FMOC-SPPS CHAIN TERMINATION

DUE TO FORMATION OF N-TERMINAL PIPERAZINE-2,5-DIONES: STRUCTURE ELUCIDATION OF A BY-PRODUCT BY NMR AND PROPOSAL FOR THE MECHANISM OF FORMATION

M. Minuth[1)], D. Rentsch[2)], and D. Samson[1)]

[1)] Bachem AG, Bubendorf, Switzerland [2)] Swiss Federal Laboratories for Materials Science and Technology (EMPA), Dübendorf, Switzerland

INTRODUCTION

Aspartimide (Asi) formation is a notorious side reaction in peptide synthesis that is well characterized and described in literature.[1-3] In addition, nucleophilic ring opening of the Asi intermediate is extensively described. In this context cyclo(-Xaa-Asp)-Yaa peptides, which can either be N-terminal 6-membered piperazine-2,5-diones (DKP) or 7-membered diazepine-2,5-diones (DKD) via Asi intermediates (Figure 1) were identified.[4] Deamidation and/or Asi-related synthetic pathways for Asp- and Asn containing peptides have been described.[5]
We observed Fmoc-SPPS chain termination at the N-terminal Xaa-Asp-Yaa motif apparently caused by formation of cyclo(-Xaa-Asp)-Yaa peptides. In the context of SPPS such Asi-related cyclizations, possibly leading to either DKDs and/or DKPs, are so far exclusively known for of Asp β-benzyl esters.[6] To the best of our knowledge there was no published study describing whether a 6- or 7-membered species or a mixture is formed and to what extent this side-reaction occurs when we initiated our investigations.[7]

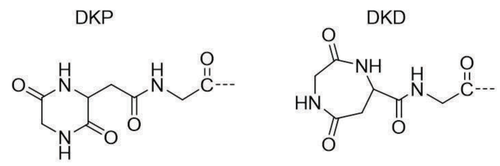


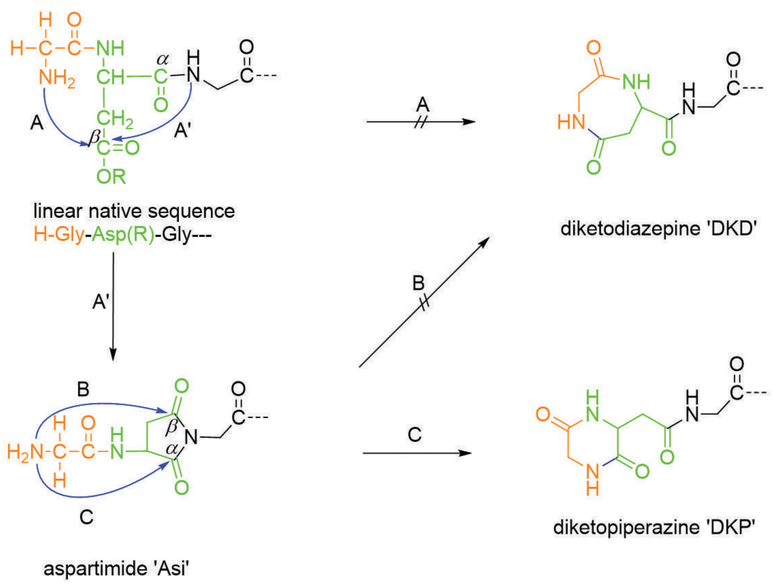
Figure 1: N-terminal 6-membered piperazine-2,5-dione (DKP) vs. 7-membered diazepine-2,5-dione (DKD).

Although ESI-MS studies of cyclic dipeptides are described in literature we were not able to discriminate between the two possible cyclic structures (i.e. 6- vs. 7- membered ring) by MS. Obviously, cyclo(-Xaa-Asp)-Yaa peptides are not amenable for further acylation. Hence, they are present as truncated sequences in the crude material of the SPPS.
We chose the model peptide Ac-Gly-Asp-Gly-Ala-Lys-Phe-NH_2 for further investigation. Its DKP analog was independently synthesized, and extensive NMR studies were performed for structural elucidation.
In addition, we investigated the impact of flanking amino acid residues on the side reaction.[7]

RESULTS AND DISCUSSION

Chain termination at Xaa-Asp-Yaa motif

During Fmoc-SPPS cyclo(-Xaa-Asp)-Yaa peptides may be formed by cyclization via nucleophilic attack of the free amino group of the Xaa residue at either the β-carboxy group of Asp and/or the α- and β-carbonyl groups of an Asi intermediate (if present) after deprotection of Fmoc-Xaa-peptide-resin. It is thus conceivable that both a 7-membered DKD following pathways A or B in Scheme 1, and/or a 6-membered DKP following pathway C can be formed.[6] However the formation of a 7-membered DKD was not observed in this study.



