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Innovations 1.16

Fmoc-Asp(OBno)-OH

The solution to the aspartimide problem in Fmoc SPPS

The most frequently encountered side reaction affecting Asp residues during solid phase synthesis is aspartimide formation, resulting from a ring-closure between the nitrogen of the α-carboxy amide bond and the β-carboxy side-chain, with loss of the ester protecting group (Figure 1) [1, 2]. It is a particularly serious problem in Fmoc SPPS as cyclization is promoted by strong bases such as piperidine and DBU used to effect Fmoc group removal [3-6].

Aspartimides are very susceptible to base-catalyzed epimerization [7] and readily undergo ring-opening reactions, leading to the formation of a variety of by-products. Attack by water yields β-aspartyl peptide and α-aspartyl peptide in a ratio of 3:1 [8]. Ring-opening by piperidine gives a mixture of α- and β-piperidides, which are characterized in MS as peaks at 67u greater than that of the expected peptide. Whilst in many cases aspartimides and α- and β-piperidides generated by this side reaction may be easily separated from the target peptide by HPLC, the β-aspartyl peptides and epimerized α-aspartyl peptide are almost impossible to remove as these frequently have same retention times as the target peptide. Furthermore, as they have the same mass as the target, the presence of these side products is hard to detect. This point is beautifully illustrated in Figure 2, which shows the HPLC profile of crude (Gly2)-GLP-2 (teduglutide) immediately after cleavage and subsequent dissolution in aqueous buffer. The aspartimides are clearly apparent in the HPLC of the crude peptide after cleavage; however, in aqueous media these rapidly ring open to the L/D-β-aspartyl and D-aspartyl peptides which co-elute with the desired peptide. Isolation of the major component would have given a "pure" peptide contaminated with approximately 15%

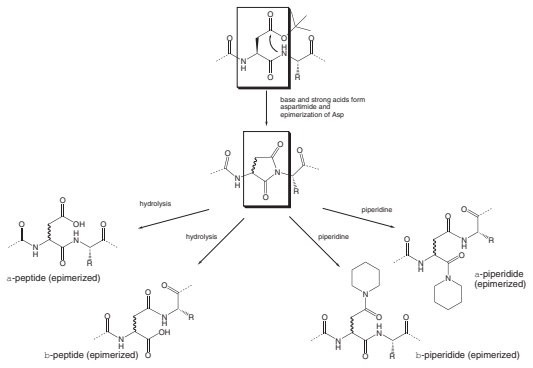

Fig. 1: Side reactions arising from aspartimide formation.

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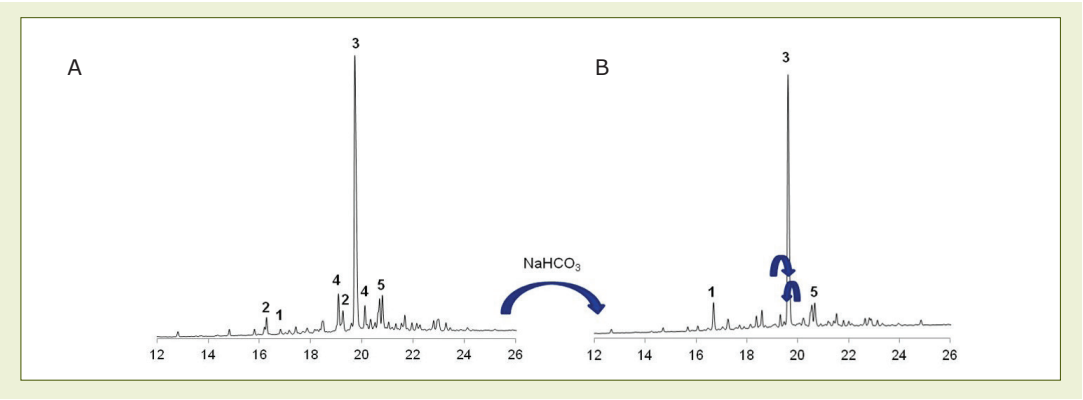


Fig. 2: HPLC profile of crude (Gly2)-GLP-2 (teduglutide) immediately after cleavage (A) and subsequent dissolution in aqueous buffer at pH 7.5 (B). 1: +56. 2: Isoacyl peptides. 3: target. 4: D/L-aspartimides. 5: D/L-α/β-piperidides.

of aspartimide related by-products. Therefore, an observation of aspartimide formation and lack of identifiable products attributable to β-aspartyl peptide and epimerized target peptide implies these by-products will be hiding underneath the product peak in the HPLC. Therefore, the implementation of synthetic strategies that minimize aspartimide formation are a prerequisite to obtaining homogenous aspartyl-containing peptides in good yields.

