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Use of a removable backbone modification strategy to prevent aspartimide formation in the synthesis of Asp lactam cyclic peptides

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Keywords

Lactam cyclic peptides | Solid-phase peptide synthesis | Aspartimide | Removable backbone modification |

Main observation and conclusion

The synthesis of an Asp lactam derivative of A-183, a selective inhibitor of Factor 7a with good anticoagulant and antithrombotic activity, is described. Our synthesis depends on the use of a removable backbone modification (RBM) strategy to prevent aspartimide formation, which thwarted all attempts to synthesize this target using direct solid-phase peptide synthesis. Validation of the RBM strategy in the synthesis of a second Asp lactam derivative was also accomplished. The RBM strategy is therefore proposed as a general method for the synthesis of Asp lactam cyclic peptides.

Comprehensive Graphic Content

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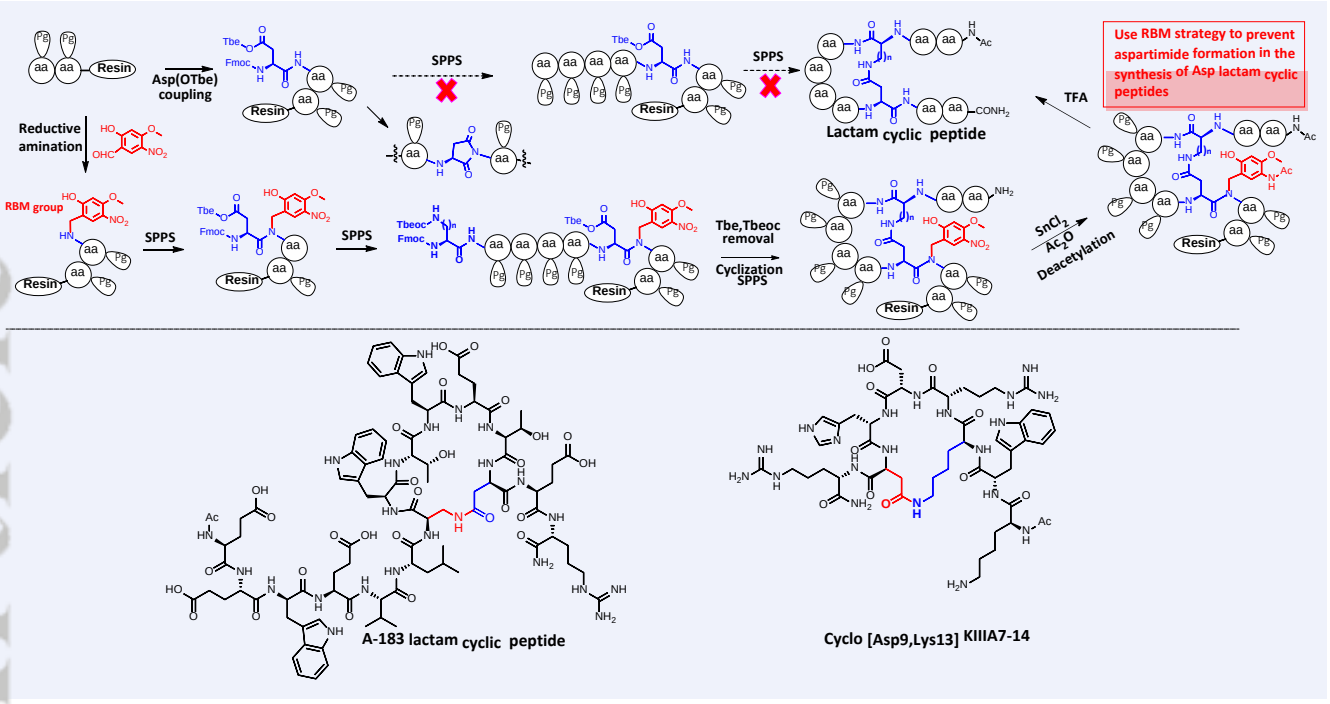

