

RESEARCH ARTICLE

The aspartimide problem persists: Fluorenylmethyloxycarbonyl-solid-phase peptide synthesis (Fmoc-SPPS) chain termination due to formation of N-terminal piperazine-2,5-diones

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Aspartimide (Asi) formation is a notorious side reaction in peptide synthesis that is well characterized and described in literature. In this context, we observed significant amounts of chain termination in Fmoc-SPPS while synthesizing the N-terminal Xaa-Asp-Yaa motif. This termination was caused by the formation of piperazine-2,5-diones. We investigated this side reaction using a linear model peptide and independently synthesizing its piperazine-2,5-dione derivative. Nuclear magnetic resonance (NMR) data of the side product present in the crude linear peptide proves that exclusively the six-membered ring is formed whereas the theoretically conceivable seven-membered 1,4-diazepine-2,5-dione is not found. We propose a mechanism where nucleophilic attack of the N-terminal amino function takes place at the α -carbon of the carbonyl group of the corresponding Asi intermediate.

In addition, we systematically investigated the impact of (a) different adjacent amino acid residues, (b) backbone protection, and (c) side chain protection of flanking amino acids. The side reaction is directly related to the Asi intermediate. Hence, hindering or avoiding Asi formation reduces or completely suppresses this side reaction.

KEYWORDS1,4-diazepine-2,5-diones, Asp-Gly motif, Asp β -carboxy protection, aspartimides, backbone protection, chain termination, diketodiazepines, diketopiperazines, Fmoc-SPPS, piperazine-2,5-diones, truncated sequences, Xaa-Asp-Yaa motif**1 | INTRODUCTION**

Aspartimide (Asi) formation is a notorious side reaction in peptide synthesis that is well characterized and described in literature.¹⁻⁶ In

addition, nucleophilic ring opening of the Asi intermediate is extensively described in literature (eg, Subirós-Funosas et al and Wade et al^{1,7}). The formation of cyclic by-products in liquid phase peptide synthesis was previously observed and described. In this context,

Abbreviations: As recommended in *J. Peptide Sci.* 1999; 5: 465-471, with the following additions and variations: Asi (according to Albericio [4]), Aspartimide; Dmb, 2,4-dimethoxybenzyl; DKD, 1,4-diazepine-2,5-diones (diketodiazepines); DKP, piperazine-2,5-diones (diketopiperazines); EDT, 1,2-ethane-dithiol; Hmb, 2-hydroxy-4-methoxy benzyl; iPrOH, isopropanol; LC-MS, liquid chromatography coupled with mass spectrometry; OMpe, 3-methylpent-3-yl ester; PG, protecting group; PS, polystyrene; RT, retention time; RRT, relative retention time; J, coupling constant

A preliminary account of this work was presented. D. Samson and G. Loidl, A novel side reaction in Fmoc-SPPS: Formation of cyclo(-Xaa-Asp)-Yaa peptides. *Proceedings of the 22nd American Peptide Symposium, San Diego 2011* 58-59 (2012).

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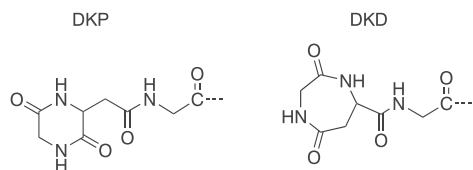


FIGURE 1 N-terminal six-membered piperazine-2,5-dione (DKP) vs seven-membered diazepine-2,5-dione (DKD)

cyclo(-Xaa-Asp)-Yaa peptides that can either be N-terminal six-membered piperazine-2,5-diones (DKP) or seven-membered diazepine-2,5-diones (DKD) via Asi intermediates (Figure 1) were identified.⁸ Deamidation and/or Asi-related synthetic pathways for Asp- and Asn containing peptides have been described.^{9–11}

We observed Fmoc-SPPS chain termination at the N-terminal Xaa-Asp-Yaa motif apparently caused by formation of cyclo(-Xaa-Asp)-Yaa peptides. These peptides were detected and quantified by LC-MS as minor components in crude material from SPPS after Trifluoroacetic acid (TFA) cleavage. In the context of SPPS such Asi-related cyclizations, possibly leading to either DKDs and/or DKPs, are so far exclusively known for Asp β -benzyl esters. To the best of our knowledge, there is no published study describing whether a six- or seven-membered species or a mixture is formed and to what extent this side-reaction occurs.^{12,13}

Although Electrospray ionization-Mass spectrometry (ESI-MS) studies of cyclic dipeptides are described in literature,¹⁴ we were not able to discriminate between the two possible cyclic structures (ie, six- vs seven-membered ring) by MS. Therefore, the nature of this side reaction was not known when we initiated our investigations.^{4,15} 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. This is in contrast to the Asi formation at the Asp-Yaa site, where chain elongation is readily possible.

We chose the model peptide Ac-Gly-Asp-Gly-Ala-Lys-Phe-NH₂ 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. Also, the impact of backbone and side chain protection is discussed.

2 | RESULTS AND DISCUSSION

2.1 | 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. Aspartimides are ubiquitous by-products in peptides with the Asp-Yaa motif synthesized via Fmoc-SPPS. It is thus

*Our attempts to synthesize the DKD analog were not successful and mainly unreacted linear peptide or intermolecular oligomers were obtained, data not shown.

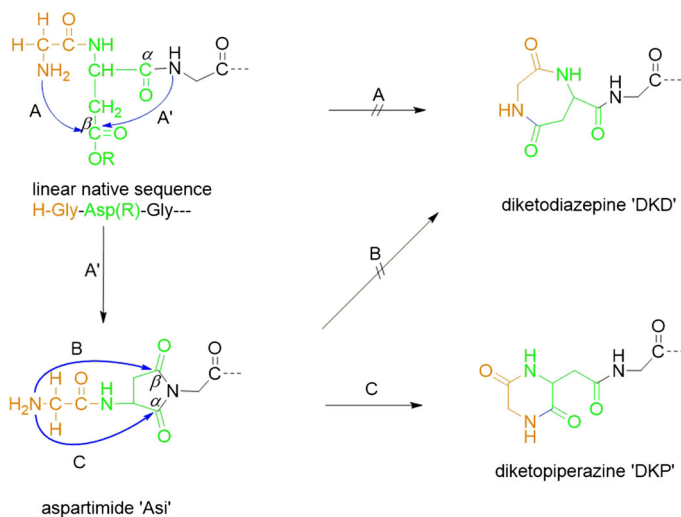
conceivable that both a seven-membered DKD following pathways A or B in Scheme 1 and/or a six-membered DKP following pathway C can be formed.¹³ However, the formation of a seven-membered DKD was not observed in this study.