Scheme 1: Xaa-Asp-Yaa motif: Xaa, Yaa = Gly for simplicity. R = Asp carboxylic acid protecting group. Alternative possible chain termination pathways A, B and C for nucleophilic attack of N-terminal amine at Asp-carboxy or at carbonyl groups of Asi intermediate. A' for nucleophilic attack of secondary amine at Asp-carboxy group. No formation of DKD was observed in this study. Stereochemistry omitted for simplification.

Influence of the flanking residues Xaa and Yaa

To investigate the impact of the neighboring amino acids in the peptide chain, two sets of peptides each consisting of twenty peptides were synthesized. One set used fixed Xaa = Gly and Yaa = 20 common amino acids and the second set used Xaa = 20 common amino acids and fixed Yaa = Gly (Figure 7).[7]

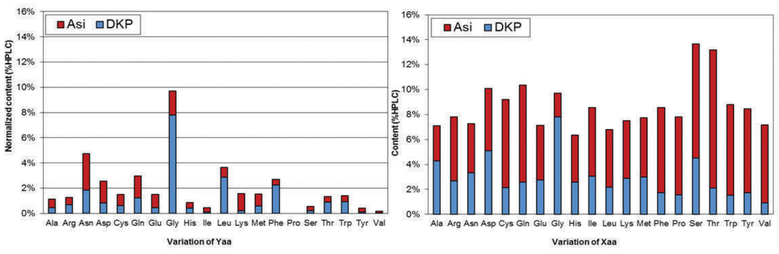
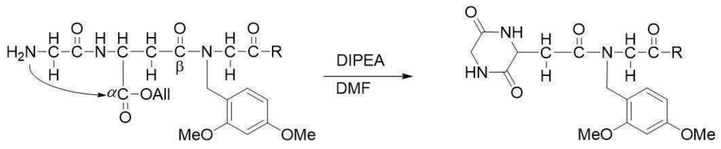


Figure 7: Amount of by-products with variation of Xaa and Yaa (sequence = Ac-Xaa-Asp-Gly-Ala-Lys-Phe-NH_2) relative to linear target.

Structure elucidation of the synthesized DKP reference compound

The DKP reference compound of the model peptide Ac-Gly-Asp-Gly-Ala-Lys-Phe-NH_2 was synthesized. Therefore, the linear precursor H-Gly-β-Asp(OAll)-(Dmb)Gly-Ala-Lys(Boc)-Phe-NH-® (Scheme 2) with a β-Asp was synthesized and cyclized on the resin. The β-Asp-OAll protection in conjunction with Gly backbone blockage excludes Asi formation and thus allows for direct and straightforward formation of the 6-membered DKP. Allyl alkoxide is obviously an excellent leaving group. Hence, cyclization occurs without additional activation under mild basic conditions. The potential competing formation of the respective 7-membered DKD is excluded by using this approach.



Scheme 2: On-resin formation of cyclo(-Gly-Asp)-(Dmb)Gly-Ala-Lys(Boc)-Phe-NH-®. Subsequent TFA cleavage from the resin/ deprotection of side chain and backbone protecting groups yielding the crude DKP reference

From the 2D NMR experiments the chemical structure including the 6-membered C-terminal ring shown in Figure 2 was unambiguously identified. The main characteristics of a 6-membered cycle is obtained from the ^1H-^{13}C long range correlations of amide protons (Figure 2), where the correlations of H-2 and H-5 to carbons C-1 and C-4 confirm the presence of this cycle. For a 7-membered ring (such as described in [6]) the correlation of H-5 to C-1 would not be observed.

