A Novel Side Reaction in Fmoc-SPPS: Formation of Cyclo(-Xaa-Asp)-Yaa Peptides

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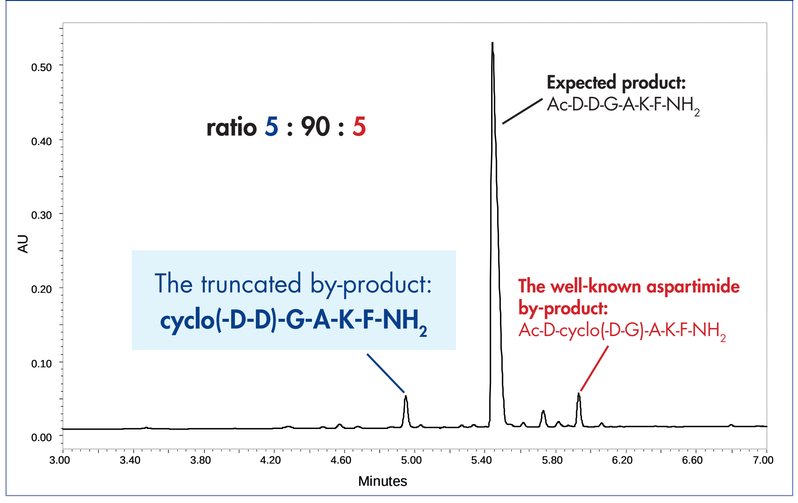
Abstract

- Chain termination during Fmoc-SPPS at the Xaa-Asp-Yaa motif is described
- Resulting by-product is most likely diketopiperazine (DKP) Independently synthesized DKP-reference substance co-elutes on HPLC
- Proposed mechanism: Truncated cyclo(-Xaa-Asp) peptides are formed via nucleophilic attack of N-terminal Xaa amino function at aspartimide intermediate (pathway C, Scheme 1)
- Diminishing aspartimide formation reduces extent of chain termination, e.g.
  - short Fmoc deprotection times
  - Asp β-carboxy protection with OMpe
  - backbone protection at Yaa, as in Asp-(Dmb)Gly

Introduction

Chain termination at the Xaa-Asp-Y aa motif caused by for mation of cyclo(-Xaa-Asp)-Yaa peptides has been identified as a side reaction during Fmoc-SPPS, which is not limited to Aspβ-benzyl esters as initially described in [1].
In contrast to the well-known aspar timide (Asi) for mation at the Asp-Yaa site [2-5], the resulting cyclo(-Xaa-Asp)-Y aa peptides are not acylated during further SPPS and are generally detected as truncated sequences only after TF A cleavage in the crude product of the SPPS (Figure 1 shows a typical HPLC trace).

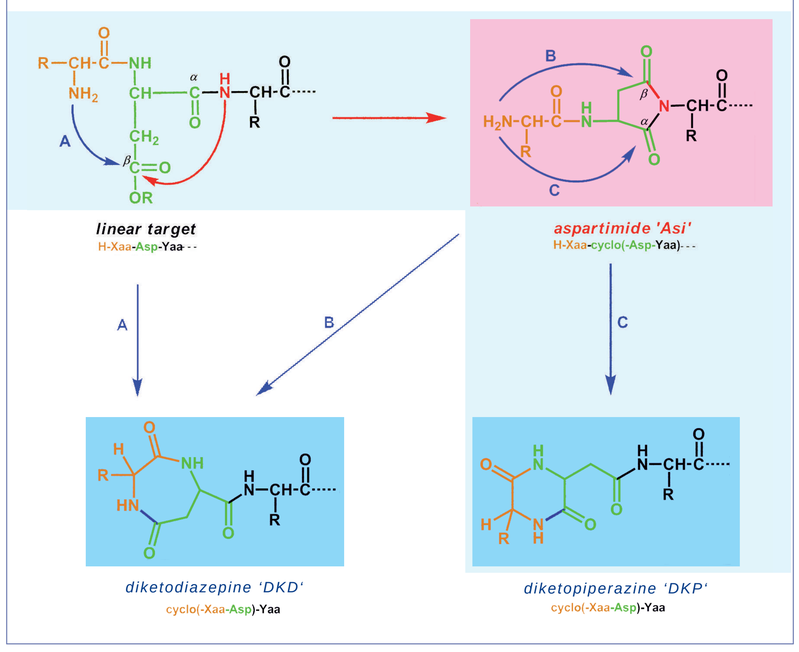
Figure 1: HPLC of crude Ac-D-D-G-A-K-F-NH$_2$