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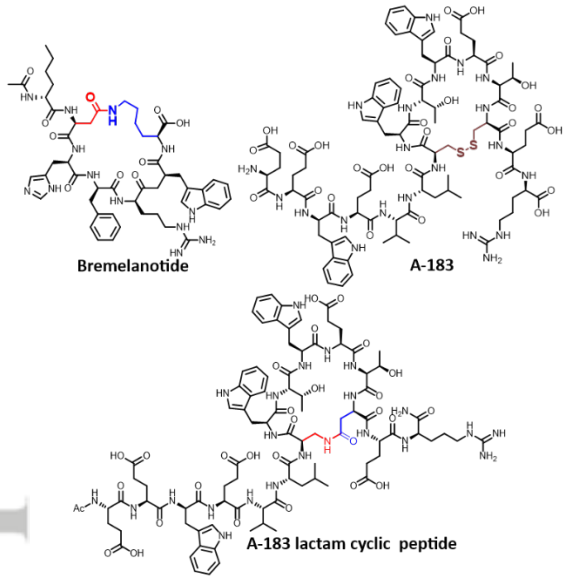
Background and Originality Content

Lactam cyclic peptides have attracted widespread attention recently due to their structural rigidity, metabolic stability, and interesting biological activity;[1-7] and the Asp-based lactam cyclic peptide drug bremelanotide (Scheme 1), a highly potent synthetic analogue of α-melanocyte-stimulating hormone, was recently approved by FDA.[8] Asp-based lactam cyclic peptides are usually synthesized by Fmoc solid-phase synthesis (SPPS),[9-15] which generally entails removal of the temporary protecting groups on the side chains of Asp and Lys (or their derivatives) prior to on-resin Asp-Lys lactam cyclization.

A-183 is a 15-residue peptide-based selective inhibitor of Factor 7a reported to exhibit good anticoagulant and antithrombotic effects,[16-18] but its metabolic stability is poor due to the presence of a disulfide bond which is prone to undergo reduction under physiological conditions. We sought to synthesize a derivative of A-183 incorporating an Asp-Dap (a Lys derivative) amide bond in place of this disulfide bond, a modification anticipated to enhance its metabolic stability, but our efforts to synthesize it through direct SPPS repeatedly failed.



Scheme 1 Structures of bremelanotide, A-183, and its lactam derivative

More careful analysis revealed that the problem stemmed from the nucleophilic attack of the β-carboxyl of the protected Asp by the amide nitrogen of the preceding residue of Asp, resulting in the formation of aspartimide by-product.[19-20] To overcome this problem, we report the use of our recently developed removable backbone modification (RBM) strategy to synthesize Asp-based lactam cyclic peptides. Both the Asp lactam derivative of A-183 and another Asp lactam cyclic peptide cyclo[Asp9,Lys13]KIIIA7-14 were successfully synthesized by the RBM strategy.

Results and Discussion

Aspartimide formation during the synthesis of an Asp-based A-183 lactam cyclic peptide derivative

We first attempted to synthesize A-183 lactam derivative by standard Fmoc-SPPS. This target incorporates Asp and Dap residues, and we planned to protect their side chains using our previously developed *tert*-butyl disulfide-based strategy for which the deprotection conditions are mild and metal-free.[13] Accordingly, 2-(*tert*-butyldisulfanyl)ethanol (Tbe) and 2-(*tert*-butyldisulfanyl)ethoxycarbonyl (Tbeoc) were installed onto the Asp and Dap side chains to give Fmoc-Asp(OTbe) and Fmoc-Dap(Tbeoc), respectively, which were then submitted to standard Fmoc SPPS using Rink amide resin (Figure 1). A linear peptide incorporating both Asp(OTbe) and Dap(Tbeoc) was expected after cleavage from the resin using trifluoroacetic acid (TFA), but no product of the expected molecular weight was detected by HPLC and ESI-MS even after repeated trials using several different SPPS conditions.

