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

Introduction

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

These cyclic by-products are formally obtained by cyclization via nucleophilic attack of the free amino group of the Xaa residue at either the β-carbonyl group of Asp or the amidic β-carbonyl group of the Asp(Yaa) 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-Gly-Ala-Lys-Phe-NH2 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/Yaa protecting group on the side reaction. Peak assignments were performed using LC-MS analysis of the crude peptides.

Figure 1: HPLC of crude Ac-D-D-G-A-K-F-NH2
In the series with constant Yaa = 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 aspartimides (red bars in Figure 3) were detected in amounts ranging from 2-13%. Hence, the nature of the residue Xaa unexpectedly influences the level of aspartimide formed at the Asp-Yaa motif. Xaa also determines the extent of subsequent truncated peptide formation via aspartimide ring opening by its N-terminal amino function.

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)-Yaa peptide (blue bars) and the corresponding aspartimides (red bars) are depicted relative to linear target.

Within the Xaa = Gly series, chain termination was most prominent with Yaa = Gly (8% of cyclo(Gly-Asp) peptide detected, see Figure 2). As expected for NαKyl amino acids, no asparagine was formed 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 termination 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 termination occurs via pathway C (see Scheme 1).

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

The peptide Ac-G-D-G-A-K-F-NH2 was synthesized using OtBu or OMpe as Asp side-chain protecting group and different reaction times for Fmoc removal. Compared to OtBu, OMpe reduces formation of aspartimide [3] and consequently of cyclo(Gly-Asp) peptide. Longer Fmoc cleavage reaction times lead to increasing levels of aspartimide formation (red lines in Figures 3 and 4) and of the truncated cyclo(Gly-Asp) peptide (blue lines in Figure 4).

Figure 4: Influence of Asp β-carbonyl Protecting Group and Fmoc Deprotection Reaction Time

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 a RAMAGE resin (150 μmol scale). Couplings were performed with amino acid derivative or AcOH and TBDMSCl/DIEA. If not stated otherwise, 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 high pressure liquid chromatography (Waters Acquity C18, 220 nm).

References