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The Aspartimide Problem in Fmoc-based SPPS. Part II

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Abstract: The sequence dependence of base-catalysed aspartimide formation during Fmoc-based SPPS was systematically studied employing the peptide models H-Val-Lys-Asp-Xaa-Tyr-Ile-OH. The extent of formation of aspartimide and related by-products was determined by RP-HPLC. Considerable amounts of by-products were formed in the case of Xaa = Asp(OtBu), Arg(Pbf), Asn(Mtt), Cys(Acm) and unprotected Thr. Aspartimide formation could be diminished by incorporation of Asp(OMpe) or by employing milder methods for Fmoc cleavage, e.g. hexamethyleneimine/N-methylpyrrolidine/HOBt/NMP/DMSO 4 : 50 : 4 : 71 : 71 (v/v/w/v/v).

Keywords: aspartimide formation; backbone protection; Fmoc-solid phase peptide synthesis; Fmoc cleavage; Asp(OMpe)

INTRODUCTION

The base-catalysed aspartimide formation is not restricted to the Asp-Gly motif [1]. The incorporation of other amino acids in place of the unhindered Gly even if they are sterically more demanding does not prevent this side reaction. The extent of aspartimide formation is determined by the bulkiness of the preceding residue and by the nature of its side-chain functionalities. The choice of the Fmoc cleavage reagent markedly influences the proportion of aspartimide generated from the desired peptide, and, therefore, has an impact on the amount of aspartimide-related by-products. Harsh cleavage conditions, i.e. the use of bases stronger than piperidine, will promote these reactions. In the case of the Asp-Gly motif, a generally applicable protocol based on Hmb backbone protection allows the reliable suppression of aspartimide formation [2,3].

In part I of this series, the influence of the Asp β-carboxy protecting group, backbone protection and Fmoc cleavage conditions on the extent of aspartimide formation for the Asp-Gly motif [3] was discussed. In continuation of this work, other amino acids prone to aspartimide formation were investigated. The corresponding hexapeptides H-Val-Lys-Asp-Xaa-Tyr-Ile-OH, by analogy to the model sequence H-Val-Lys-Asp-Gly-Tyr-Ile-OH [4], were prepared accordingly and in this study, results for Xaa = Ala, Arg, Asn, Asp, Cys, Cys(Acm), His, Ser, Thr, Tyr, Val are presented.

MATERIALS AND METHODS

ESMS spectra were recorded in the positive mode with a Finnigan MAT LCQ mass spectrometer. Analytical RP-HPLC chromatograms were obtained employing a Merck-Hitachi chromatograph consisting of: pump L-6200, UV-detector L-4000, integrator D-2500, column thermostat L-5025.

Solid-phase Synthesis of H-Val-Lys-Asp^3-Xaa^4-Tyr-Ile-OH

Solid-phase syntheses were performed on Wang resin. Fmoc was used for N^{α} -protection, and the

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Abbreviations: As recommended in *J. Peptide Sci.* 1999; **5**: 465–471, with the following additions and variations: DEPBT, 3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4-(3H)-one); Hmb, 2-hydroxy-4-methoxybenzyl; OMpe, 3-methylpent-3-yl ester; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofurane-5-sulphonyl.

