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Synthesis of aspartimide-free protected peptides on base-labile functionalized resins

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Abstract

Aspartimide prone sequence containing protected peptides are successfully synthesized in solid phase by using the bifunctional linker *N*-[(9-hydroxymethyl)-2-fluorenyl] succinamic acid (HMFS) in combination with morpholine as the cleavage reagent. Access to high purity peptide synthons opens a straightforward way to the synthesis of large proteins by convergent strategies. © 2000 Elsevier Science Ltd. All rights reserved.

Formation of aspartimides is a severe side reaction that still remains unresolved in the chemical synthesis of aspartic acid containing peptides and proteins.[1,2] Although it is catalyzed both by acids and bases, the extent of this intramolecular cyclization is much higher in basic conditions.[2b] Aspartimides are susceptible to opening by nucleophiles. In aqueous milieu, hydrolysis reverts them back to the deprotected aspartate form, although the isomer containing a β-amide bond tends to be the main byproduct. When piperidine is present (i.e. in Fmoc/^{t}Bu synthesis) mixtures of the corresponding amides, β- and α-piperidides, are obtained (Fig. 1).[2e,f] Generally speaking, aspartimide formation is sensitive to steric, electronic and conformational factors, as well as to the nature of the protecting group of the β-carboxy function.[2a] Several methods have been described to palliate the consequences of this side reaction, although most of them can only be applied in the Fmoc/^{t}Bu methodology. Thus, mild acids such as HOBt or 2,4-dinitrophenol have been used as additives in the piperidine reagent.[2d] However, this approach is not convenient when the peptide is attached to the resin by means of a base-labile linker as it is released into solution and contaminated with great amounts of those additives. The protection of the amide group with Hmb [*N*-(2-hydroxy-4-methoxybenzyl)] is also really effective at suppressing this side reaction, but it is not compatible with the Boc/Bzl synthetic

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Abbreviations: Boc, *tert*-butyloxycarbonyl; Bzl, benzyl; ClZ, 2-chlorobenzyloxycarbonyl; DIEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; ESMS, electrospray mass spectrometry; Fmoc, 9-fluorenylmethyloxycarbonyl; cHex, cyclohexyl; HMFS, *N*-[(9-hydroxymethyl)-2-fluorenyl]succinamic acid; HPLC, high performance liquid chromatography; PEGA, co(polyethilenglycol-acrylamide) resin; TBAF, tetrabutylammonium fluoride ^{t}Bu, *tert*-butyl.

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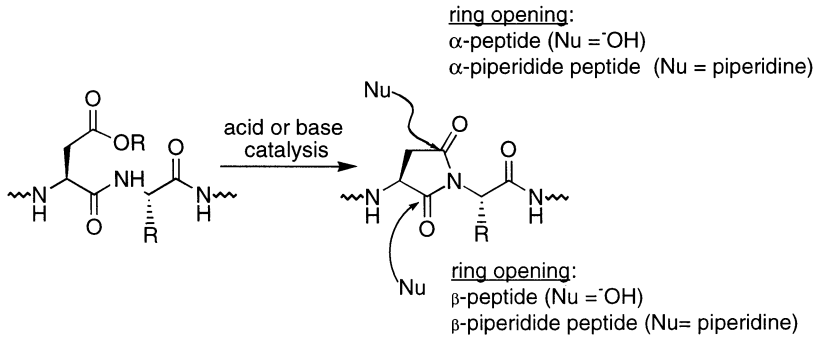

Figure 1. Aspartimide formation in peptides and subsequent ring opening

methodology.[2g] In this article, we describe an efficient methodology to eliminate the formation of aspartimides in the synthesis of Boc/Bzl protected peptides.