2.2 | Structure elucidation of the synthesized DKP reference compound

The DKP reference compound of the model peptide Ac-Gly-Asp-Gly-Ala-Lys-Phe-NH₂ 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 six-membered DKP. Allyl alkoxide is obviously an excellent leaving group. Hence, cyclization occurs without additional activation under mild basic conditions. Please note that the potential competing formation of the respective seven-membered DKD is excluded by using this approach.

The chemical structure of the DKP is shown in Figure 2. All ¹H, ¹³C, and ¹⁵N NMR chemical shifts for the independently synthesized reference compound were unambiguously assigned via the corresponding 1D and 2D NMR experiments (a detailed NMR resonance assignment with all correlations observed is summarized in the Supporting Information). On the basis of the observed spin systems, the assignment of resonances to the individual amino acids Phe,⁶ Lys,⁵ Ala,⁴ and Gly³ towards the C-terminus was straightforward. With the aid of crucial ¹H-¹³C HMBC correlations to the amide carbons and of cross signals of neighboring amide protons in the ROESY NMR spectra, the linkages between the four amino acid residues (Gly-Ala-Lys-Phe) were clearly identified. In the NMR spectra of the linear peptide sequence, all chemical shifts identified for these four amino acid residues are almost identical to the chemical shifts evaluated for the DKP product (see data in the Supporting Information). For the linear peptide, the NMR data of the N-terminal acetylated Gly¹ and the Asp² residue were unambiguously assigned as well. In the case of the purified DKP reference material NMR resonances assignable to three amide, two methylene, and one methine groups were identified, all groups belonging to the originally present Gly¹ and Asp² residues. Apparently, both signals of the acetyl protecting group originally identified for the linear peptide were absent. In addition, it was clear from the DQF-COSY NMR spectrum that the detected methine group was directly bound to one of the methylene groups, whereas the remaining methylene group was present solitary.

From the 2D NMR experiments, the chemical structure including the six-membered C-terminal ring shown in Figure 2 was unambiguously identified. The main characteristics of a six-membered cycle is obtained from the ¹H-¹³C long range correlations of amide protons (Figure 3, where the correlations of H-2 and H-5 to carbons C-1 and C-4 confirm the presence of this cycle. For a seven-membered ring (such as described in Süli-Vargha et al¹³), the correlation of H-5 to C-1 would not be observed. Although the long range correlation of H-9 to C-7 is rather weak, it is a further proof that the methylene



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 diazepine-2,5-diones (DKD) was observed in this study. Stereochemistry omitted for simplification.

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 piperazine-2,5-diones (DKP) reference compound is not shown

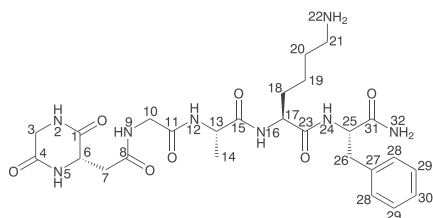


FIGURE 2 Chemical structure of the piperazine-2,5-diones (DKP) with assignments of positions used for ^1H , ^{13}C , and ^{15}N chemical shifts

group C7 must be exocyclic (for a full list of long range correlations, see the Supporting Information). All long range correlations of NH protons to the directly bound amide carbons are usually strong (eg, prominent cross signals of H-12 \rightarrow C-11, Figure 3), but only weak correlations were observed for H-2 \rightarrow C-1 or H-5 \rightarrow C-4, respectively. This could be associated with the six-membered ring structure (ring strain). Consistently with the identification of a similar DKP ring system, no (or only a weak) HMBC correlation from an endocyclic amide proton onto the directly bound amide carbon was reported¹⁶ (see Supporting Information). All additional ^1H - ^{13}C heteronuclear multiple bond correlation (HMBC) correlations of protons of interest to amide carbons further confirm the formation of a cyclic product of residues Gly1 and Asp2 (H-3 \rightarrow C-1, C-4; H-6 \rightarrow C-1, C-4, C-8; H-7 \rightarrow C-1, C-8; H-10 \rightarrow C-8, C-11, see the Supporting Information).

The formation of the six-membered ring of the DKP was further confirmed by correlations observed in the ^1H - ^{15}N HMBC NMR experiments. From the ^1H and ^{13}C NMR assignments, the resonances attributed to Gly,³ Ala,⁴ Lys,⁵ and Phe⁶ were already known. Subsequently,

the amide nitrogen atoms were assigned (positions 9, 12, 16, 22, 24, and 32, see Figure 2), and, therefore, the two remaining nitrogen atoms must belong to positions 2 and 5. 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 4, the exocyclic protons H-7 correlate to N-5 of the cycle and to N-9 of residue Gly.³ In aqueous solution, additionally, the correlations of the methine proton H-6 to exactly the same nitrogens N-2 and N-5 as for H-3 of the solitary methylene group were observed (see the Supporting Information). This is a further proof for the presence of the six-membered cycle.

The most prominent by-product in the crude product mixture of the model peptide[†] has been identified by LC-MS (M-60, m/z = 575.29 u, approximately 4 % peak area in HPLC, see Figure 5). Minor cleavage related impurities (<1% area by HPLC) were identified but are disregarded in this context.

HPLC coelution with the independently synthesized and purified DKP reference compound with the corresponding truncated sequence (see black HPLC trace overlay in Figure 5) indicates that chain termination occurs via pathway C (Scheme 1). Unfortunately, the attempts to synthesize the seven-membered DKD were not successful due to dominating intermolecular reactions instead of the intended intramolecular cyclization (see, eg, Moure et al¹⁷). We synthesized the structure described in the literature¹³ as a seven-membered DKD and could show by NMR experiments that the six-membered DKP structure is formed (data shown in the supporting information).

[†]No potential m/z signal for the analogous DKD was found.