following groups were chosen for protecting side-chain functionalities: Boc for Lys, tBu for Thr and Tyr, OtBu and OMpe for Asp, Pbf for Arg, Acm and Trt for Cys, Trt for His, Mtt for Asn. The standard protocol for Fmoc cleavage consisted of two treatments (5 and 10 min) with piperidine in DMF 1 : 4 (v/v), up to the incorporation of the Fmoc-Asp derivative. Subsequently, the conditions for Fmoc cleavage were varied (Tables 1–4). All couplings were performed with a threefold excess of Fmoc amino acid derivative, TBTU and collidine in DMF for 1 h at ambient temperature. Conversion was monitored by the Kaiser test and the 2,4,6-trinitrobenzenesulfonic acid test. The resulting hexapeptides were cleaved from the resin with 95% aqueous TFA at room temperature for 1 h followed by precipitation with ice-cold tert. butylmethylether. Cleavage of the Cys(Trt)-containing peptides was performed with 95% aq TFA/EDT 95 : 5 (v/v). The crude products were characterized by analytical RP-HPLC and ESMS. HPLC-chromatograms of the peptides were obtained following a run on a 4.6 × 250 mm Bakerbond-column C_{18} 300Å in a buffer system containing 95 mM H_3PO_4 and 90 mM Et_3N in water (pH 2.3), buffer A: 10% CH_3CN, buffer B: 60% CH_3CN; linear gradient: 0 to 30% B in 45 min; flow 1 ml/min; detection at 220 mn.

Fmoc-Asp(OtBu)-Hmb-Asp(OtBu)-OH

Hmb-Asp(OtBu)-OH (32.5 g, 100 mmol) was suspended in DMF (410 ml) and water (41 ml) at room temperature followed by addition of DIPEA (17.1 ml, 100 mmol). After addition of Fmoc-Asp(OtBu)-OSu (50.8 g, 100 mmol) in portions, the suspension was stirred for 1 h, then the pH was adjusted to 7.2 with DIPEA and the reaction was stirred overnight. The reaction was monitored by TLC (chloroform/MeOH/AcOH/water; 70 : 42 : 0.5 : 10). TLC analysis showed an incomplete conversion. After two recouplings each with Fmoc-Asp(OtBu)-OSu (25.4 g, 50.0 mmol) followed by adjusting the pH to 7.0 with DIPEA and stirring overnight, the reaction mixture was diluted with water (400 ml) and EtOAc (400 ml). HCl 5.5 M (65 ml) was used to adjust the pH to 1–2. The organic layer was extracted three times with water (200 ml), dried with Na_2SO_4 and evaporated to yield an oily residue. After repeated co-evaporation with toluene, the residual oil started to crystallize. The suspension was diluted with toluene (100 ml). The crystals were filtered off and washed with toluene. The mother liquor was evaporated to dryness and, after flash chromatography on SiO_2 (toluene/dioxan/AcOH; 93 : 5 : 2), a second crop of product was obtained. The total yield of the desired product was 40.8 g (56.8%) of the desired product. TLC (benzene/dioxan/AcOH; 95 : 25 : 4): r_f 0.27; ESIMS (negative mode): 717.0 (MH^-), 1434.9 (M_2 H^-); ^{1}H-NMR ($CDCl_3$): δ 1.40 (9H, s, OtBu), 1.41 (9H, s, OtBu), 2.60–2.67 (2H, m, β-CH), 2.79–2.84 (1H, m, β-CH), 3.11–3.15 (1H, dd, J = 5.4, 17.4, β-CH), 3.70 (3H, s, OCH_3), 4.14–4.24 (2H, m, OCH_2(Fmoc)), 4.29–4.36 (2H, m, 1H, m, α-CH, H-9(Fmoc)), 4.50–4.65 (1H, m, NCH_2(Hmb)), 4.75–4.89 (1H, m, NCH_2(Hmb)), 5.27–5.39 (1H, m, α-CH), 6.23–6.31 (1H, d, J = 10.6, NH), 6.21–6.42 (2H, m, arom.), 7.19–7.28 (3H, m, arom.), 7.33–7.38 (2H, m, arom.), 7.50–7.57 (2H, m, arom.), 7.71–7.72 (2H, J = 7.4, arom.), 9.25 (1H, s, PhOH). The target compound was checked for racemization and only <0.3% of D-Asp was detected.