Sequence dependence of aspartimide formation

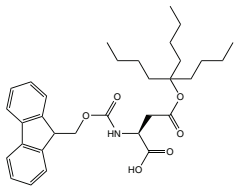
The extent of aspartimide formation is highly dependent on the nature of the amino acid at the C-terminus of the aspartyl residue (Table 1). Moreover, it is also sensitive to the conformation and hence sequence of the peptide.

Table 1: Asp-Aaa sequences prone to aspartimide formation under base treatment. + low; ++++ high [2, 4, 6, 15]

Aaa	Propensity
Ala	+
Arg(Pbf)	++
Asp(OtBu)	+++
Asn(Trt)	+++
Cys(Acm)	++
Cys(Trt)	+
Gly	++++

Peptides containing the Asp-Gly sequence are particularly prone to aspartimide formation, which is estimated to occur to the extent of approximately 0.5% per Fmoc deprotection cycle [9]. Obviously, the problem is most serious in sequences containing more than one site of potential aspartimide formation and in long peptides, as the degree of aspartimide formation is dependent on the total exposure time to piperidine. It is also exacerbated by the use of DBU as this base has been shown to more effectively promote aspartimide formation than piperidine [10].

Fmoc-Asp(OBno)-OH



Fmoc-Asp(OBno)-OH was developed to provide a simple and generic solution to aspartimide formation in Fmoc SPPS [10, 11]. It is an hindered variant of Fmoc-Asp(OtBu)-OH in which the steric bulk of the t-butyl group is increased by linear homologation, to help shield the aspartyl β-carbonyl group and thereby reduce the formation of aspartimide derived by-products.

The utility of Fmoc-Asp(OBno)-OH at preventing aspartimide formation in Asp-Asn and Asp-Arg sequences was demonstrated in Innovations 02/14. In this innovation, we test the efficacy of this new reagent against more problematic Asp-Gly cotanining sequences. Again the model peptide scorpion toxin II (H-Val-Lys-Asp-Gly-Tyr-Ile-OH) [13] was used as the test sequence. Peptides were prepared using either Fmoc-Asp(OtBu)-OH, Fmoc-Asp(OMpe)-OH [14], or Fmoc-Asp(OBno)-OH for introduction of the Asp residue. Prior to TFA cleavage the resins were treated with 20% piperidine in DMF for 18h, to simulate the effects of approximately 100 deprotection cycles.

Figure 3 shows the HPLC profile of the total crude product obtained for H-Val-Lys-Asp-Gly-Tyr-Ile-OH. For the peptide prepared with Fmoc-Asp(OtBu)-OH, the desired product was a minor component, with piperidides and aspartimides being the major components, resulting from approximately 2.23% aspartimide/deprotection cycle (Table 2). With Fmoc-Asp(OBno)-OH, formation of aspartimide by-products was dramatically reduced to only 0.14% per piperidine deprotection step (Table 2). This result is very significant since this value is lower than that for the impurities typically found in preparations of commercially available Fmoc-amino acids. Fmoc-Asp(OBno)-OH, thus, represents a viable solution to the problems of aspartimide formation for Asp-Gly peptides in Fmoc SPPS

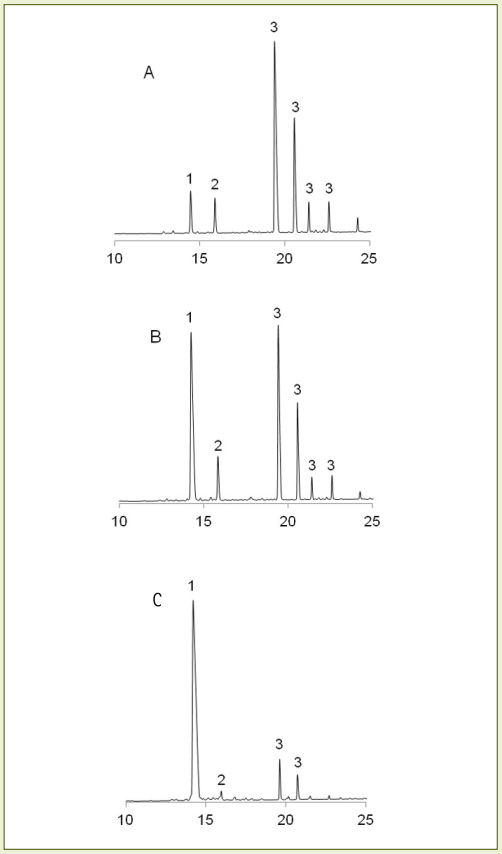


Fig. 3: HPLC profiles of H-Val-Lys-Asp-Gly-Tyr-Ile-OH prepared with (A) Fmoc-Asp(OtBu)-OH, (B) Fmoc-Asp(OMpe)-OH and Fmoc-Asp(OBno)-H (C), after 18h 20%piperidine in DMF treatment. 1: Product; 2: piperidides; 3: aspartimide.

