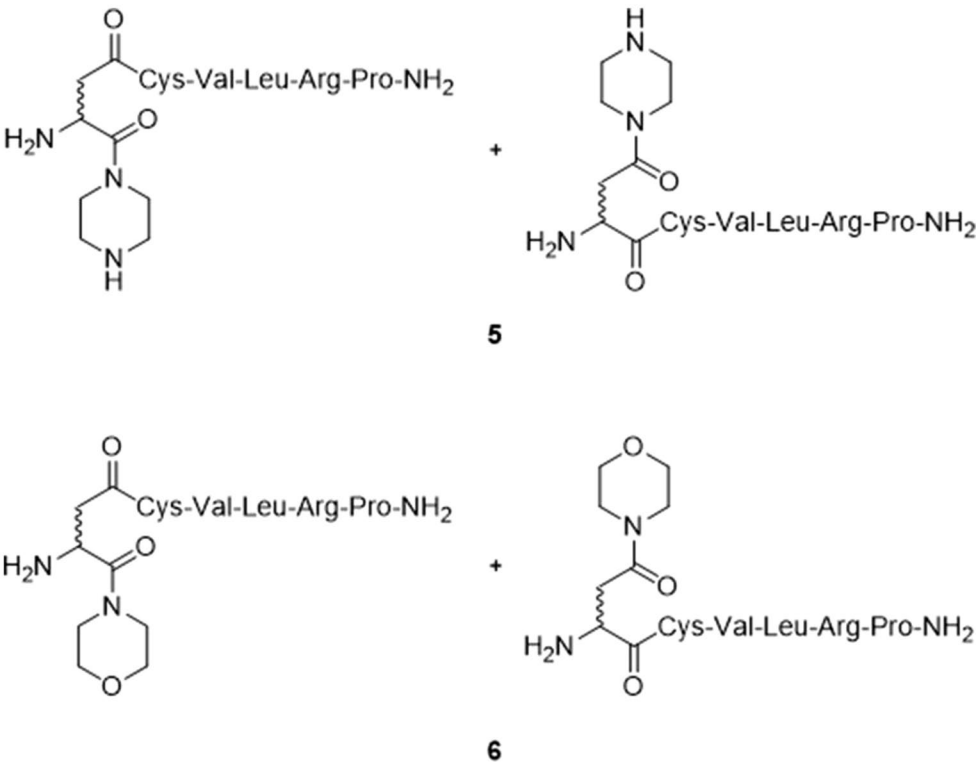

Fig. 4 Piperazine (**5**) and morpholine (**6**) derivatives formation

Scheme 1 Competition between N-hydroxylamine and amide backbone proton abstraction

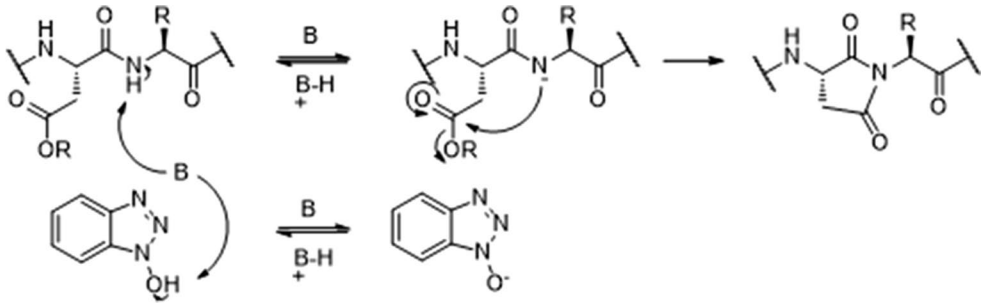

Table 2 Formation of piperdinyl derivatives during the synthesis of peptides **7**–**14**

Peptides	Sequence	Piperidinyl derivate
7	Asp(OAll)-Cys-Val-Leu-Arg-Ala-NH_2	++
8	Asp(OAll)-Cys-Val-Leu-Ala-Pro-NH_2	+++
9	Asp(OAll)-Cys-Val-Ala-Arg-Pro-NH_2	++
10	Asp(OAll)-Cys-Ala-Leu-Arg-Pro-NH_2	++
11	Asp(OAll)-Ala-Val-Leu-Arg-Pro-NH_2	++
12	Asp(OAll)-Leu-Pro-Arg-Val-Cys-NH_2	–
13	Asp(OAll)-Val-Arg-Pro-Cys-Leu-NH_2	–
14	Asp(OAll)-Pro-Cys-Val-Leu-Arg-NH_2	–

– no adduct;' +++ formed in quantitative yield after 3 h base treatment

However, in our case, the addition of HOBt to piperidine solution (0.1 M) did not avoid the formation of piperidinyl derivative (Table 1, entry f).

Sequence

The nature of the neighboring amino acid located at the C-terminus of the aspartic acid (Asp-X) and the amino acidic sequence can influence the aspartimide formation (Lauer 1995).