RESULTS AND DISCUSSION

In all cases studied, aspartimide formation was promoted by applying harsher Fmoc cleavage conditions (DBU/piperidine/DMF 1 : 20 : 79, v/v/v instead of piperidine/DMF 1 : 4, v/v). Rapid removal of those bases after the cleavage step by immediately neutralizing the excessive base by washings with HOBt in DMF resulted only in a slight improvement. To investigate the influence of repetitive contact with the Fmoc cleavage reagent, e.g. to verify implications for the synthesis of a longer peptide, prolonged base treatment was applied. In general, this treatment led to a significant amount of by-products, especially for the more base-labile hexapeptides. As already established for the Asp-Gly motif [3], replacement of the β-tert.butyl ester of Asp by the bulkier β-3-methyl-3-pentyl ester (OMpe) [5] could impede cyclization.

Asp-Val had a lower tendency towards cyclization than Asp-Ala. Moreover, side-chain functionalities may promote this side reaction, even when they are blocked by rather bulky protecting groups. A range of β-functionalized amino acids showed a considerable propensity for aspartimide formation with the adjacent Asp(OtBu), especially Cys(Acm) and unprotected Thr. In the first instance, the results obtained on the less sensitive Asp-Xaa combinations are presented.

Synthesis of H-Val-Lys-Asp^3-Xaa^4-Tyr-Ile-OH with Various Derivatives in Position 4

Xaa = *Ala, His, Ser, Tyr, Val.* Aspartimide formation was negligible during the SPPS of the corresponding

hexapeptides if the base piperidine was employed for Fmoc removal (Table 1) even if the duration of the treatment was extended.

Amongst these analogues, the combination Asp(OtBu)-Ala showed a pronounced tendency towards aspartimide formation, when subjected to harsher Fmoc cleavage conditions for a longer period (Table 2, Entry No 2). In this case, the cycle was readily reopened by piperidine leading to piperidide formation. In general, prolonged treatment with piperidine/DMF generated only small amounts of by-products, whereas the peptides were sensitive to prolonged treatment with the DBU-containing cleavage reagent.

The Val analogue was expected to represent one of the most stable residues due to the large, β-branched side chain and thus, this amino acid was viewed as a 'standard'. According to the prolonged DBU treatment, the residues were rated according to their increasing potential to form aspartimide related side products. As a consequence, the series Val < Ser < Tyr < His < Ala starting with the most stable 'standard' has emerged.

Aspartimide formation seems to represent less of a problem if a bulky side-chain is present in the position of Xaa. The position of the residue His in this series may be understood as follows. The 1-protected imidazole ring of His is well known to enhance racemization in the case of activation prior to coupling. Therefore, an influence on aspartimide formation was suspected. According to our results, the latter reaction was not catalysed to a great extent. However, both effects of the His side chain, bulkiness and catalytic activity, may counteract each other and, thus lead to a certain lability of the His residue with respect to aspartimide formation.

Table 1 Extent of Formation of Aspartimide and Piperidide during Syntheses of H-Val-Lys-Asp^3-Xaa^4-Tyr-Ile-OH (Xaa = Ala, His, Ser, Tyr or Val). Focus on Piperidine Treatment

Entry no	Fmoc-Xaa-derivative	Time (min)	Desired product (%)	Aspartimide (%)	Piperidide (%)
1	Ala	45	99.0	nd	nd
2	Ala	225	95.7	1.4	1.1
3	His(Trt)	45	96.3	nd	nd
4	His(Trt)	225	93.6	1.3	0.3
5	Ser(tBu)	45	97.2	nd	nd
6	Ser(tBu)	225	96.9	nd	nd
7	Tyr(tBu)	45	97.7	nd	nd
8	Tyr(tBu)	225	95.6	1.5	1.6
9	Val	45	97.7	nd	nd
10	Val	225	95.2	nd	nd

Conditions of SPPS see Materials and Methods. Fmoc removal was performed with piperidine/DMF (1 : 4), 5 and 10 min, at room temperature. Percentages of desired product and by-products were determined by RP-HPLC, as reported in Materials and Methods. nd, not detectable (<0.3%).