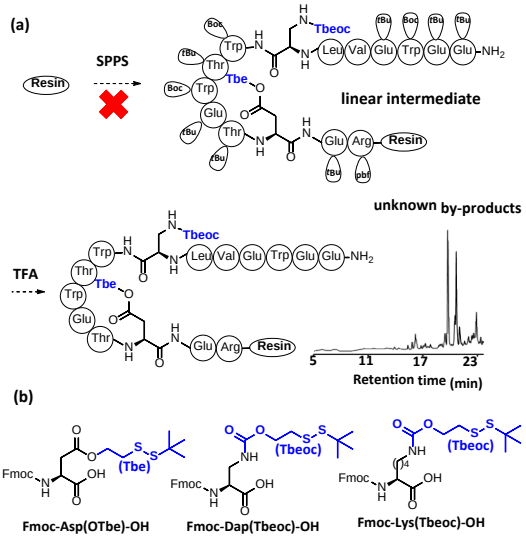


Figure 1. (a) Direct SPPS of the linear peptide of A-183 derivative. (b) Chemical structure of 2-(*tert*-butyldisulfanyl)ethanol protected amino acids.

To identify the problem, we carefully monitored the peptide chain elongation process. As shown in Figure 2a, sequential coupling of Arg(Pbf), Glu(O*t*Bu), and Asp(OTbe) onto the Rink amide resin gave intermediate **1**, which gave rise to a single peak with the correct molecular weight by HPLC / ESI-MS analysis after cleavage using TFA. Next, we coupled Fmoc-Thr(O*t*Bu) onto **1** and again cleaved the product from the resin. HPLC / ESI-MS analysis showed that target product **2** was obtained, as well as a by-product with a molecular weight of 166 units less than that of **2** (Figure 2a). Further coupling of Glu(O*t*Bu), Trp(Boc) and Thr(O*t*Bu) onto **2** followed by cleavage and analysis showed that no target product **3** had been formed, but a by-product having a molecular weight 166 units less than **3** had been synthesized instead. Noting that the difference in molecular weight between **3** and by-product could be accounted for by loss of 2-(*tert*-butyldisulfanyl)ethanol (MW 166), we hypothesized that the Asp(OTbe) had converted to the corresponding aspartimide under the basic (piperidine) conditions used for Fmoc deprotection.

To confirm this hypothesis, we synthesized the tripeptide Asp(OTbe)-Pro-Arg(Pbf) **4**, which is similar to the sequence in **2**, but incorporating a Pro residue in place of the Glu(O*t*Bu) and then added Thr(O*t*Bu) to **4** to generate **5**. After cleavage of **5** by TFA, HPLC and ESI-MS analysis showed the formation of a single product with the expected mass. Sequential addition of Glu(O*t*Bu), Trp(Boc) and Thr(O*t*Bu) to **5** yielded **6**, which also

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gave rise to a single peak with the expected molecular weight by HPLC / ESI-MS after cleavage. Therefore, replacement of the Glu(O*t*Bu) with Pro successfully disabled aspartimide formation, presumably because the Pro residue does not contain an amide nitrogen capable of nucleophilic attack on the β-carboxyl moiety of the Asp.[21-23]