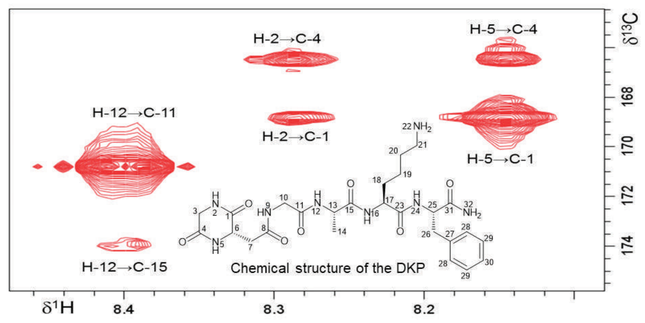


Figure 2: Region showing correlations of amide protons to amide carbons of the DKP reference with assignment of crucial ^1H-^{13}C HMBC NMR correlations from H-2 and H-5 to C-1,4 (spectrum recorded with selection of 4 Hz H-C coupling constant).

The formation of the 6-membered ring of the DKP was further confirmed by correlations observed in the ^1H-^{15}N HMBC NMR experiments (Figure 3). The correlations found for methylene protons H-3 to nitrogens N-2 and N-5 indicate the presence of a cycle. As highlighted by circles in Figure 3 the exocyclic protons H-7 correlate to N-5 of the cycle and to N-9 of residue Gly[3]. This is a further proof for the presence of the 6-membered cycle.

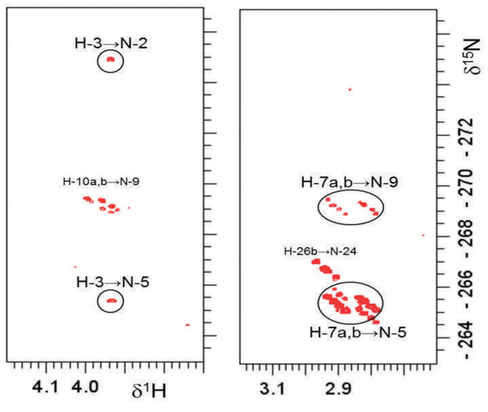


Figure 3: Regions of interest with assignment of crucial ^1H-^{15}N HMBC NMR correlations for the DKP reference. The spectra were recorded in DMF-d_7 with selection of 4 Hz (left) and 2 Hz (right) long range couplings.

Structure elucidation of 6-membered DKP in crude native peptide

Most of the NMR resonances identified for the linear peptide sequence and for the synthesized compound are also observed in the spectra of the crude native peptide. DKP product are almost identical, of course with exception of the N-terminal regions of the sequences. Figure 4 shows an overlay of ^1H-NMR spectra of the crude native peptide and the synthesized 6-ring DKP reference compound. The signals of the reference compound are also observed in the spectra of the crude native peptide.

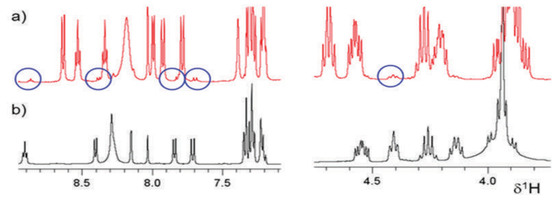


Figure 4: ^1H-NMR of a) crude native peptide and b) 6-ring peptide in DMF with signals of the 6-membered ring peptide by-product indicated in the spectrum of the crude native peptide.

HSQC NMR spectra of the crude native peptide and of the purified DKP reference material are shown in Figure 5. It can be clearly observed that cross peaks assignable to positions 3, 6 and 7 of the DKP are also present in the spectrum of the crude native peptide. Additionally, slightly less split cross peaks are observed for positions 10, 13 and 17 assigned to residues Gly[3], Ala[4] and Lys[5], respectively.

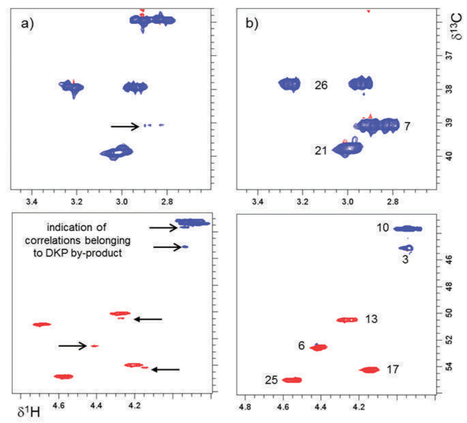


Figure 5: Enlarged regions of ^1H-^{13}C HSQC NMR spectra recorded in DMF-d_7 of a) crude native peptide with indication of residual resonances of the DKP by-product and b) synthesized purified DKP product with assignment of correlations.