Initially, we decided to check the contribution of each residue to the by-product formation through an alanine scanning analysis (peptides **7**–**11**, Table 2). As described above, all peptides were synthesized on a rink-amide resin using allyl as aspartic acid protecting group and were incubated with piperidine at different times. As shown in Table 2, these changes did not show a significant effect to suppress aspartimide formation resulting, after 16 h piperidine treatment, in the formation of the piperidinyl adducts.

In addition to evaluate if the specific sequence, rather than the amino acid composition, is critical for by-product formation, we synthesized three scramble peptides (**12**–**14**, Table 2). These peptides, containing the three aliphatic residues of the original sequence (Ile, Val, and Pro) contiguous to the aspartic residue, could provide a steric and conformational impairment to the initial nucleophilic attack of the nitrogen atom to the side-chain carboxyl group of aspartic acid. As shown in Table 2, in these cases, the formation of side products was not observed (see supporting info), indicating that the conversion of Asp into aspartimide units could be conformation-dependent.

β-Carboxyl protecting group

Other influential factor in the formation of aspartimide-based side compounds is the nature of the β-carboxyl protecting group of Asp (Subirós-Funosas 2011). Thus, we analyzed the comportment of three Asp β- protecting group: two highly sterically hindered ODmab [4-{N-[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl] amino}benzyl] and 2-PhiPr (β-(2-phenylisopropyl) and the bulky but less voluminous tert-butyl group (Fig. 5).

As shown in Table 3, the β-protection of Asp as ODmab (Table 3, entry a) does not result in improved prevention of the side reactions and the results are comparable to that achieved by β-allyl ester protection (Table 1, entry a). The use of bulky tert-butyl (Table 3, entry b) and 2-PhiPr (Table 3, entries b and c, respectively) groups has shown greater efficacy than the above-mentioned protection strategies in preventing this side reaction in basic media. In fact, for entry b (Table 3), we have not observed the formation of aspartimide and piperidinyl derivatives that are formed in very small amounts for entry c (Table 3).

As above described we also assessed the influence of the protecting group on the side chain of the adjacent amino acid on the nucleophilic attack initiated by amide backbone nitrogen atom, using tert-butyl group and 2-PhiPr as protector groups of Cys and Asp, respectively (Table 3, entry d). In this case, this orthogonal protection increased the formation of side products. These results indicate a possible electronic interaction between the aromatic ring of Trt and 2-PhiPr favoring a steric hindrance able to avoid the initial aspartimide formation (Fig. 6).

Although the use of the tBu protecting group on the aspartic acid residue gave better results, this does not give us the possibility to have an orthogonal protection, so we carried out the synthesis of our linear peptide on a Rink-amide