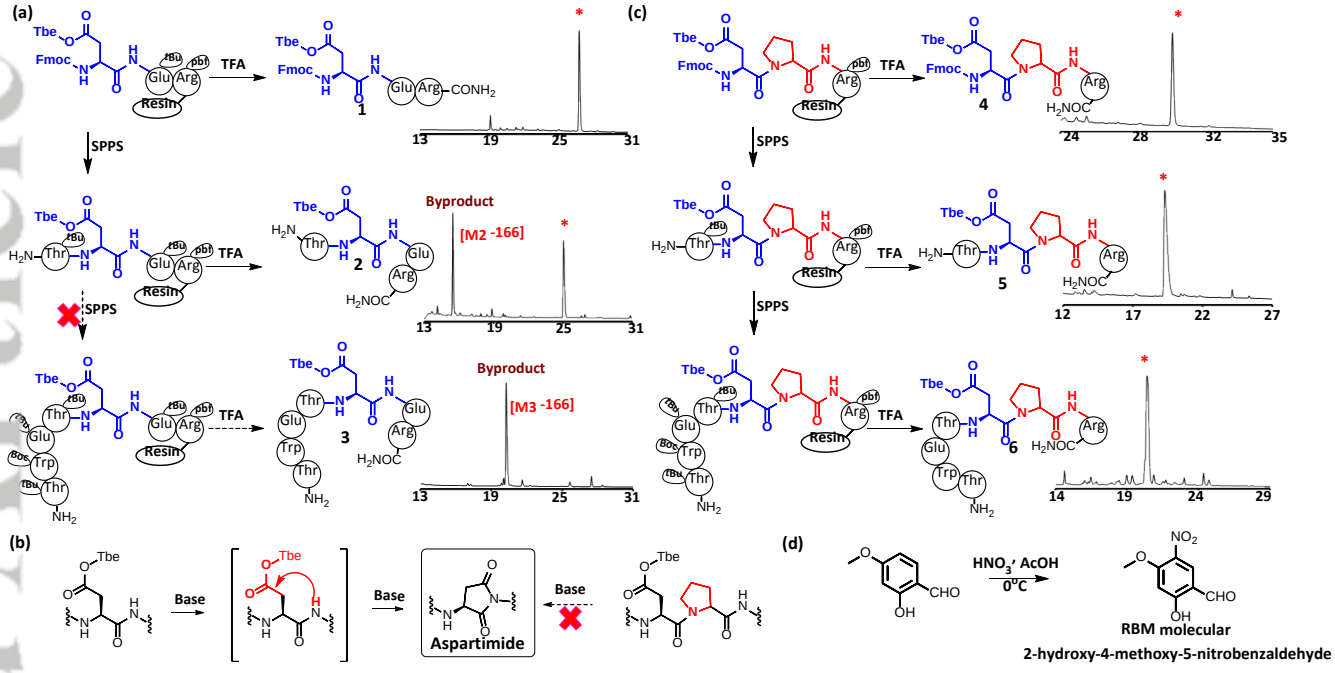

Figure 2. Aspartimide formation during the synthesis of the A-183 derivative. (a) The aspartimide-forming side reaction occurred during the synthesis of A-183. (b) Base can promote aspartimide formation and Asp-Pro sequence can prevent aspartimide formation. (c) Asp-Pro sequence can prevent aspartimide formation. (d) Synthesis route of the RBM molecule.

Synthesis of Asp-based A-183 lactam cyclic peptides derivative through an RBM strategy

To definitively implicate the backbone amide nitrogen in the aspartimide formation side reaction, we decided to protect it, to prevent its nucleophilic attack.[19] However, due to the steric bulk of the Asp and Glu residues flanking it on either side, the direct introduction of such a protecting group was anticipated to be challenging. Recently we developed a method to disrupt the formation of secondary structure by nascent peptides during SPPS and protein ligation by protecting selected amide nitrogen atoms with the RBM building block 2-hydroxy-4-methoxy-5-nitrobenzaldehyde.[24-31] This strategy is applicable to any secondary amide nitrogen regardless of its steric encumbrance because the building block is attached by reductive amination when the amide nitrogen is still a free amine. The amino acid that is next in the peptide sequence is attached to the phenolic group of the building block, whereupon the protected peptide is obtained by *para*-nitrophenol induced intramolecular O-to-N acyl transfer that is a highly efficient process applicable to bulky amino acids.