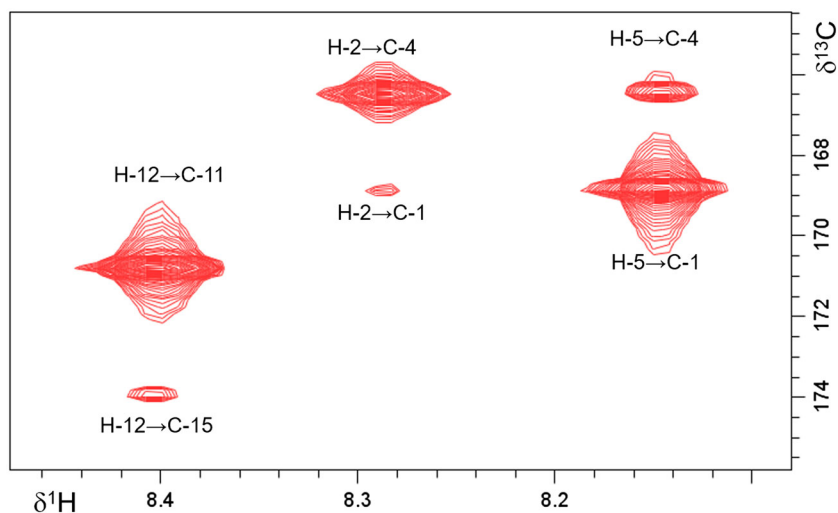


FIGURE 3 Region showing correlations of amide protons to amide carbons of the piperazine-2,5-diones (DKP) reference with assignment of crucial ^1H - ^{13}C HMBC NMR correlations from H-2 and H-5 to C-1,4 (spectrum recorded with selection of 4 Hz H-C coupling constant)

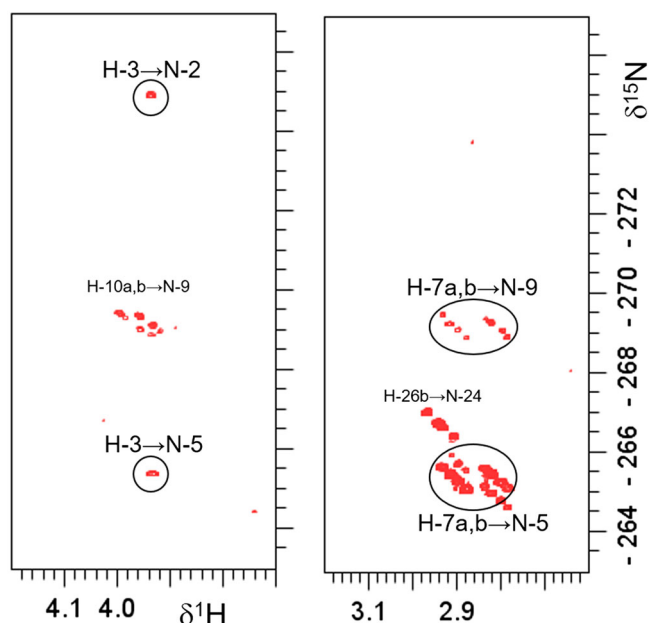


FIGURE 4 Regions of interest with assignment of crucial ^1H - ^{15}N HMBC NMR correlations for the piperazine-2,5-diones (DKP) reference. The spectra were recorded in DMF- d_7 with selection of 4 Hz (left) and 2 Hz (right) long-range couplings

Most of the NMR resonances identified for the linear peptide sequence and for the synthesized DKP product are almost identical (in the 2D NMR spectra as well), of course with exception of the N-terminal regions of the sequences. Figure 6 shows an overlay of ^1H -NMR spectra of the crude native peptide and the synthesized six-ring DKP reference compound. The signals of the reference compound are also observed in the spectra of the crude native peptide.

In Figure 7, enlarged regions of the HSQC NMR spectra of the crude native peptide and of the purified DKP reference material are shown. It can be clearly observed (Figure 7A), 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. 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 (see Figure 8). Signal integration with respect to the ^1H NMR resonance of H-25 at 4.58 ppm resulted in ≈ 4 to 5 mol-% of the DKP by-product in the crude peptide mixture, which is well aligned with the analytical HPLC peak area relative to main peak of 4%. A similar amount was determined from the ^1H NMR data recorded in aqueous solution (integrated ^1H NMR spectra shown in Supporting Information).

It is noteworthy that we did not observe any additional sets of resonances in the NMR spectra of the crude peptide mixture showing noticeable signal intensities (Limit of quantitation (LOQ) at approximately 1%), which could be assigned to a species with a terminal seven-membered ring.

Following the identification of the six-membered ring by-product in the crude mixture of the linear peptide, we made efforts to verify the presence of this species already on-resin prior to cleavage from the resin. In Figure 9B, a section of the HSQC HR-MAS NMR spectrum of the resin bound peptide is shown. It is evident that not all observed correlations are detected at exactly the same ^1H and ^{13}C chemical shifts as in the spectra of the crude linear peptide (Figure 9C). The DMF swollen on-resin species (a) are still protected by t-butoxy groups at residues Asp² and Lys⁵ (effects mainly on positions 7 and 21), (b) the peptide is still linked to the tricyclic amide linker resin (influence mainly on position 25), and (c) the presence of the polystyrene back-bone additionally can slightly modify chemical shifts. All resonances unambiguously assignable to specific positions of the linear peptide (see Supporting Information) are denoted in Figure 9, and the correlations of the six-membered ring by-product are marked by arrows. In the spectral regions shown in Figure 9, an additional correlation in this region of interest (marked by circles,

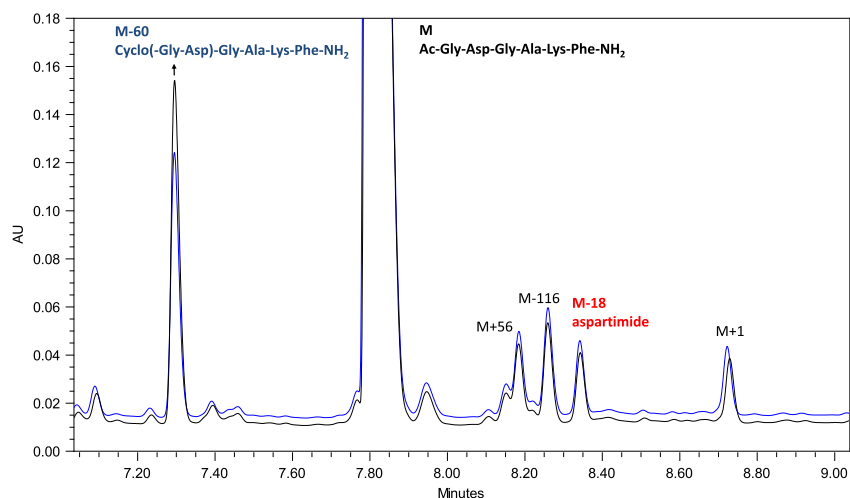


FIGURE 5 HPLC UV trace overlay of crude Ac-Gly-Asp-Gly-Ala-Lys-Phe-NH₂ (blue trace, linear target retention time [RT] 7.85 min) and same crude spiked with piperazine-2,5-diones (DKP) reference (black trace, RT 7.30 min). Peak assignments by liquid chromatography coupled with mass spectrometry (LC-MS) (mass relative to target M)

