Suppression of Piperidine-mediated Side Product Formation for Asp(OBu^t)-containing Peptides by the Use of *N*-(2-hydroxy-4-methoxybenzyl) (Hmb) Backbone Amide Protection

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Piperidine-mediated aspartimide and subsequent piperidide formation, during Fmoc-based synthesis of sensitive aspartyl (β-*tert*-butyl ester, OBu^t)-containing peptides, is completely suppressed by the use of Hmb protection of the aspartyl amide bond.

The amino acid residue aspartic acid (Asp) is the source of numerous undesired peptide transformations, arising during both synthesis and purification, due to its susceptibility to dehydration, transpeptidation and epimerization.[1,2] Such reactions can occur under both acid and base conditions, and have been extensively studied in the Boc-benzyl approach to peptide synthesis.[1-5]

The β-*tert*-butyl ester side-chain protection of aspartic acid, routinely used in the fluoren-9-ylmethoxycarbonyl (Fmoc) approach to peptide synthesis[6] has shown good stability in countless peptide syntheses, towards the repetitive piperidine–DMF cycle used for N^{α}-Fmoc removal. Thus, Asp(OBu^t) has appeared to provide sufficient protection towards aspartimide (cyclic imide) formation and subsequent reactions under basic conditions. However, recently Dolling *et al.*[7] have described a sequence containing Asp(OBu^t)–Asn(Trt) which was very susceptible to piperidine modification. In addition, the complete stability of β-*tert*-butyl ester protection in Asp–Gly-containing sequences has previously been questioned.[8] In the light of these findings, we have re-examined these troublesome sequences, confirming that piperidine modification does occur. Here, we wish to report the complete piperidine stability of the aspartyl (OBu^t) amide bond in these sequences during synthesis, by the use of *N*-(2-hydroxy-4-methoxybenzyl) amide bond protection **1**.

The formation of aspartimide peptides occurs under base-catalysed conditions (*e.g.* piperidine) through abstraction of the aspartyl amide nitrogen proton, and subsequent nucleophilic attack of the β-carboxyl (Scheme 1). The main factors governing the ease of this reaction are the base employed,[5,8] the β-carboxyl protecting group used[8] and the *C*-terminal amino acid residue of the aspartyl bond.[4] Once formed, nucleophilic ring opening by the base can occur, giving a mixture of α- and β-substituted peptides (Scheme 1). Additionally, the aspartimide is very vulnerable to base-catalysed epimerization, presumably through the α-proton abstraction–enolization mechanism.[9]

The extent of the aforementioned reactions was examined by Nicolas *et. al.*[8] using the 1–6 fragment from scorpion *Androctonus australis Hector* (toxin II), **2**. We have re-examined this sequence, using **2a**, Asp(OBu^t), and **2b** Asp(OBu^t), combined with Hmb-protection of the aspartyl–glycyl amide bond, and examined the stability of the protected

H-Val-Lys-Asp-Gly-Tyr-Ile-OH **2**

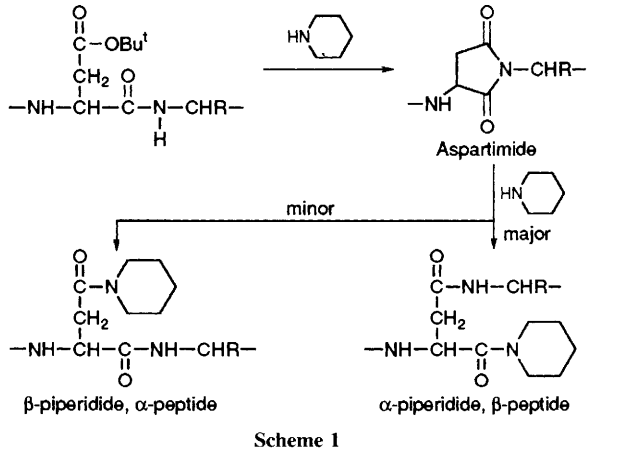
resin-bound sequences to piperidine–DMF treatment.† Sequence **2a** gave upon peptide–resin cleavage,‡ six peaks on HPLC analysis.§ Each peak was isolated and characterised by electrospray mass spectrometry (ES-MS) (Table 1), amino acid analysis and sequencing. As expected,[3] a predominance of α-piperidide (*i.e.* β-peptide) was found from sequence analysis. The tentative assignments of L- and D-Asp stereoisomers is derived from the work of Schon *et al.*[3] An overall modification of 71% of the peptide in 24 h corresponds to 0.5% per standard 10 min piperidine–DMF cycle.

Sequence **2b** gave upon peptide–resin cleavage,‡ exclusively unmodified peptide, even after 24 h treatment with piperidine–DMF. These results illustrate that the amide proton is essential for aspartimide formation, and that aspartyl amide alkylation with the Fmoc/*tert*-butyl compatible Hmb[10] group, completely suppresses this reaction. A potential problem with this approach is the pseudo Asp–Pro nature of the protected aspartyl bond. This has previously been shown to be particularly labile to acid-catalysed chain cleavage.[11] We have not however observed any evidence for trifluoroacetic acid lability of the Asp(OBu^t)–(Hmb)Gly peptide bond. Considering that aspartimide formation, like other secondary reactions, appears to be sequence dependant, it may well be that such rearrangements are less prone to occur in other Asp–Gly sequences.

