Piperidine-mediated Side Product Formation for Asp(OBu^t)-containing Peptides

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A sequence- and conformation-dependent, unexpected high extent of aspartimide and piperidide formation is observed for the synthesis of Asp(OBu^t)-containing peptides using piperidine in the Fmoc-chemistry.

The undesired formation of aspartimide peptides by strong acid or strong base is a well known, sequence-dependent side reaction in peptide synthesis using the Boc–benzyl strategy.[1–7] Because of the stability of the β-*tert*-butyl ester of aspartic acid against piperidine–DMF, routinely used fluoren-9-ylmethoxycarbonyl (Fmoc)-deprotecting reagent, the application of Asp(OBu^t) in the Fmoc-chemistry seemed to be a sufficient protection towards imide formation and subsequent reactions.[3] Surprisingly, our approach to synthesise the partial sequence of coat protein phage MS2,[8] MASNFTQFVLDNGGTGDVTVAPSNF **I**, by means of the Fmoc-strategy (Fmoc-deprotection with 50% piperidine–DMF for 20 min/step) failed completely. We obtained a peptide product with a mass difference of +67 (ES-MS‡) with respect to the desired product. In contrary, syntheses of the *N*-terminal 12-*mer* peptide and the *C*-terminal 14-*mer* peptide **II** were carried out without any problem. This indicates that conformational aspects or amino acid residues, Asp or Asn, at the junction of both fragments may be responsible for the observed problems for the whole sequence. Thus, starting with peptide **II** we studied the stepwise elongation (Table 1). Assuming that the side product formation might be caused by piperidine, the peptide-resins were finally treated with piperidine–DMF for 5 h imitating conditions of a further peptide elongation. Whereas for peptide **II** with *N*-terminal Asn(Trt) the piperidine treatment does not affect the desired peptide, we obtained for peptide **III**, with an additional *N*-terminal Asp(OBu^t), an increase in side product formation; this increase was even more drastic after the incorporation of the next two amino acid residues. For peptide **V** the observed side product **VII** was characterized by ES-MS [*M*(V): 1662.8; *M*(found): 1729.5] and showed again a mass difference of +67 with respect to the desired product. This can be interpreted as shown in Scheme 1, assuming an aspartimide formation at Asp(OBu^t) followed by aminolysis with piperidine yielding the corresponding piperidides; especially the β-piperidide may be formed preferably in DMF.[6] As expected for a β-piperidide the sequence analysis of the side product **VII** has shown the release of *N*-terminal Leu and Val, but no release of Asp or of any other amino acid.

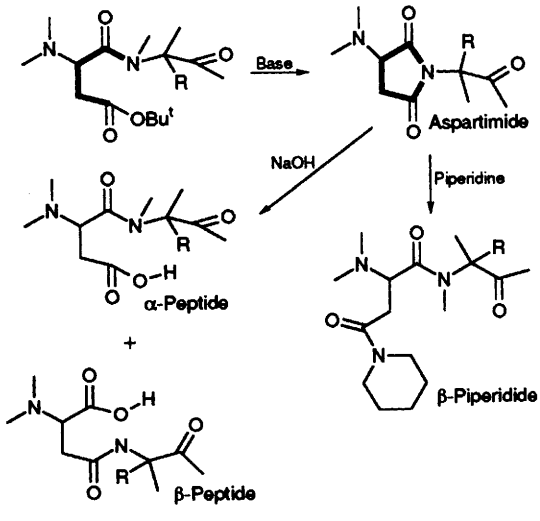
The results show that the tendency of side product formation depends not only on the nature of the amino acid at the carboxylic site of Asp(OBu^t), but also on *N*-terminal elongation. The increasing tendency for aspartimide formation by the *N*-terminal elongation might be explainable by the formation of a supporting conformation of the growing peptide, since it becomes stronger with increasing distance of the incorporated amino acid (comparison of peptides **IV** and **VI** in Table 1).