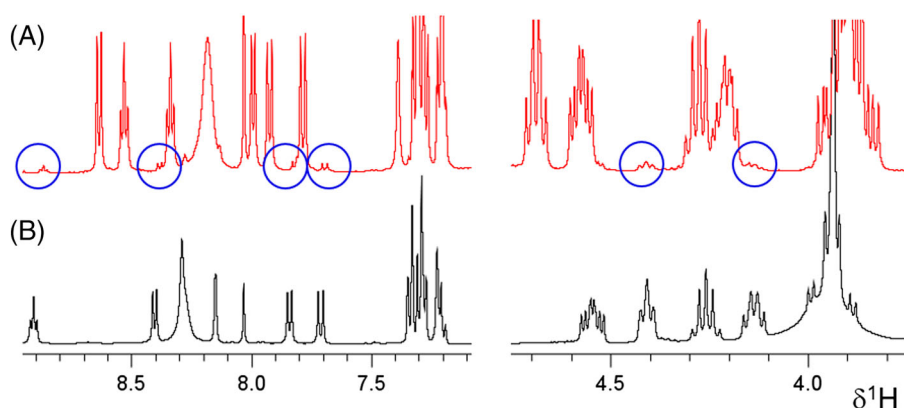


FIGURE 6 ¹H-NMR of (a) crude native peptide and (b) six-ring peptide in DMF with signals of the six-membered ring peptide by-product indicated in the spectrum of the crude native peptide

Figure 9A,B) could be attributed to the tricyclic amide linker resin. In summary, it is clear from our HR-MAS NMR investigation, that the formation of the six-ring by-product already takes place on-resin and that the by-product formation is not a consequence of the reaction conditions during the cleavage of the peptide from the solid support or of the deprotection step.

Although we have not proven that solely the DKP is formed in peptides other than our model peptide, the fact that both NMR data and coelution of the reference compound only show the DKP in case of our model peptide, we conclude that this Asi-related side reaction leads to N-terminal DKP.

After 5 years of storage of the peptide resin from this experiment, the same amount of DKP was found by analytical HPLC. Hence, it can be excluded that the modification occurs on the peptide-resin after the SPPS is completed (data not shown).

2.3 | Influence of the flanking residues Xaa and Yaa in the Ac-Xaa-Asp-Yaa-Ala-Lys-Phe-NH₂ sequence

It is well-known that side reactions such as Asi formation are highly dependent on the peptide sequence^{3,6,18} and on the Asp β-carboxy

protecting group.^{1,2,4,6} The peptide sequence Ac-Xaa-Asp-Yaa-Ala-Lys-Phe-NH₂ has been defined to further investigate the influence of different parameters such as the flanking amino acid residues Xaa and Yaa, the Fmoc cleavage conditions, and the Asp carboxy protecting group on the level of side reaction.

To investigate the impact of the neighboring amino acids in the peptide chain, two sets of peptides each consisting of 20 peptides (general sequence Ac-Xaa-Asp-Yaa-Ala-Lys-Phe-NH₂) were synthesized. One set used fixed Xaa = Gly and Yaa = 20 common amino acids (Figure 10), and the second set used Xaa = 20 common amino acids and fixed Yaa = Gly (Figure 11). In all cases, we found a similar impurity profile with the DKP by-product eluting at relative retention time (RRT) 0.9 and the Asi by-product eluting at RRT 1.1 in the analytical HPLC system. The detected amounts of DKP (blue bars) and the corresponding Asi (red bars) are depicted relative to linear target. The results indicate that the DKP by-products are formed via Asi intermediates. The detected sum of these two may be an indicator of the sequence-specific susceptibility to this side reaction whereas the individual levels of Asi and DKP could also depend on process variables such as, eg, basic and acidic conditions,^{7,8} temperatures, and water content in organic media. In

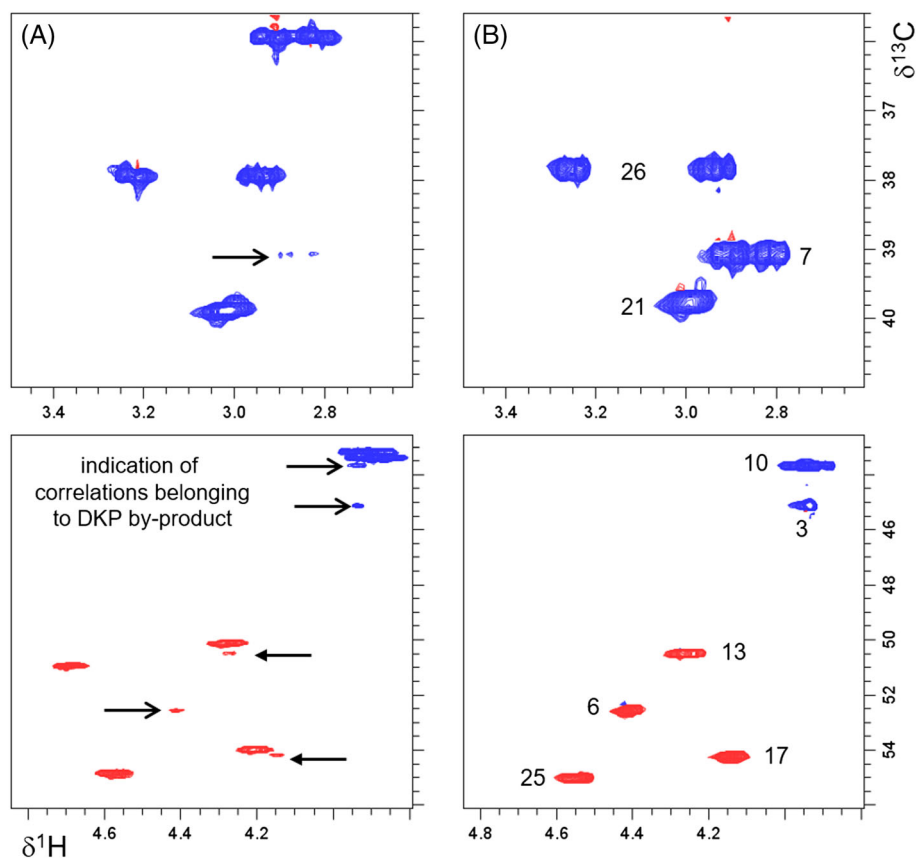


FIGURE 7 Enlarged regions of ^1H - ^{13}C HSQC NMR spectra recorded in DMF-d_7 of (a) crude native peptide with indication of residual resonances of the piperazine-2,5-diones (DKP) by-product and (b) synthesized purified DKP product with assignment of correlations (chemical structure with atom numbering shown in Figure 2)