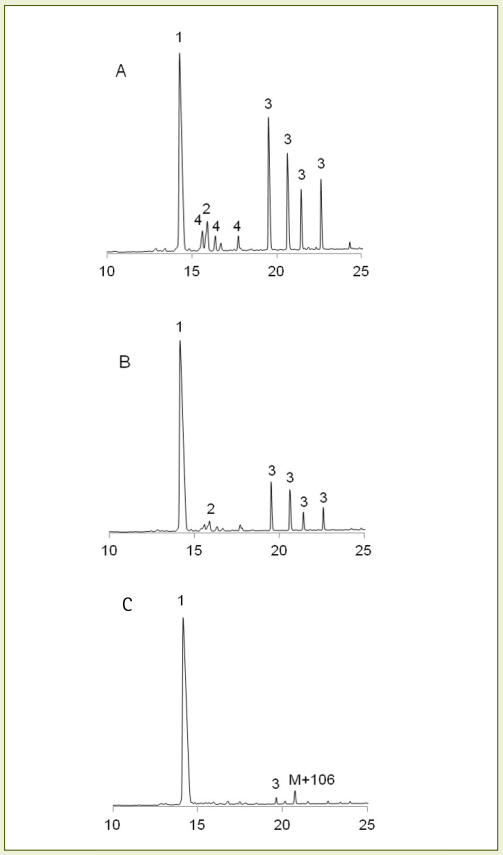


Fig. 4: HPLC profiles of H-Val-Lys-Asp-Gly-Tyr-Ile-OH prepared with (A) Fmoc-Asp(OtBu)-OH, (B) Fmoc-Asp(OMpe)-OH and Fmoc-Asp(OBno)-H (C), after 18h treatment with 0.1 M Oxyma Pure in 20% piperidine. 1: target peptide. 2: D/L-aspartimide. 3: D/L-α/β-piperidide. 4: Impurities resulting from Oxma Pure

Effect of acidic modifier

Addition of acidic modifers to the piperidine solution used for Fmoc removal has been shown to be effective in suppressing aspartimide formation [15], presumably by reducing the ionization of the Asp-Aaa amide bond. The most frequently used modifiers are 0.1M HOBt [15], 0.1 – 1M Oxyma Pure [16] and 0.1 M formic acid [17] in 20% piperidine/DMF. We were, therefore, interested to compare the efficacy of this method with our approach, and to evaluate the effects of combining the use of an acidic modifier with the use of OBno aspartyl protection. To this end, the peptidyl resins of the model peptide was exposed to 0.1 M and 1M solution of Oxyma Pure in 20% piperidine for 18 h, and the crude products cleaved with TFA and analyzed by HPLC. For the Asp(OtBu)-Gly sequence, 0.1M and 1M Oxyma Pure in 20% piperidine reduced aspartimide formation to 0.67% and 0.46%, respectively, per deprotection cycle (Table 2). However, the use of Oxyma Pure led to the formation of additional impurities; these were most prominent with 0.1M Oxyma Pure in 20% piperidine/DMF (Figure 4A). For the peptide prepared with Fmoc-Asp(OBno)-OH, virtually no aspartimide related by-products were formed after treatment with 0.1 M or 1 M Oxyma Pure in 20% piperidine/DMF. This is a remarkable result, considering the exposure is equivalent to 100 deprotection cycles. Interestingly, the by-products associated with Oxyma Pure were also eliminated, indicating that these impurities arise from some reaction between the acidic modifier and aspartimide.

Table 2: Calculated % aspartimide (Asu) formation/ deprotection cycle for H-Val-Lys(Boc-Asp(OR)-Aaa-Tyr(tBu)-Ile-Wang resin treated with 20% piperidine in DMF or Oxyma Pure/20% piperidine in DMF.

Conditions	(OR)	% Asu/cycle
20% piperidine	tBu	2.23
20% piperidine	Mpe	0.77
20% piperidine	Bno	0.14
20% piperidine + 0.1M Oxyma Pure	tBu	0.67
20% piperidine + 0.1M Oxyma Pure	Mpe	0.24
20% piperidine + 0.1M Oxyma Pure	Bno	0.04
20% piperidine + 1M Oxyma Pure	tBu	0.46
20% piperidine + 1M Oxyma Pure	Mpe	0.16
20% piperidine + 1M Oxyma Pure	Bno	0.02

Conclusion

The new β-tributylmethyl ester of aspartic acid, Bno, provides excellent protection from aspartimide formation during the stepwise Fmoc SPPS of the classic Asp-Gly containing scorpion toxin II model peptide. The routine use of Fmoc-Asp(OBno)-OH should therefore provide a simple and robust solution to the problem of aspartimide formation in Fmoc SPPS..

Ordering Information

Cat.No.	Product	Contents	Price
852418	Fmoc-Asp(OBno)-OH	1 g	POA
NEW		5 g	POA

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