To implement the above RBM strategy to protect the amide backbone between Asp(OTbe) and Glu(O*t*Bu), we first synthesized the RBM building block 2-hydroxy-4-methoxy-5-nitrobenzaldehyde from commercially available materials (Figure 2d).[24] The RBM group was then installed onto the amino group of Glu(O*t*Bu) on the solid-phase by reductive amination to obtain intermediate **7**. Coupling of Asp(OTbe) to **7** led to the formation of a phenol ester intermediate which underwent simultaneous O-to-N acyl transfer to generate the desired amide **8**. Sequential condensation of Dap(Tbeoc), Trp(Boc), Thr(O*t*Bu), Trp(Boc), Glu(O*t*Bu), and Thr(O*t*Bu) with **8** afforded **9**. HPLC and ESI-MS analysis of the product obtained by cleavage of **9** from the resin with TFA showed a single peak with the expected molecular weight and no detectable aspartimide by-products.

Removal of the side chain protecting groups of Asp(OTbe) and Dap(Tbeoc) in **9** was accomplished using 2-mercaptoethanol/DIPEA in 40% H_2O/DMF. The exposed carboxyl and amino groups of the Asp and Dap side chains were then cyclized on resin using PyAOP/HOAT/N-methylmorpholine (NMM) to give a lactam **10**. Sequential addition of the remaining amino acids by standard Fmoc-SPPS gave the full-length product **11**. The RBM group became an acid-sensitive group by reduction of its NO_2 group to NH_2 using $SnCl_2$ on the solid phase, followed by acetylation of the NH_2 group using acetic anhydride on the solid phase. Finally, TFA cleavage was carried out to cleave the peptide from the resin to yield Asp lactam cyclic peptide **13**. HPLC and ESI-MS analysis demonstrated the purity and correct molecular weight of the peptide **13** (total yield 25.7%). Collectively the above observations demonstrated that the RBM strategy could effectively prevent aspartimide formation, and enable the efficient synthesis of the Asp-based A-183 amide cyclic peptide derivative.

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Synthesis of Cyclo [Asp9,Lys13] KIIIA7-14 derivative

To assess the versatility of our RBM strategy for the synthesis of other Asp-based lactam cyclic peptides, we next synthesized cyclo [Asp9,Lys13] KIIIA7-14, an Asp lactam derivative of the potent analgesic KIIIA.[31] We first attempted its synthesis using direct Fmoc SPPS under standard conditions, but again observed significant aspartimide formation (Figure S11). However, by installing the RBM group between Asp(OTbe) and Arg(Pbf) (Figure 4), we obtained **14**, without observable aspartimide formation (Figure 4). Further Asp-Lys cyclization was conducted and all the protecting groups including RBM were removed. HPLC and ESI-MS analysis demonstrated the purity and correct molecular weight of the final peptide **17** (total yield 35.4%). Thus the RBM strategy could constitute a generally practicable approach to prevent aspartimide formation during synthesis of the Asp-based lactam cyclic peptide derivatives.