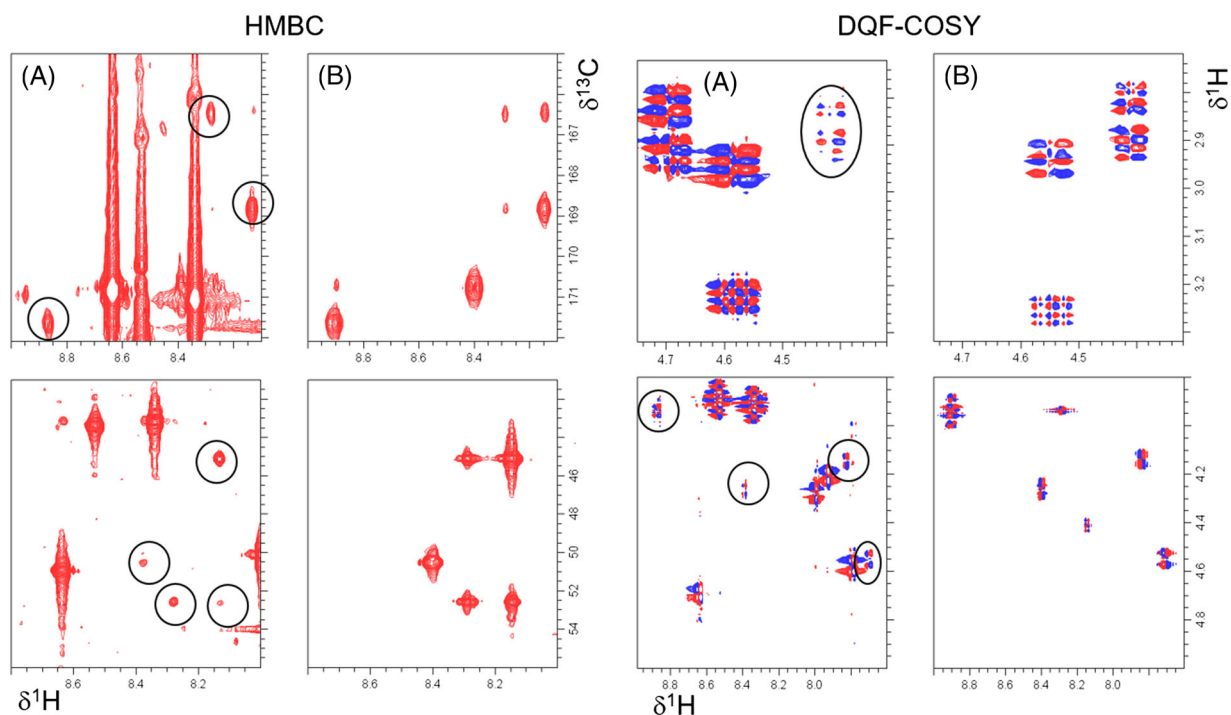


FIGURE 8 Enlarged regions of ^1H - ^{13}C HMBC and ^1H - ^1H DQF-COSY NMR spectra recorded in DMF-d_7 of (a) crude native peptide with indication of residual resonances assignable to the piperazine-2,5-diones (DKP) by-product and (b) synthesized purified DKP reference

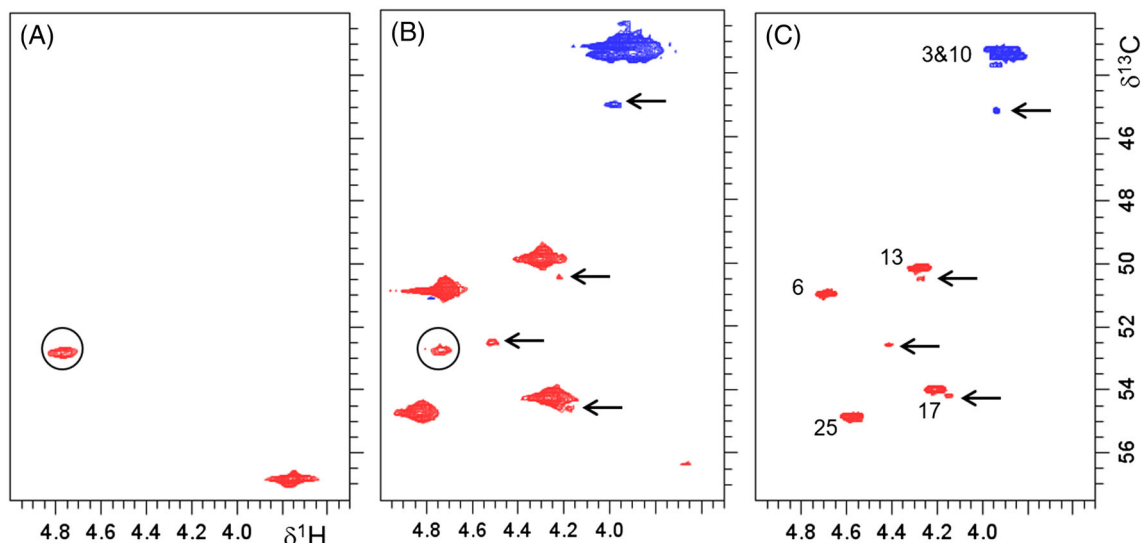


FIGURE 9 Enlarged regions of interest of ^1H - ^{13}C HR-MAS HSQC NMR spectra recorded in DMF-d_7 of (a) tricyclic amide linker, (b) on-resin peptide, and (c) crude peptide with assignment of resonances of the main product. Resonances assigned to the six-ring product in (b) and (c) are labeled by arrows, resonances assignable to the tricyclic amide linker resin in the HR-MAS NMR spectra are marked with circles. The additional correlation at 3.76/56.8 ppm in (a) is assigned to the α amide position of phenylalanine