Table 2 Extent of Formation of Aspartimide and Piperidide during Syntheses of H-Val-Lys-Asp^3-Xaa^4-Tyr-Ile-OH (Xaa = Ala, His, Ser, Tyr or Val). Focus on DBU Treatment

Entry no	Fmoc-Xaa-derivative	Time (min)	Desired product (%)	Aspartimide (%)	Piperidide (%)
1	Ala	45	93.9	3.2	1.0
2	Ala	225	39.5	10.7	43.1
3	His(Trt)	45	96.0	nd	nd
4	His(Trt)	225	66.2	12.7	9.4
5	Ser(tBu)	45	95.9	nd	nd
6	Ser(tBu)	225	80.6	3.0	11.9
7	Tyr(tBu)	45	94.4	2.3	nd
8	Tyr(tBu)	225	75.3	10.5	12.9
9	Val	45	96.1	nd	nd
10	Val	225	87.9	nd	1.9

Conditions of SPPS see Materials and Methods. Fmoc removal was performed with DBU/piperidine/DMF 1 : 20 : 79 (v/v/v), 5 and 10 min, at room temperature. Percentages of desired product and by-products were determined by RP-HPLC, as reported in Materials and Methods. nd, not detectable (<0.3%).

Xaa = *Arg, Cys(Acm), Cys*. The following amino acid replacements had a more severe impact on aspartimide formation.

In principle, Arg(Pbf)-containing peptides should behave similarly to Arg(Pmc)-containing sequences with respect to potential Asp related side-reactions. For the latter case, increased aspartimide formation was already reported [1].

In our hands, the motif Asp(OtBu)-Arg(Pbf) was quite unstable towards prolonged piperidine treatment (Table 3, Entry No 2) or contact with DBU (Table 4), though the aspartimide cycle was not cleaved as readily as the aspartimide resulting from the sequence motif Asp(OtBu)-Asn(Mtt) (see below). For the HPLC profiles of typical crude products as

Table 3 Extent of Formation of Aspartimide and Piperidide during Syntheses of H-Val-Lys-Asp^3-Xaa^4-Tyr-Ile-OH [Xaa = Arg, Cys or Cys(Acm)]. Focus on Piperidine Treatment

Entry no	Fmoc-Xaa derivative	Time (min)	Desired product (%)	Aspartimide (%)	Piperidide (%)
1	Arg(Pbf)	45	93.8	2.1	nd
2	Arg(Pbf)	225	71.7	18.1	4.8
3	Cys(Acm)	45	81.3	2.3	1.3
4	Cys(Acm)	225	47.6	19.9	16.8
5	Cys(Trt)	45	95.5	0.2	0.3
6	Cys(Trt)	225	92.1	1.6	2.4

Conditions of SPPS see Materials and Methods. Fmoc removal was performed with piperidine/DMF (1 : 4), 5 and 10 min, at room temperature. Percentages of desired product and by-products were determined by RP-HPLC, as reported in Materials and Methods. nd, not detectable (<0.3%).

displayed in Figure 1, the main peaks were identified by LCMS. The presence of the δ-NH guanidino function may explain the increased tendency of Arg derivatives for cyclization. As in the case of His, the behaviour of Arg(Pbf) suggests a catalytic involvement of the guanidino moiety, despite its bulky and electron-withdrawing protecting group.

The Asp(OtBu)-Cys(Acm) motif turned out to be extraordinarily sensitive towards bases (Tables 3 and 4). Even limited piperidine treatments performed during the synthesis of H-Val-Lys-Asp-Cys(Acm)-Tyr-Ile-OH led to a variety of by-products, amongst them the piperidides and D/L-aspartimide. These side products were identified by LCMS. Figure 2 documents the increased stability of the model peptide synthesized using Cys(Trt) protection. According to HPLC, the quality of the crude product following synthesis with Acm-protected Cys was less than 50%. By contrast, the crude peptide obtained after incorporation of Fmoc-Cys(Trt)-OH showed a superior purity of >90%. In addition, the combination Asp(OtBu)-Cys(Trt) withstood prolonged piperidine treatment much better than the corresponding motif with Cys(Acm).