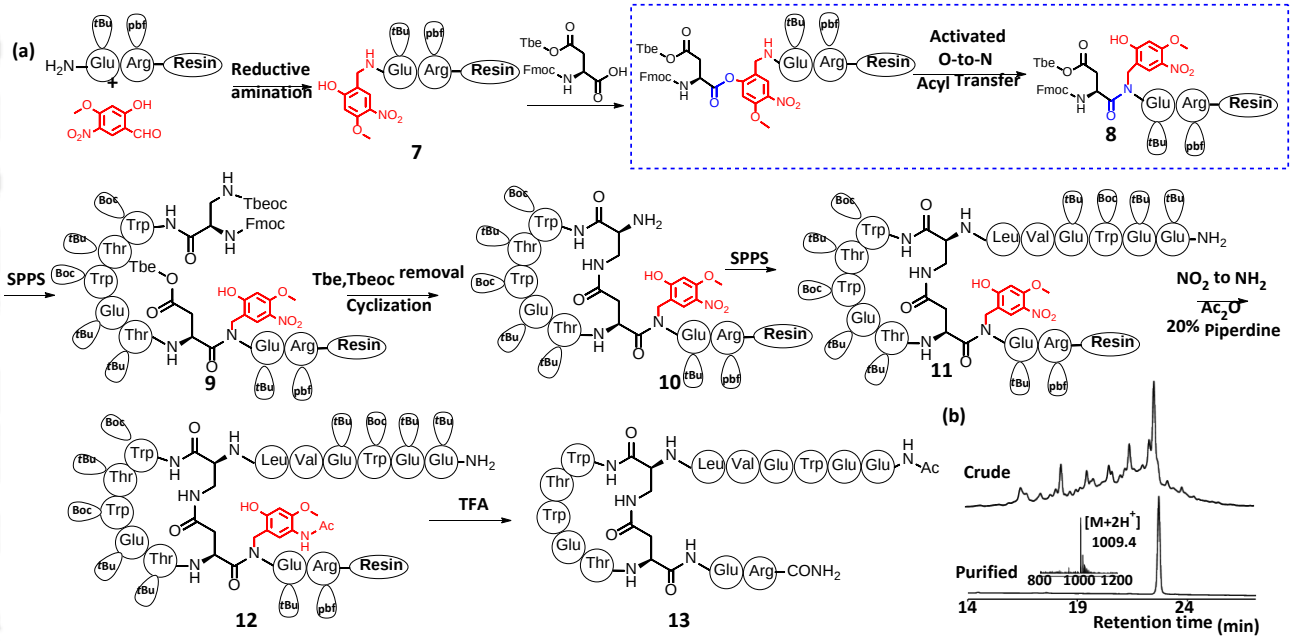

Figure 3. (a) Synthesis of A-183 derivative using RBM strategy. Specially, when hydroxyl group was acylated, RBM group could not be cleaved by TFA. After the acetylated hydroxyl group on the RBM group was deacetylated with 20% piperidine /DMF, the RBM group could be completely removed. (b) RP-HPLC traces of the crude and purified **13** and ESI-MS of purified **13**.

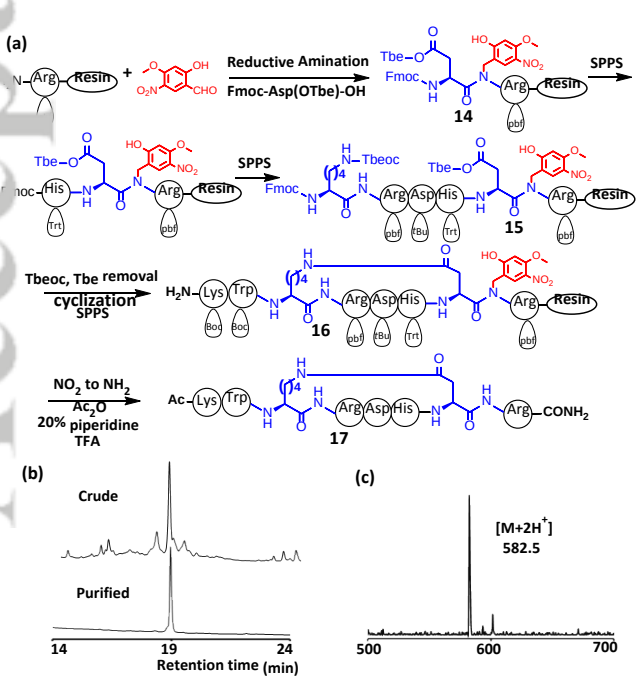

Figure 4. (a) Synthesis of Cyclo [Asp9,Lys13] KIIIA7-14 using the RBM strategy. (b) RP-HPLC traces of crude and purified **17**. (c) ESI-MS of purified **17**.

The RBM strategy can also prevent aspartimide formation associated with commercially available Asp(OAllyl)

Finally, we tested whether aspartimide formation was due to the use of 2-(*tert*-butyldisulfanyl)ethanol as the Asp protecting group by attempting the direct SPPS of A-183 using commercially available Fmoc-Asp(OAllyl) instead of Fmoc-Asp(OTbe). As shown in Figure 5, we first synthesized **18** by direct Fmoc-SPPS. As expected, coupling of Thr(O*t*Bu) to **18** to obtain **19** was accompanied by significant formation of a by-product with a molecular weight of 58 mass units less than **19**, presumably corresponding the conversion of Asp(OAllyl) to aspartimide. However, by using the RBM group to protect the Glu(O*t*Bu) before the Asp(OAllyl), we were able to obtain **21**, which was coupled with Thr(O*t*Bu) to give **22** without observable formation of an aspartimide-containing byproduct.