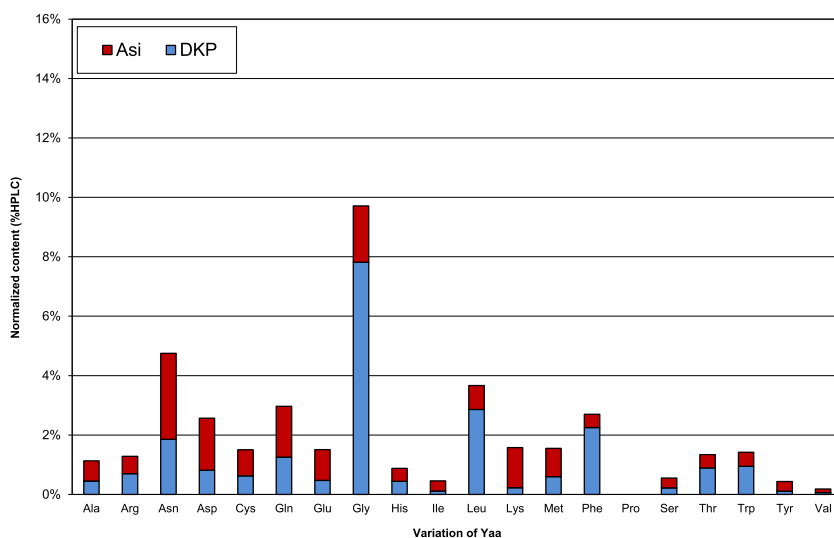


FIGURE 10 Amount of by-products with variation of Yaa (sequence = Ac-Gly-Asp-Yaa-Ala-Lys-Phe-NH₂) relative to linear target

some crude products, we found substantial amounts of the according β -Asp peptides. It is noteworthy that nucleophilic ring opening of Asi also occurs by hydrolysis leading to linear α - β -Asp peptides.^{4,7} The latter is a competing reaction to the herein discussed DKP formation and diminishes the observable Asi level to a certain extent.

In the Xaa = Gly series, chain termination is most prominent with Yaa = Gly (8% of cyclo-(Gly-Asp) peptide detected, see Figure 10). Yaa = Gly is especially prone to Asi formation.^{4,7} As expected for N-alkyl amino acids bearing no acidic backbone amide proton, no Asi by-products were found for Yaa = Pro or when Yaa = Gly was incorporated as backbone-protected Fmoc-(Dmb)Gly-OH (data not shown^{4,19}). Consequently, no cyclo-(Gly-Asp) peptides were observed

in these cases, and it is obvious that chain termination via pathway A in Scheme 1 can be excluded. When incorporating pseudo proline dipeptides in Yaa position, the same effect as for Pro is achieved, and Asi-related chain termination is avoided.²⁰⁻²²

The set of experiments with Yaa = Gly is the "worst case motif" for Asi formation. Highest levels of DKP were detected for Xaa = Gly, Asp, Ser, and Ala (blue bars in Figure 11). Additionally, the corresponding Asi (red bars in Figure 11) was detected in amounts up to 13% depending on Xaa.

Hence, not only adjacent Yaa but also the residue Xaa has a certain contribution to the observed level of Asi at the Xaa-Asp-Yaa motif and consequently of N-terminal DKP. A similar observation was previously described for DKD formation from Asp β -benzyl esters.¹³

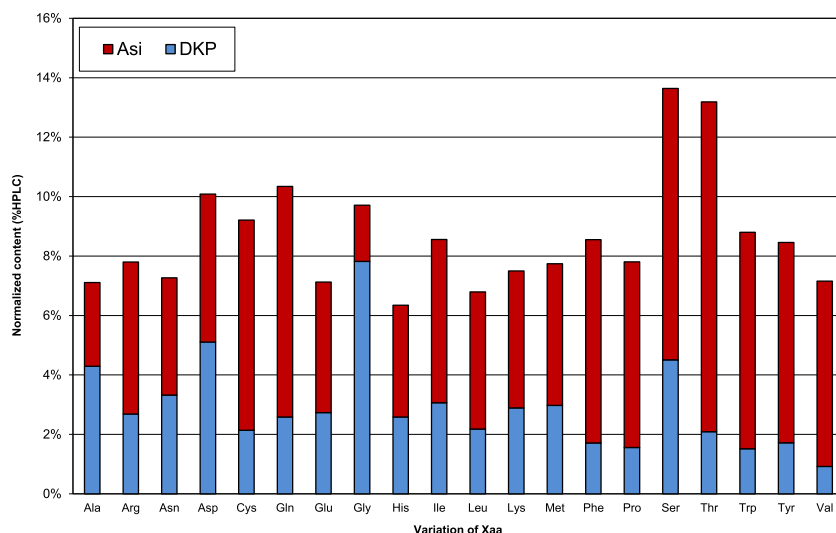


FIGURE 11 Amount of by-products with variation of Xaa (sequence = Ac-Xaa-Asp-Gly-Ala-Lys-Phe-NH₂) relative to linear target

2.4 | Influence of Asp-carboxy protecting groups and Fmoc deprotection conditions

The peptide Ac-Gly-Asp-Gly-Ala-Lys-Phe-NH₂ was synthesized using different Asp protecting groups (β -PG: [OtBu] vs [OMpe]; α -PG: -OtBu) and different reaction times for Fmoc removal.

It is noticeable that the formation of DKP and Asi is increasing linearly with increasing Fmoc deprotection time. The effect is shown in Table 1.

Compared with OtBu, OMpe²³ considerably reduces formation of Asi² and consequently of DKP. In the series, the extent of cyclization is β -PG = (OAll) > (OtBu) > (OMpe) (ie, coupling of α -carboxy of Asp) and even remarkably higher with α -OtBu or α -OAll protection (ie, coupling of β -carboxy of Asp). A similar trend is also known for Asi formation.^{2,4,24} Recently, the OMpe protecting group was surpassed by β -tri-alkylmethyl esters (alkyl = ethyl/propyl/butyl) for Asi

suppression.²⁵ Longer Fmoc cleavage reaction times lead to increasing levels of Asi or of DKP, respectively. Approximately constant levels of the sums of cyclic side products and linear target are observed, which is aligned with the proposal that DKP is formed via ring opening of the Asi intermediates (see Table 1).