The use of DBU rapidly decreased the amount of the desired product, as outlined in Table 4, Entries No 4 and 5.

Hence, when synthesizing peptides containing the Asp-Cys motif, Cys in this particular position should not be incorporated as the Acm-protected derivative.

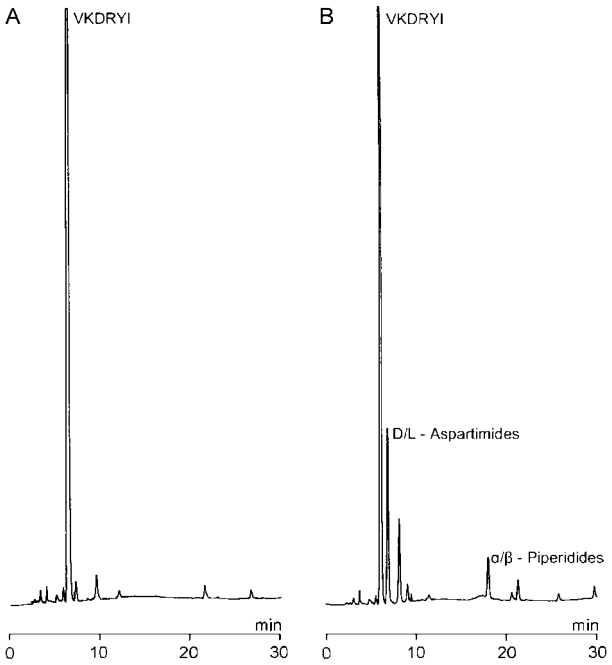

Figure 1 Analytical HPLC-profiles of crude products obtained after synthesis of VKDRYI. A: SPPS using piperidine/DMF 1 : 4 for Fmoc cleavage, B: Additional treatment with piperidine/DMF for 3 h.

Table 4 Extent of Formation of Aspartimide and Piperidide during Syntheses of H-Val-Lys-Asp^3-Xaa^4-Tyr-Ile-OH [Xaa = Arg, Cys or Cys(Acm)]. Focus on DBU Treatment

Entry no	Fmoc-Xaa-derivative	Time (min)	Desired product (%)	Aspartimide (%)	Piperidide (%)
1	Arg(Pbf)	45	73.7	14.4	1.9
2	Arg(Pbf)	225	5.3	31.5	54.9
3	Cys(Acm)	45	56.7	27.1	7.6
4	Cys(Acm)	225	nd	nd	61.9
5	Cys(Trt)	45	86.8	5.5	2.6
6	Cys(Trt)	225	43.0	8.8	35.5

Conditions of SPPS see Materials and Methods. Fmoc removal was performed with DBU/piperidine/DMF 1 : 20 : 79 (v/v/v), 5 and 10 min, at room temperature. Percentages of desired product and by-products were determined by RP-HPLC, as reported in Materials and Methods. nd, not detectable (<0.3%).

In the case of trityl protection, aspartimide related problems may also arise when synthesizing long peptides. For the synthesis of peptides containing two or more disulfide bridges with the intention of