Further evidence for the presence of the DKP by-product in the crude native peptide are the correlation signals somewhat outstanding of the main resonances detectable in the HMBC and DQF-COSY NMR spectra appearing at exactly the same positions as in the spectra recorded for the independently synthesized DKP reference (Figure 6).

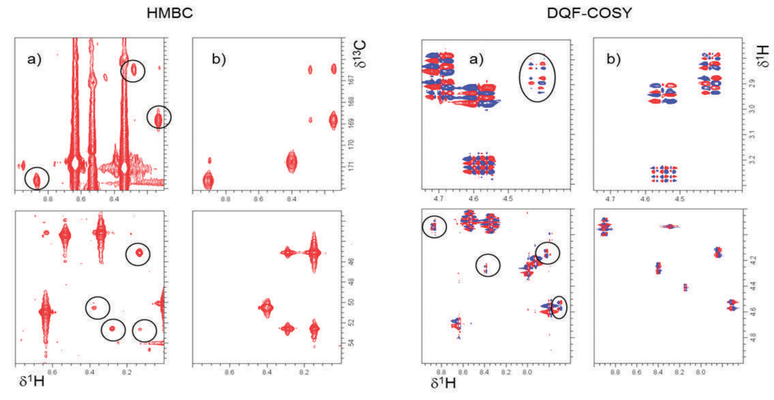


Figure 6: Enlarged regions of ^1H-^{13}C HMBC and ^1H-^1H DQF-COSY NMR spectra recorded in DMF-d_7 of a) crude native peptide with indication of residual resonances assignable to the DKP by-product and b) synthesized purified DKP reference.

CONCLUSIONS

- Chain termination during Fmoc-SPPS at the Xaa-Asp-Yaa motif is described
- NMR data of the model peptide Ac-Gly-Asp-Gly-Ala-Lys-Phe-NH_2 peptide and its DKP analog clearly indicate that the by-product is an N-terminal DKP.
- Formation of the 6-membered ring of the DKP was further confirmed by NMR structure elucidation.
- 7-membered DKD is not found
- Truncated DKP is formed via nucleophilic attack of the N-terminal Xaa amino function at the α-carbonyl of the Asi intermediate

Diminishing aspartimide formation reduces extent of chain termination, e.g.
- Short Fmoc deprotection times
- Acidic modifiers[8]
- Asp β-carboxy protection with OMpe or trialkylcarbinol based protecting groups[10]
- Backbone protection at Yaa[9]

REFERENCES

[1] Subirós-Funosas R, El-Faham A, Albericio F, *Tetrahedron*. **2011;** *67*(45): 8595-8606.
[2] Mergler M, Dick F, *J. Pept. Sci.* **2005;** *11*(10): 650-657.
[3] Bodanszky M, Kwei JZ, Peptides. *Int. J. Pept. Protein Res.* **1978**; *12* (2) 69-74.
[4] Schön I, Kisfaludy L, *Int. J. Pept. Protein Res.* **1979;** *14*(5): 485-494.
[5] Curnis F, Cattaneo A, Longhi R, et al., *J. Biol. Chem.* **2010;** *285*(12): 9114-9123.
[6] Süli-Vargha H, Schlosser G, Ilaš J, *J. Pept. Sci.* **2007;** *13*(11): 742-748.
[7] Samson D, Rentsch D, Minuth M, Meier T, Loidl G, *J Pep Sci.* **2019;** e3193. https://doi.org/10.1002/psc.3193
[8] Subirós-Funosas R, El-Faham A, Albericio F, *Pept. Sci.* **2012;** *98*(2), 89-97.
[9] Packman LC, *Tetrahedron Lett.* **1995;** *36*(41), 7523-7526.
[10] Behrendt R, Huber S, White P, *J. Pept. Sci.* **2016;** *22*(2), 92-97.