TABLE 2 Influence of water content during Fmoc cleavage (2 × 15 min). (Experiments were performed independently of results shown in Figure 9 and Figure 10 resulting in slightly differing results for piperazine-2,5-diones [DKP] and Asi content)

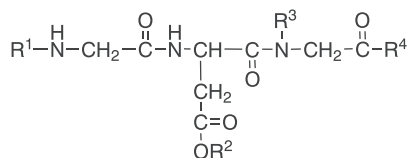
Water content in pip/DMF (%)	% Area HPLC			
	DKP	Linear target sequence	Asi	Sum of linear and cyclic products
0.7 ± 0.2	5.9	89.3	1.2	96.4
5.3 ± 0.4	13.2	80.0	1.8	95.0

TABLE 1 Asp protecting group (PG) and Fmoc cleavage duration variations

Fmoc cleavage duration/min	PG	% Area HPLC			
		DKP	Linear target sequence	Asi	Sum of linear and cyclic products
1 × 10'	α -OtBu	28.0	31.8	23.9	83.7
1 × 10'	β -(OtBu)	2.3	81.6	1.4	85.3
1 × 10'	β -(OMpe)	0.8	83.9	0.7	85.4
2 × 10'	α -OtBu	51.0	14.8	17.4	83.2
2 × 10'	β -(OtBu)	5.4	77.4	1.9	84.7
2 × 10'	β -(OMpe)	1.6	82.4	0.7	84.7
3 × 10'	α -OtBu	69.3	3.8	9.4	82.5
3 × 10'	β -(OtBu)	9.0	74.6	2.3	85.9
3 × 10'	β -(OMpe)	2.7	81.8	0.8	85.3
4 × 10'	α -OtBu	77.1	1.0	3.0	81.1
4 × 10'	β -(OtBu)	14.3	67.8	2.6	84.7
4 × 10'	β -(OMpe)	4.1	80.9	0.9	85.9

TABLE 3 Coupling of Ac-Gly-OH building block and Asp protecting group (PG) variations

Conditions	PG	% area HPLC			Sum of linear and cyclic products
		DKP	Target	Asi	
Fmoc-Gly-OH coupling + piperidine treatment + acetylation	α -OtBu	58.9	5.4	14.4	78.7
	β -(OtBu)	7.1	71.6	7.7	86.4
	β -(OMpe)	1.9	79.1	7.2	88.2
Ac-Gly-OH coupling	α -OtBu	0.6	55.8	23.2	79.6
	β -(OtBu)	0.6	80.4	8.8	89.8
	β -(OMpe)	0.6	82.6	7.9	91.1
Ac-Gly coupling + piperidine treatment	α -OtBu	0.3	2.0	42.2	44.5
	β -(OtBu)	0.4	47.4	26.3	74.1
	β -(OMpe)	0.4	69.1	10.1	79.6



R1 = any amino acid (or derivative) residue
 R2 = OMpe
 R3 = backbone amide protecting group such as Hmb or Dmb
 R4 = amino acid incorporated as pseudoproline derivative (if possible)

FIGURE 12 Xaa⁻¹-Xaa-Asp-Yaa-Zaa motif (Xaa, Yaa = Gly for simplicity). Usage of protecting groups (R² and R³) as well as pseudoprolines during SPPS can reduce or avoid aspartimide (Asi)-related chain termination

To investigate the role of water content in 20% piperidine/DMF mixtures, two control experiments were performed. Two samples of the peptide resin Fmoc-Asp (OtBu)-Gly-Ala-Lys (Boc)-Phe-NH- were treated separately with 20% piperidine/DMF solutions containing 0.7% and 5.3% water, respectively. After Fmoc-Gly-OH coupling a second, Fmoc removal with the same respective water contents in 20% piperidine/DMF followed. The % area of DKP and Asi by-products in both crude samples after final acetylation and cleavage from resin is given in Table 2. It is noticeable that higher water content leads to formation of more cyclic by-products.

A control experiment was performed applying varying conditions for N-terminal Ac-Gly incorporation and with different Asp protecting groups. Ac-Gly-OH instead of Fmoc-Gly-OH followed by Fmoc removal and final acetylation was coupled onto H-Asp (OtBu)-Gly-Ala-Lys (Boc)-Phe-NH- with and without final 20% piperidine/DMF treatment in order to simulate high pH stress due to final Fmoc deprotection.

As expected, almost no DKP is formed in case of coupling Ac-Gly-OH. However, Asi is found, especially when treating the peptide after coupling of Ac-Gly-OH with 20% piperidine/DMF in order to simulate proceeding SPPS (Table 3). This finding supports the formation pathway of DKP via the Asi intermediates as the N-terminal nucleophilic attack at Asi is impossible by using the Ac-Gly building block. It also shows that DKP is formed after Fmoc deprotecting following the Xaa coupling step, whereas Asi is formed throughout all SPPS cycles following Asp coupling.

3 | CONCLUSION

Chain termination during Fmoc-SPPS at the Asi-prone Xaa-Asp-Yaa motif is described. NMR data of the model peptide Ac-Gly-Asp-Gly-Ala-Lys-Phe-NH₂ peptide and its DKP analog clearly indicate that the by-product is an N-terminal DKP. Hence, we conclude that the truncated DKP is formed via nucleophilic attack of the N-terminal Xaa amino function at the α -carbonyl of the Asi intermediate. The extent of this side reaction depends on (a) the peptide sequence (with Glycine as adjacent residues as a worst case, see Figure 10 and Figure 11), (b) the Asp side chain protecting group, and (c) Fmoc deprotection duration (Table 1). Hold times under basic conditions (Fmoc cleavage), temperature, and water content in organic media used (data not shown) have been found to promote Asi and consequently N-terminal DKP formation.

In conclusion, we found that chain termination can be overcome by reducing Asi formation eg, by shortened Fmoc deprotection duration, acidic modifiers,²⁶ and/or modifications of the Asp building block. Three strategies to reduce Asi formation during SPPS are the usage of the OMpe protecting group for Asp, the usage of a backbone amide protecting group²⁷ (Figure 12), and the usage of pseudoprolines.