During the peptide chain extension process to give **23**, HPLC and ESI-MS analysis demonstrated the purity and correct molecular weight of target products (Figure 5b). Next, the nitro group of RBM was reduced to an amino group, which was subsequently acetylated by acetic anhydride. The resulting peptide was cleaved from resin, and HPLC and ESI-MS analysis demonstrated the purity and correct molecular weight of **24**. Thus, we concluded that the aspartimide formation side reaction was not limited to 2-(*tert*-butyldisulfanyl)ethanol protection, but also possible with other ester

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protection groups, and that our RBM strategy could be used to prevent the aspartimide side reaction when using Asp(OAllyl).

Conclusions

Asp-based lactam cyclic peptide A-183 and cyclo [Asp9,Lys13] KIIIA7-14 lactam cyclic peptides have been successfully synthesized using an RBM strategy, wherein a 2-hydroxy-4-methoxy-5-nitrobenzaldehyde group is appended to the residue immediately before Asp(OTbe), to prevent aspartimide formation.[32-36] This RBM strategy may constitute a simple, efficient, and general method for the synthesis of Asp-based lactam cyclic peptides, which we established to be inaccessible by direct Fmoc SPPS.

Supporting Information

The supporting information for this article is available on the WWW under https://doi.org/10.1002/cjoc.2021xxxxx.