In the course of the chemical synthesis of the B domain of *Staphylococcus aureus* protein A, we encountered this side reaction during the preparation of the C-terminal protected fragment 227–233: Boc-Asn-Asp(OcHex)-Ala-Gln-Ala-Pro-Lys(ClZ)-COOH. According to the sequence of the peptide, it was *not* anticipated that the presence of an Asp–Ala pair could pose any major chemical problem during the synthesis. The heptapeptide was initially assembled using a Boc/Bzl protection scheme on a 4-hydroxymethyl-3-nitrobenzamide derivatised PEGA resin in order to obtain the protected peptide by nucleophilic displacement (Fig. 2).[3,4] Treatment of the peptide resin with a hydroxide ion source [LiOH in DMF:H_2O (1:1) or TBAF·$3H_2O$ in DMF][3,5] proved to be totally unsuccessful. Analysis of the crude products obtained by HPLC (Fig. 3(a)) and HPLC-ESMS revealed the total absence of the target product; only byproducts lacking a cyclohexyl group ($m/z = 993.4$, M−100, and $m/z = 1011.4$, M−100+18) were detected. These results suggested the formation of the peptide aspartimide and the subsequent hydrolysis of the succinimide ring to yield the partially deprotected peptide.

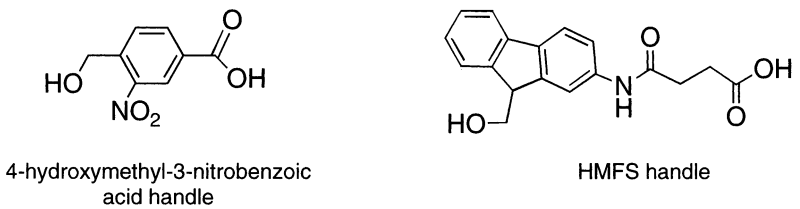

Figure 2. Structure of the 4-hydroxymethyl-3-nitrobenzoic acid and HMFS handles

We then decided to use the bifunctional linker *N*-[(9-hydroxymethyl)-2-fluorenyl]succinamic acid (HMFS).[6] The main advantage of this handle lies in its *fine tuned* lability that enables the release of protected peptides from the solid support with a mild base treatment (typically, piperidine or morpholine in DMF for 0.5–1 h). Assembly of the target sequence was carried out on a HMFS-PEGA-resin following the same Boc/Bzl protocol as in the previous case. Cleavage with piperidine (1:4 in DMF) afforded the expected peptide as the major product (Fig. 3(b)), although a significant amount of byproducts (7% in 15 min, 20–25% in 1 h) was also present. As before, the aspartimide was also detected. However, the main byproducts were in this case the related piperidides, derived from the opening of the succinimide ring by piperidine ($m/z =$ 1078.6, M−100+85). In addition, it was observed that the percentage of piperidides rose substantially on long treatments (>50% in 6 h).