A particularly good example of the sequence-dependent nature of piperidine-mediated aspartimide formation has

Table 1 Modification of H-Val-Lys(Boc)-Asp(OBu^t)-Gly-Tyr(Bu^t)-Ile-Pepsyn KA upon 24 h treatment with 20% piperidine–DMF, prior to cleavage

Peak HPLC Retention time/min	Peak %	ES-MS	Inference
13.75	29	694	Unmodified peptide
15.64	8	676	Aspartimide
20.71	40	761	α-Piperidide
22.33	16	761	D-α-Piperidide
23.18	3.5	761	β-Piperidide
24.76	3.2	761	D-β-Piperidide



Scheme 1

recently been presented by Dolling *et al.*[7] They describe an attempted synthesis of a segment of coat protein phage MS 2[12] by Fmoc chemistry, which gave a predominant product with m_w +67 (*i.e.* a piperidide). A test segment¶ of the sequence was prepared **3a**, and shown to contain 45% piperidide‖ after piperidine treatment. However, simply extending the *N*-terminal sequence a further 2 residues **3b**, and repeating the

H-Asp(OBu^t)-Asn(Trt)-X-Resin **3a**

H-Leu-Val-Asp(OBu^t)-Asn(Trt)-X-Resin **3b**

piperidine stability test, now gave 70% piperidide. We confirmed these results, also repeating the syntheses without trityl protection of the asparagine residue *C*-terminal of aspartic acid **3c**, and with Hmb protection of the aspartyl–asparaginyl amide amide bond** **3d**.

H-Leu-Val-Asp(OBu^t)-Asn-X-Resin **3c**

H-Leu-Val-Asp(OBu^t)-(Hmb)Asn(Trt)-X-Resin **3d**

Resin **3c** and **3d** were equilibrated with piperidine–DMF as before, prior to cleavage. Interestingly, **3c** contained 55% piperidide, compared to 70% for **3b**, the only difference being trityl protection of the asparagine at the susceptible bond in **3b**. Peptide **3d** containing Hmb protection of the aspartyl amide bond gave no evidence for aspartimide formation, containing >90% correct material in the crude peptide. Dolling *et al.*[7] provide a further example with the modification of an Asp(OBu^t)–Gln(Trt)-containing peptide, where a small change in the nature of the *C*-terminal residues renders this originally prone motif perfectly stable. These results highlight the subtle nature of the factors affecting aspartimide formation.

Martinez and Bodanszky[13] describe the efficient suppression of aspartimide formation by the use of additives such as 1-hydroxybenzotriazole or 2,4-dinitrophenol to the Fmoc-deprotecting reagent. Whilst this prevents undesired aspartyl transformations, continuous flow Fmoc deprotection data,[6] an invaluable tool for monitoring the progression of a synthesis, will be lost. Incorporation of aspartyl amide protection into the peptide sequence not only prevents piperidine-mediated transformations, but also retains the ability for Fmoc-deprotection data collection, and inhibits the potential formation of peptide β-sheet structures,[14] a major factor contributing to peptide impurities generated during solid phase peptide syntheses.

In conclusion, sensitive cases such as **2**, containing an Asp–Gly sequence at the start of a long synthesis may generate significant amounts of modified peptide. In these instances, along with other particularly susceptible cases, *e.g.* Asp–Ser/Thr/Asn/Gln,[4] the use of aspartyl amide protection should provide superior crude products.

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Footnotes

† Our syntheses described here were performed on functionalised and cross-linked polydimethylacrylamide supported in macroporous Kieselguhr (Pepsyn KA) resin. Syntheses were conducted on an LKB Biolynx automated peptide synthesiser, under standard conditions.[6]

The Hmb-protected glycine residue was incorporated *via* the *N*,*O*-bis-Fmoc-*N*-(2-hydroxy-4-methoxybenzyl)glycine pentafluorophenyl ester deivative[15] with 1-hydroxybenzotriazole activation, and coupled to the tyrosine residue for 2 h. The following aspartic acid residue was double coupled (2 × 2 h), as the OPfp ester, to the *N*-terminal (Hmb)Gly residue.

Resin-bound peptides were equilibrated with 20% piperidine in DMF for 24 h prior to cleavage.

‡ Peptide–resins were cleaved with trifluoroacetic acid–phenol (95 : 5, v/v) for 90 min.

§ Determined by C8 RP-HPLC.

¶ Where X = GGTGDVTVAPSNF-Pepsyn KA, with appropriate side-chain protection.

‖ Resin-bound peptides were equilibrated with 50% piperidine in DMF for 5 h prior to cleavage, which was effected with trifluoroacetic acid–water (95 : 5, v/v) for 2 h.

** The Hmb-protected asparagine residue was incorporated *via* the *N*,*O*-bis-Fmoc-*N*-(2-hydroxy-4-methoxybenzyl)(N^{ω}-trityl)asparagine pentafluorophenyl ester derivative[15] with 1-hydroxybenzotriazole activation, and coupled to the glycine residue for 2 h. The following aspartic acid residue was coupled off machine in a flask, in the minimum of dichloromethane as the *N*-carboxyanhydride (10 equiv. 24 h) to the *N*-terminal (Hmb)Asn(Trt) residue.

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