We obtained evidence for a conformational influence on side product formation by synthesising certain analogues of the corticotropin-releasing hormone, CRH (SQEPPISLDLTFHLLREVLEMTKADQLAQQAHSNRKLLDIA-NH_2), with D-amino acid replacements. Among others, we synthesised CRH and the analogue with D-Leu^{27} and D-Ala^{28}, next to Asp(OBu^t)^{25}-Gln(Trt)^{26}, using standard Fmoc-chemistry in the continuous flow mode.§ The synthesis of CRH turned out well yielding the desired product according to the amino acid analysis and ES-MS [*M*(calc): 4671; *M*(found): 4670]. For the analogue D-Leu^{27}-D-Ala^{28}-CRH the synthesis yielded repeatedly a main product with a mass difference of −18 [*M*(f): 4652]. Additionally, we found a side product with a mass difference of + 67 [*M*(found): 4737]. These findings indicate the formation of the corresponding aspartimide peptide (−18) and some of the corresponding piperidide. Owing to the two possible routes of a nucleophilic attack to an aspartimide, the treatment of this crude product with aqueous NaOH led consistently to the formation of two products with the mass of the desired product [*M*(found): 4670] indicating the formation of the corresponding α- and β-peptides, and to the remaining piperidide in accordance with HPLC/ES-MS studies (not shown here). Surprisingly, changing the configuration of amino acids in the same manner at the carboxylic site of Asp(OBu^t)^9-Leu^{10} in CRH, synthesising D-Thr^{11}-D-Phe^{12}-CRH, no (or only traces) corresponding aspartimide was observed, even when Leu in position 10 was additionally replaced by Gln(Trt). Although we do not know details of the structure resulting from the D-amino acid replacement under the conditions of solid-phase synthesis, the results clearly

Table 1 Piperidide formation on -Asp(OBu^t)-Asn(Trt)- containing peptide sequences[a]

No.	Sequence[b]	Content of desired product in crude product (%)[c]	Ratio of desired product : piperidide product
II	NGG-X	85.1	No piperidide
III	DNGG-X	53.9	1.44
IV	VDNGG-X	46.6	1.44
V	LVDNGG-X	19.8	0.51
VI	VLVDNGG-X	16.8	0.34

[a] Synthesis on Wang-resin with Fmoc-aa-OH/TBTU/HOBt (6 equiv.) and NMM (12 equiv.), Fmoc-deprotection with 20% piperidine–DMF for 15 min, treatment of final peptide resin for 5 h with 50% piperidine–DMF; final cleavage from resin with TFA–H_2O (95 : 5) for 2 h. [b] X: -TGDVTVAPSNF. [c] Determined by reversed-phase-HPLC; peak characterization by ES-MS.



Scheme 1 Base-catalysed aspartimide formation and nucleophilic opening by piperidine or NaOH (aq)

show a conformational influence on aspartimide formation dependent of the position of the replacement. Furthermore, it was demonstrated that the aspartimide formation can cause a complete failure of Fmoc-chemistry using piperidine.

In 1978 Martinez and Bodanszky[4] described an efficient suppression of aspartimide formation caused by an equimolar amount of tertiary amine. The addition of an equimolar quantity of substituted phenols and/or hydroxybenzotriazole results in prevention of aspartimide formation by buffereing the amine as we believe. Surprisingly, the addition of only a relatively small amount of hydroxybenzotriazole or 2,4-dinitrophenol to the Fmoc-deprotecting reagent (0.1 mol dm^{-3} to 20% piperidine–DMF) was almost sufficient for an efficient suppression of the aspartimide formation and can be recommended for syntheses of sensitive sequences using Fmoc-chemistry.

Mrs Annerose Klose is thanked for her skillful technical assistance. Drs Bienert and Beyermann also thank the Fonds der Chemsichen Industrie for financial support.

Received, 16th November 1993; Com. 3/06862E

Footnotes

† HPLC conditions: Polyencap A300, 5 μm, 250 × 4 mm i.d. (Bischoff Analysentechnik GmbH, Germany), mobile phase A; 0.07 mol dm^{-3} phosphate buffer ph 2.3, B: 50% A–50% MeCN, linear gradient 20–80% B in 40 min, 1 ml min^{-1}, detection at 220 nm.

‡ ES-MS obtained on quadrupole mass spectrometer TSQ 700 (Finnigan MAT with electrospray ion source (positive ion mode, syringe infusion with 1 μl min^{-1}, in MeOH–H_2O 1:1 containing 1% acetic acid).

§ TentaGel SRAM, single couplings with 0.3 mol dm^{-3} Fmoc-aa-OH/TBTU with DIEA 2 equiv. for 20 min, deprotection with 25% piperidine–DMF for 10 min, final cleavage with TFA–5% phenol, 5% H_2O, 2% triisopropylsilan for 3 h, protecting groups: Asn,Gln, His(Trt), Arg(Pmc), Asp,Glu(OBu^t), Lys(BOC), Thr,Ser(Bu^t).

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