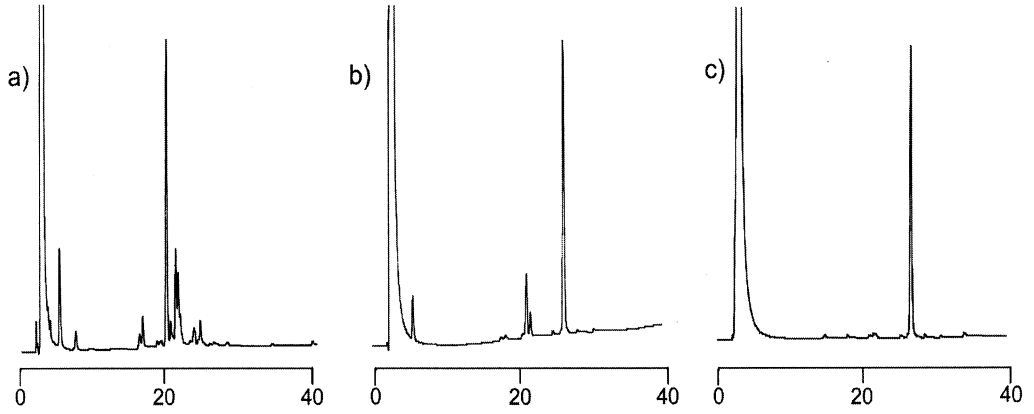

Figure 3. HPLC traces corresponding to crude Boc-Asn-Asp(OcHex)-Ala-Gln-Ala-Pro-Lys(ClZ)-OH synthesized under different conditions: (a) synthesis on a 4-hydroxymethyl-3-nitrobenzamide-PEGA resin and cleavage with TBAF·3H_2O for 45 min: no target product ($t_R \sim 27$ min) was detected; major identified byproducts were the partially deprotected peptide Boc-Asn-Asp-Ala-Gln-Ala-Pro-Lys(ClZ)-OH at $t_R \sim 20$ min and the peptide aspartimide at $t_R \sim 22$ min; (b) synthesis on HMFS-PEGA resin and cleavage with piperidine–DMF (1:4) for 1 h: the expected protected peptide is the major product ($t_R \sim 27$ min) although piperidides are also obtained ($t_R \sim 22$–23 min); (c) synthesis on HMFS-PEGA resin and cleavage with morpholine–DMF (1:4) for 1 h: the expected protected peptide is essentially the only product obtained. HPLC elution conditions: (A) H_2O–0.045% TFA; (B) MeCN–0.036% TFA; gradient: 20%B for 5 min, then 20–100% in 50 min

In order to minimize the effect of side reactions, cleavage with the milder base morpholine (in DMF, 1:4, 1 h) was assayed. The desired protected peptide was then obtained in excellent yields and without any trace of aspartimide related byproducts (Fig. 3(c)). Even after 6 h of treatment with morpholine, no byproducts were detected. These results suggest that amide proton abstraction is a key step in aspartimide formation,[2d] and that the strength of the base (morpholine's pK_a is 8.3, whereas piperidine's pK_a is 11.1) should be carefully chosen to generate the fluorenyl anion but preventing the amide from being deprotonated.

A final test to demonstrate the extent and factors influencing this side reaction was carried out by treating a protected heptapeptide sample with morpholine in aqueous conditions (morpholine–H_2O–DMF (1:4:5), apparent pH ~ 9–10). Conversion to the aspartimide and the corresponding hydrolysis byproducts was fast and prominent. The kinetic evolution of this reaction system

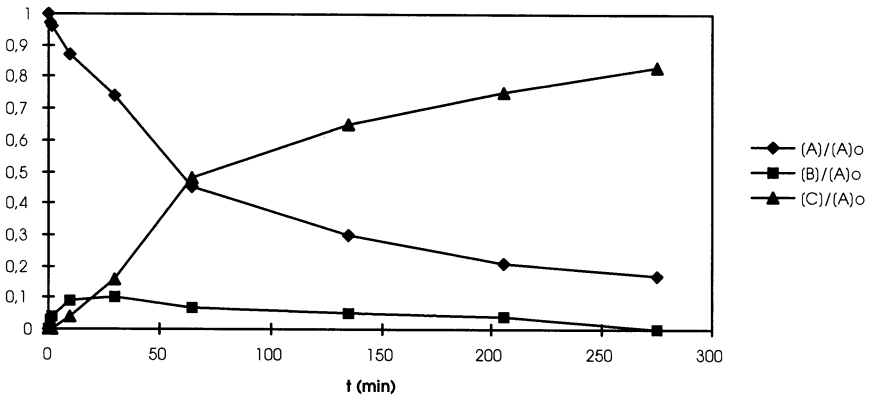

Figure 4. Evolution of the different aspartimide related byproducts of Boc-Asn-Asp(OcHex)-Ala-Gln-Ala-Pro-Lys(ClZ)-OH (A, ◆) in morpholine–H_2O–DMF (1:4:5). B (■) corresponds to the aspartimide peptide and C (▲) is the partially protected peptide Boc-Asn-(α–β)Asp-Ala-Gln-Ala-Pro-Lys(ClZ)-OH in its α and β forms

(simplified to an A→B→C system) is shown in Fig. 4. This result confirmed those obtained with TBAF·3H_2O and LiOH reagents, and suggests that strong nucleophilic bases with little hindrance such as the hydroxide anion are quite deleterious for this kind of Asp containing peptides.

In conclusion, our results confirm that the Boc/Bzl/HMFS protection scheme in combination with anhydrous morpholine as the cleavage reagent is an excellent strategy for the preparation of protected peptides free from aspartimide related byproducts. We believe this approach opens a straightforward way to the synthesis of large proteins by convergent strategies.[6,7]

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