These cyclic by-products are for mally obtained by cyclization via nucleophilic attack of the free amino group of the Xaa residue at either the β-carboxy group of Asp or theα- and β-carbonyl groups of the Asi intermediate after deprotection of Fmoc-Xaa-resin. Thus, it is conceivable that both a 7-membered ring (diketodiazepine, 'DKD') following pathways A or B in Scheme 1 and / or a 6-membered ring (diketopiperazine, 'DKP') following pathway C in Scheme 1 can be formed.

The peptide sequence Ac-Xaa-Asp-Yaa-Ala-Lys-Phe-NH$_2$ has been used to investigate the influence of different parameters such as the flanking amino acid residues Xaa and Yaa, the Fmoc cleavage conditions and the Asp β-carboxy protecting group on the side reaction. Peak assignment was performed using LC-MS analysis of the crude peptides.

Scheme 1: Possible chain termination pathways A, B and C



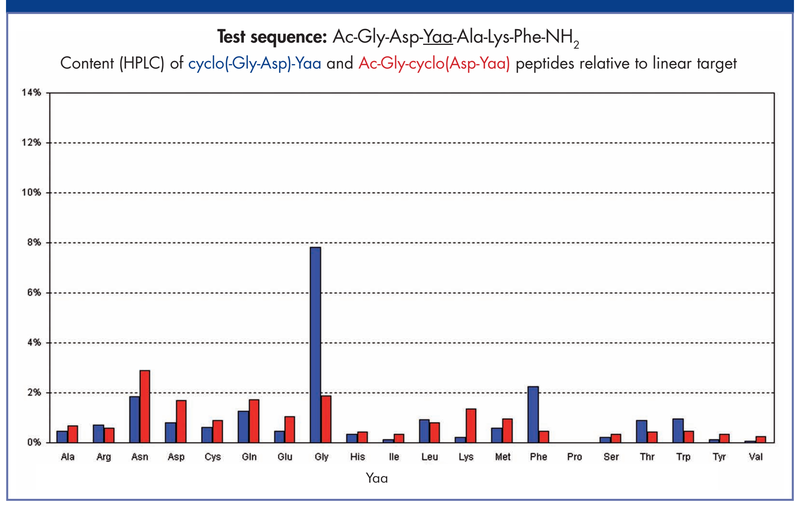
Influence of the Flanking Residues Xaa and Yaa

Two peptide series were synthesized with Xaa = Gly and Yaa = all 20 natural amino acids (Figure 2) or Xaa = all 20 natural amino acids and Yaa = Gly (Figure 3). The obtained amounts of cyclo(-Xaa-Asp)-Y aa peptide (blue bars) and the corresponding aspartimides (red bars) are depicted relative to linear target.

Within the Xaa = Gly series, chain ter mination was most prominent with Yaa = Gly (8% of cyclo(-Gly-Asp) peptide detected, see Figure 2). As expected for N-alkyl amino acids, no aspar timide was found for Yaa = Pro or when Yaa = Gly was incorporated as backbone-protected Fmoc-(Dmb)Gly-OH. Furthermore, no cyclo(-Gly-Asp) peptides were observed in these cases. Thus, chain ter mination via pathway A in Scheme 1 can be excluded.