4 | MATERIALS AND METHODS

4.1 | General automated SPPS

All peptides, unless described otherwise, were synthesized on tricyclic amide linker resin (150 μ mol scale, substitution B₀ = 0.65 mmol/g) using an automated peptide synthesizer (Symphony from Protein Technology Inc.). All reactions were carried out at room temperature. Couplings were performed with Fmoc protected amino acid derivatives or AcOH (2 eq., 300 μ mol) and TBTU/DIPEA (1.8 eq., 275 μ mol/3 eq., 450 μ mol) in DMF (5 mL). All derivatives and reagents were obtained from Bachem AG, Bubendorf, Switzerland. Fmoc was removed using 20 % (v/v) piperidine in DMF (2 \times 15 min, 2 \times 3.75 mL), and unless stated differently, Asp was introduced as Fmoc-Asp (OtBu)-OH. Crude peptides were obtained after cleavage with

TFA/water/EDT 92:5:3 (v/v) and analyzed by Ultra High Performance Liquid Chromatography (UHPLC) (Waters Acquity C18, 1.7 μm ; linear gradient of Acetonitrile (ACN) in 0.1% TFA; flow 0.4 mL/min, $\lambda = 220$ nm). All LC-MS spectra were acquired in positive ion mode with a Bruker microTOF ESI-MS mass spectrometer coupled to a Dionex Ultimate 3000 UHPLC system. Peak assignment was performed using LC-MS analysis of the crude peptides (see Supporting Information). Aspartimide side product peaks were assigned by their mass -18 μ relative to the linear target sequence (less intense isobaric peaks, if present, and piperidides were disregarded for simplicity).

4.2 | Manual SPPS

The Ac-Gly-Asp-Gly-Ala-Lys-Phe-NH₂ peptides were synthesized at a 150 μmol scale using 20 % (v/v) piperidine in DMF (2 \times 15 min) with a varying water content of 0.7% and 5.3% for Fmoc removal starting from Fmoc-Asp (OtBu)-Gly-Ala-Lys (Boc)-Phe-NH-. Fmoc-Gly-OH (2 eq., 300 μmol) coupling was performed with Oxyma/DIC (3.1 eq., 465 μmol /2.7 eq., 405 μmol). Remaining conditions matched the general automated SPPS.

4.3 | Synthesis of Cyclo(-Gly-Asp)-Gly-Ala-Lys-Phe-NH₂ trifluoroacetate salt (DKP) reference compound

Fmoc-Gly- β -Asp (OAll)-(Dmb)Gly-Ala-Lys (Boc)-Phe-NH- was synthesized on Xanthylenyl linker resin (7.5 mmol scale, substitution B₀ = 0.40 mmol/g) using an automated peptide synthesizer (PTI Sonata). Couplings were performed with amino acid derivative (except Fmoc-Asp-OAll) (2 eq., 15 mmol) and TBTU/DIPEA (1.9 eq., 14 mmol/3 eq., 23 mmol) in DMF. For Fmoc-Asp-OAll, HATU was used instead of TBTU; 24 g of peptide resin were obtained.

An aliquot of Fmoc-Gly-Asp-OAll-(Dmb)Gly-Ala-Lys (Boc)-Phe-NH- (10.0 g, approximately 3 mmol) was swollen in DMF (3 \times 100 mL). Fmoc was cleaved with 20% piperidine in DMF for a total of 30 minutes. After washing with DMF and *i*PrOH (100 mL each), DIPEA was added dropwise until a basic pH value was obtained (estimated using wetted pH indicator paper). The mixture was gently agitated for 60 hours. The resin was washed with DMF and *i*PrOH (100 mL each) and dried under vacuum to obtain 9.0 g TFA cleavage and precipitation yielded the crude DKP with 87% purity (UHPLC, Waters Acquity C18, 1.7 μm ; linear gradient of ACN in 0.1% TFA; flow 0.4 mL/min, $\lambda = 220$ nm), which was subsequently purified using preparative RP-HPLC on C18 and a gradient of ACN in 0.1% (v/v) TFA. The purified TFA salt was obtained after lyophilization with 99% HPLC purity (0.53 g overall gross yield, TFA content by HPLC 16.4%, net peptide content estimated by elemental analysis 77.4% [N theory 19.50%; N found 15.09%], 0.7 mmol, approximately 24% yield). ESI-MS: [M+H]⁺ found 575.29 μ , theory 575.29 μ .

4.4 | Varying flanking residues Xaa and Yaa

The peptide sequence Ac-Xaa-Asp-Yaa-Ala-Lys-Phe-NH₂ has been used to investigate the impact of the neighboring amino acids in the

peptide chain, two sets of peptides each consisting of 20 peptides (general sequence Ac-Xaa-Asp-Yaa-Ala-Lys-Phe-NH₂) 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 (see Figure 10 and Figure 11 in the Results and Discussion section). DKP and Asi side products in peptide crudes were identified via LC-MS and corresponding content determined with HPLC (see data in the Supporting Information).

4.5 | NMR experiments

¹H, ¹³C, and ¹⁵N NMR spectra were recorded at 400.1, 100.6, and 40.5 MHz on a Bruker Avance 400 NMR spectrometer (Bruker Biospin AG, Fällanden, Switzerland). The 1D ¹H and ¹³C NMR spectra, and the ¹H-¹³C HSQC, ¹H-¹³C HMBC, ¹H-¹⁵N HSQC, ¹H-¹⁵N HMBC, ¹H-¹H DQF-COSY, TOCSY, and ROESY 2D correlation NMR experiments were performed at 298 K on a 5-mm BBO CryoProbe Prodigy equipped with z-gradient applying 90° pulse lengths of 11.4 (¹H), 10.0 (¹³C), and 13.5 μs (¹⁵N). The NMR experiments of the crude peptide and of the purified DKP reference compound were recorded in DMF-d₇, D₂O, or H₂O/D₂O (9/1) solutions using Bruker standard pulse programs and parameter sets selecting coupling constants of 145 Hz (HSQC), 8 or 4 Hz (¹H-¹³C HMBC), and 95, 4, or 2 Hz, respectively (¹H-¹⁵N HSQC and HMBC). Mixing times of 120 and 250 ms were applied in the TOCSY and ROESY NMR experiments, respectively. On-resin material was investigated using a HR-MAS NMR probe applying spinning rates of 4000 Hz. To remove excess solvents resins were washed five times with CDCl₃ and one time with DMF-d₇. The slightly dried resins (approximately 20 mg) were filled into 4 mm zirconia rotors (active volume of 50 μL) and swollen in DMF-d₇. The ¹H and ¹³C chemical shifts were referenced to the remaining resonances of the solvent DMF at 2.75 and 29.76 ppm or externally referenced relative to the signals of 3-trimethylsilyl 2,2,3,3-tetrauterio sodium propionate dissolved in D₂O at -0.09 and -1.7 ppm, respectively. The ¹⁵N NMR spectra were referenced to an external sample of nitromethane at 0.0 ppm. Coupling constants *J* are reported, in Hz and for ¹H NMR data, coupling patterns are described as s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad. For ¹³C NMR data s = quaternary carbon, d = CH, t = CH₂, q = CH₃ and w = weak HMBC correlation.

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SUPPORTING INFORMATION

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