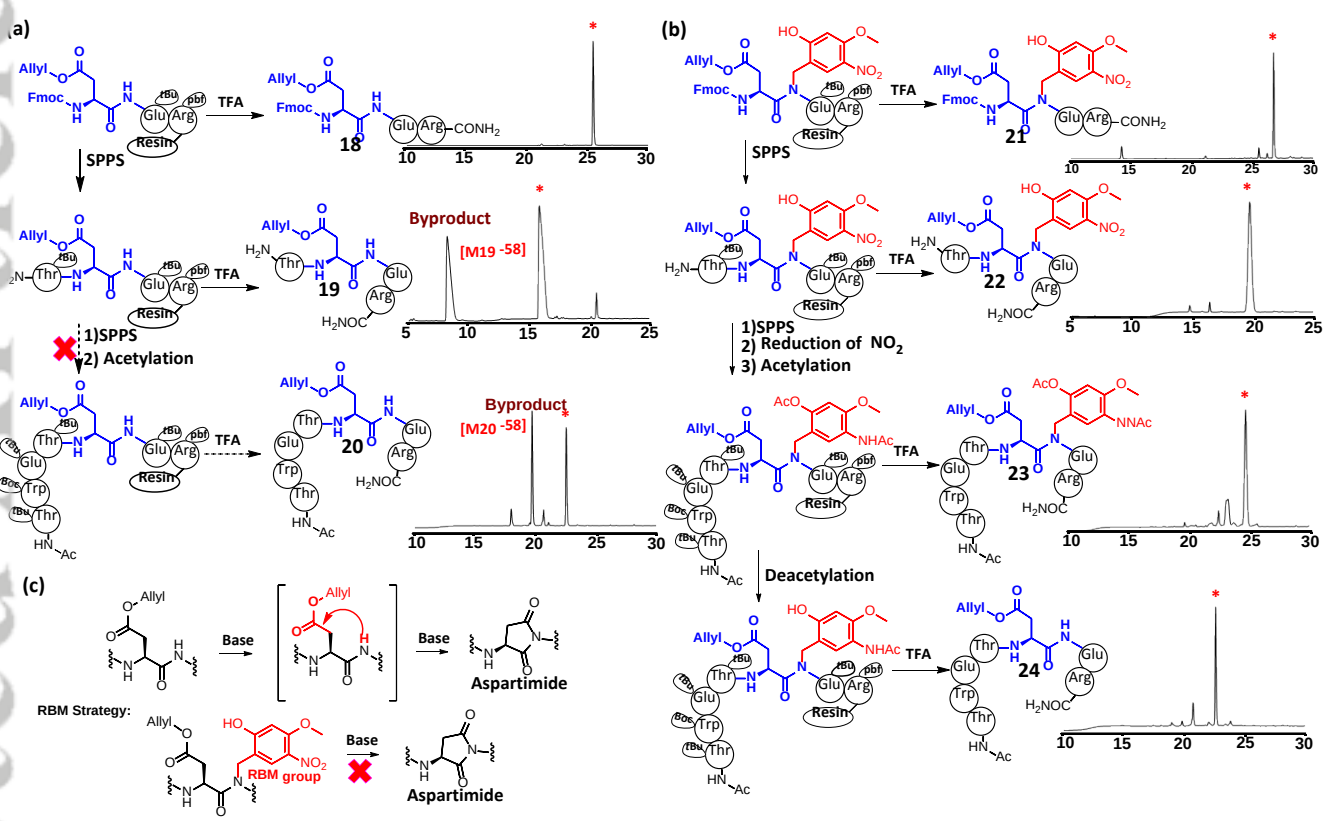

Figure 5. (a) Aspartimide formation occurred during the synthesis of the A-183 derivative **13** using Asp (OAllyl). (b) The RBM strategy also prevented aspartimide formation when using Asp(OAllyl) in place of Asp(OTbe). (c) Proposed mechanism of RBM-prevented aspartimide formation during SPPS.

Acknowledgement

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Use of a removable backbone modification strategy to prevent aspartimide formation in the synthesis of Asp lactam cyclic peptides

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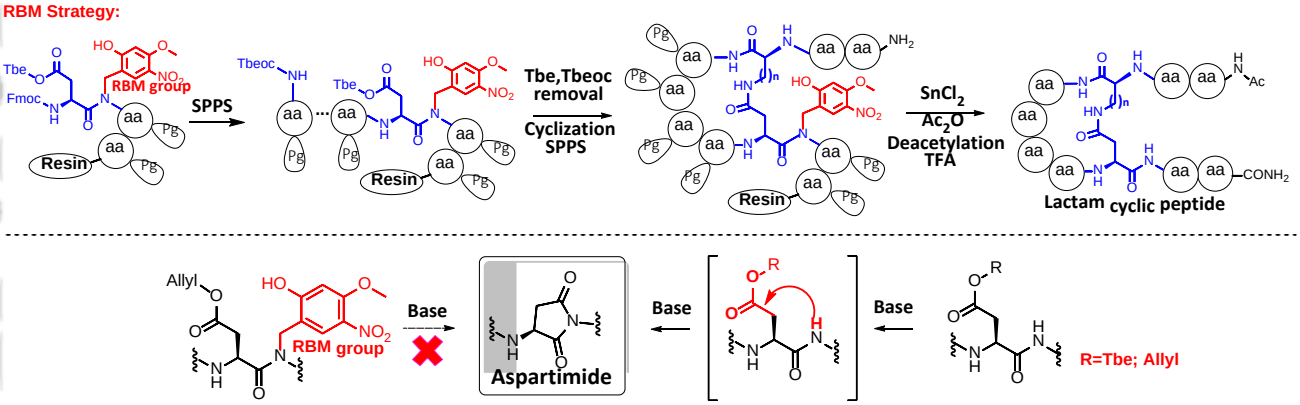

Our synthesis depends on the use of an RBM strategy to prevent aspartimide formation, which thwarted all attempts to synthesize the Asp-based lactam cyclic peptide A-183 and cyclo [Asp9,Lys13] KIIIA7-14 lactam cyclic peptides using direct SPPS.

Accepted Article

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