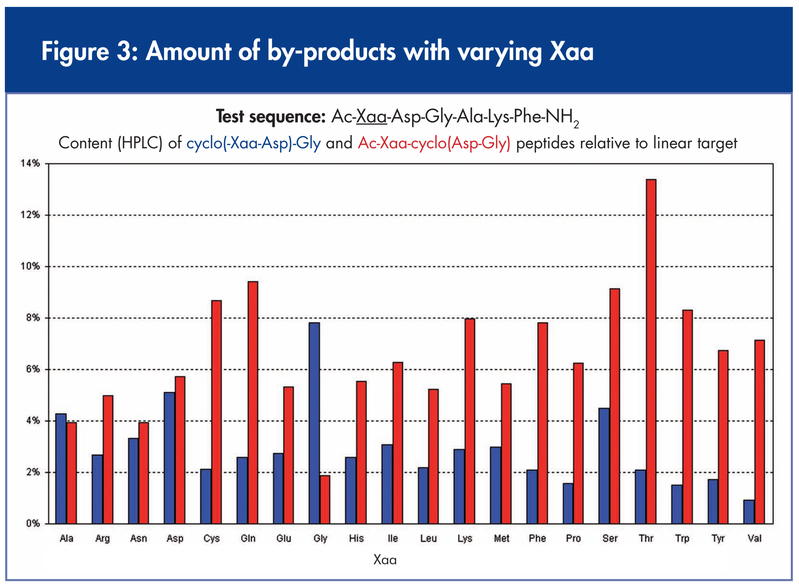
The diketopiperazine cyclo(-Gly-Asp)-Gly peptide was synthesized independently. HPLC co-elution of this reference compound with the corresponding truncated sequence indicates that chain ter mination occurs via pathway C (see Scheme 1).

Figure 2: Amount of by-products with varying Yaa



In the series with constant Y aa = Gly, the 'worst case motif ' for aspartimide formation, highest levels of cyclo(-Xaa-Asp) peptides were obtained for Xaa = Gly and Asp (blue bars in Figure 3). Additionally, the corresponding aspar timides (red bars in Figure 3) were detected in amounts ranging from 2-13%. Hence, the nature of the residue Xaa unexpectedly infl uences the level of aspar timide formed at the Asp-Yaa motif. Xaa also determines the extent of subsequent truncated peptide formation via aspar timide ring opening by its N-ter minal amino function.

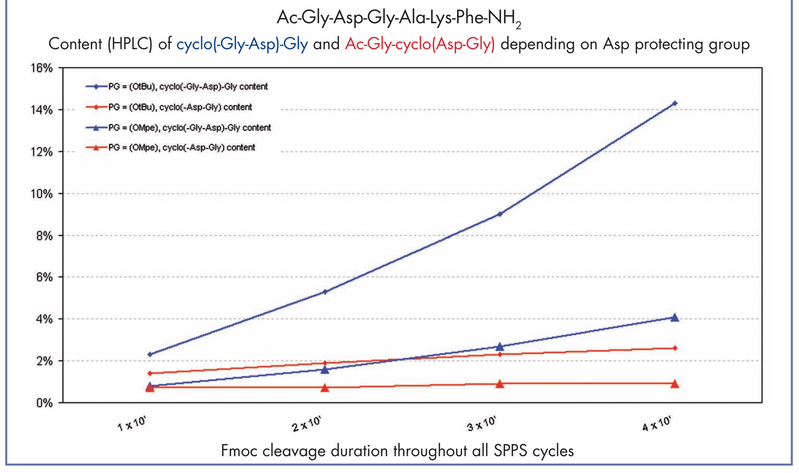
Figure 3: Amount of by-products with varying Xaa



Influence of Asp β-carboxy Protecting Group and Fmoc Deprotection Reaction Time

The peptide Ac-G-D-G-A-K-F-NH $_2$ was synthesized using OtBu or OMpe as Asp side-chain protecting group and different reaction times for Fmoc removal. Compared to OtBu, OMpe reduces for mation of aspartimide [3] and consequently of cyclo(-Gly-Asp) peptide. Longer Fmoc cleavage reaction times lead to increasing levels of aspar t-imide (red lines in Figure 4) and of the truncated cyclo(-Gly-Asp) peptide (blue lines in Figure 4).

Figure 4: Influence of Asp(PG) and Fmoc cleavage duration



A detailed evaluation of the reaction mechanism yielding cyclo(-Xaa-Asp)-Yaa peptides with structure elucidation of these truncated by-products and the influence of residue Xaa on aspartimide formation is ongoing and will be presented elsewhere in due course.

Experimental Procedures

All peptides were synthesized on Ramage resin (150 µmol scale). Couplings were per formed with amino acid derivative or AcOH and TBTU/DIPEA. If not stated other wise, Asp was introduced as Fmoc-Asp(OtBu)-OH and Fmoc was removed using 20% piperidine in DMF (2x15 min). The crude peptides were obtained after cleavage with aqueous TFA and analyzed with rapid HPLC (Waters Acquity C_{18}, 1.7 µm; linear gradient of ACN in 0.1% TF A; flow 0.4 mL / min, λ = 220 nm).

References

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