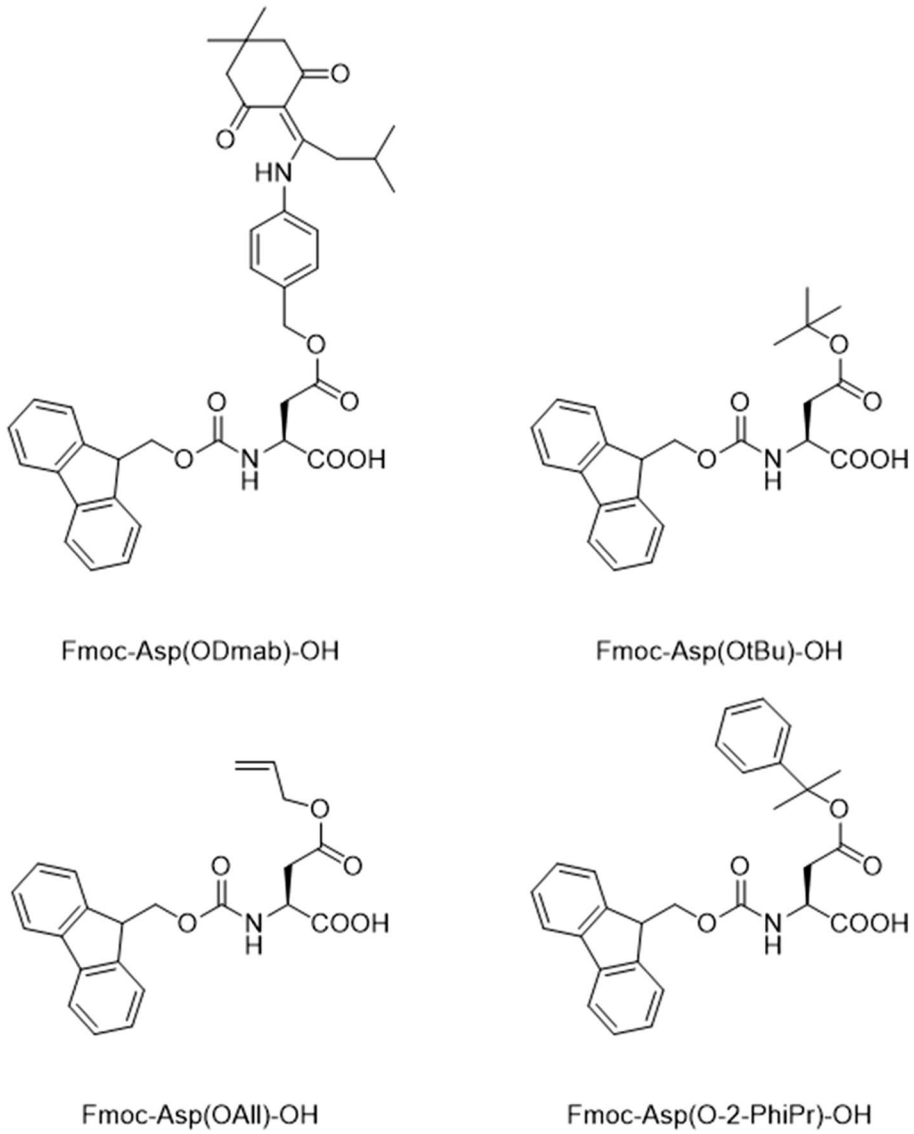

Fig. 5 Structure of Asp β-carboxyl protecting groups used in this work

Table 3 Influence of β-carboxyl protecting group on by-product formation

Entries	Protecting group of Asp	Protecting group of Cys	Type of resin	Fmoc deprotection (for 5 + 25 min)	By-product
a	ODmab	Trt	Rink amide	25% piperidine/DMF	+++
b	OtBu	Trt	Rink amide	25% piperidine/DMF	−
c	O2-PhiPr	Trt	Rink amide	25% piperidine/DMF	+
d	O2-PhiPr	tBu	Rink amide	25% piperidine/DMF	+++

− no adduct, +++ formed in quantitative yield after 3 h base treatment

resin using the amino acids N^{α}-Fmoc-Asp(2-PhiPr)-OH and N^{α}-Fmoc-Lys(Mmt)-OH as lactam precursors. After linear assembly, the 2-PhiPr and the Mmt groups were removed and the macrocyclic lactam ring formation was mediated. In these conditions, the peptide yield was 30%.

Conclusion

The synthesis of the lactam-bridged peptide, S[KHSSLD] CVLRP proved to be difficult yielding by-products such as aspartimide and α-piperidinyl derivatives according to LC–MS and NMR analyses.

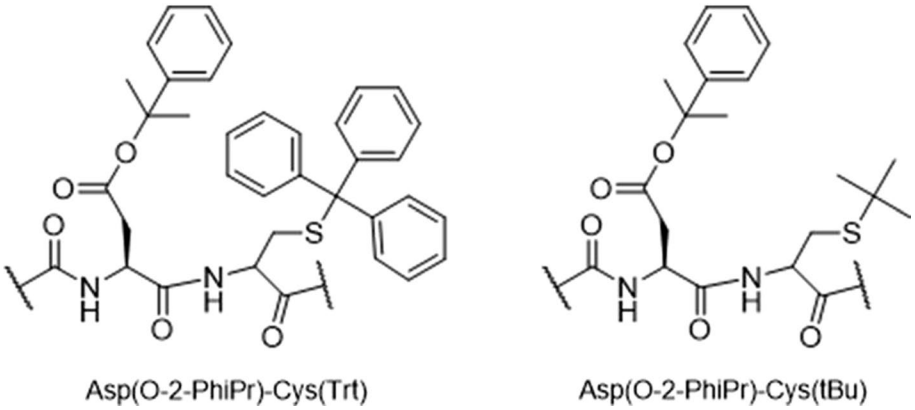

Fig. 6 Influence of cysteine β-protecting group on aspartimide formation

A systematic study clearly revealed that the conformation adopted from the precursor peptide (DCVLRP) promotes the formation of these undesired by-products. Moreover, we also observed a strong influence of the side-chain protecting group nature of the neighboring aspartic and cysteine amino acids on the initial cyclization process. Use of Asp(tBu) and Cys(Trt) avoided the by-product generation, even if this protocol was not suitable for our synthetic approach.

The most effective orthogonal strategy for minimization of aspartimide formation during the synthesis of the lactam-bridged peptide, S[KHSSLD]CVLRP was (i) 2-PhiPr side-chain protection of aspartate; (ii) Trt side-chain protection of cysteine; and (iii) 4-methoxytrityl for lysine side chain.

Our results indicate that the aspartimide formation depends not only on the nature of the neighboring amino acid located at the C-terminus of the aspartic acid but also on the protecting groups used and on the conformation adopted by the peptide backbone.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Informed consent All authors listed have contributed to conception, design, synthesis, gathering, analysis, or interpretation of data and have contributed to the writing and intellectual content of the article. All authors gave informed consent to the submission of this manuscript.

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