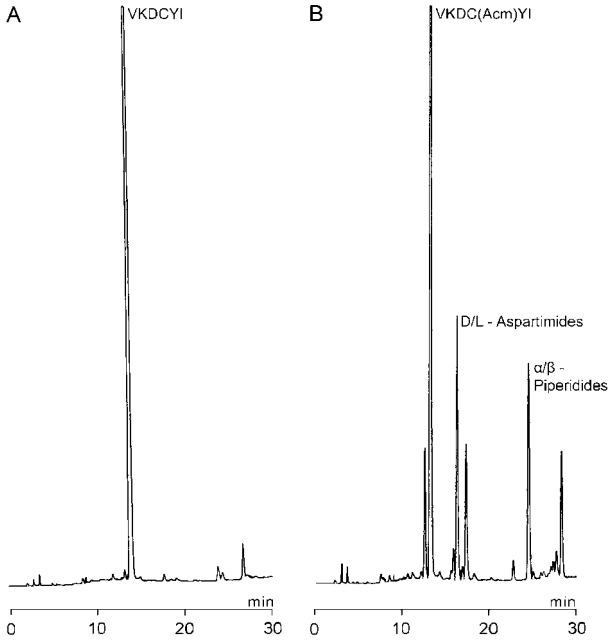

Figure 2 Analytical HPLC-profiles of crude products obtained after synthesis of VKDCYI using piperidine/DMF 1 : 4 for Fmoc removal. The peptide resin was treated with base for an additional period of 3 h. A: Synthesis performed with Fmoc-Cys(Trt)-OH, B: Synthesis performed with Fmoc-Cys(Acm)-OH.

forming those bonds consecutively, the presence of an Asp-Cys sequence limits the range of feasible synthetic strategies.

Xaa = *Asp*, *Asn*. Both amino acid derivatives are known to enhance aspartimide formation [6]. According to our observations, the motif Asp(OtBu)^3-Asn(Mtt)^4 seems to be more sensitive towards bases (Tables 5 and 6). Considerable amounts of aspartimide were formed in the presence of DBU (Table 6, Entry No 5). As expected, the beneficial effect of the bulkier Asp β-carboxyl protecting group OMpe could also be demonstrated, especially if prolonged base treatment was applied.

The HPLC-profiles displayed in Figure 3, demonstrate the increased stability of the combination Asp(OMpe)^3-Asp(OtBu)^4 towards DBU. Even though the Asp(OtBu)-Tyr(tBu) motif is quite stable towards bases (see Table 1, Entry No 8), prolonged treatment with DBU produced aspartimide-piperidides including Asp^3, Asp^4 bis-piperidides.

In the case of the Asp-Gly motif, backbone protection proved to be the most efficient method for suppressing aspartimide formation [3]. For further

Table 5 Extent of Formation of Aspartimide and Piperidide during Syntheses of H-Val-Lys-Asp^3-Xaa^4-Tyr-Ile-OH (Xaa = Asp or Asn). Focus on Piperidine Treatment

Entry no	Fmoc-Xaa-derivative	Time (min)	Desired product (%)	Aspar-timide (%)	Piperidide (%)
1	Asp(OtBu)	45	98.0	0.7	nd
2	Asp(OtBu)[a]	45	97.3	nd	nd
3	Asp(OtBu)	225	90.2	nd	3.9
4	Asp(OtBu)[a]	225	94.1	nd	1.5
5	Asn(Mtt)	45	96.9	1.0	nd
6	Asn(Mtt)[a]	45	97.0	nd	nd
7	Asn(Mtt)	225	79.7	6.6	11.7
8	Asn(Mtt)[a]	225	91.8	2.6	3.4

Conditions of SPPS see Materials and Methods. Fmoc removal was performed with piperidine/DMF (1 : 4), 5 and 10 min, at room temperature. Percentages of desired product and by-products were determined by RP-HPLC, as reported in Materials and Methods. nd, not detectable (<0.3%).

[a] Derivative for incorporation of Asp^3 : Fmoc-Asp(OMpe)-OH.

Table 6 Extent of Formation of Aspartimide and Piperidide during Syntheses of H-Val-Lys-Asp^3-Xaa^4-Tyr-Ile-OH (Xaa = Asp or Asn). Focus on DBU Treatment

Entry no	Fmoc-Xaa-derivative	Time (min)	Desired product (%)	Aspar-timide (%)	Piperidide (%)
1	Asp(OtBu)	45	68.5	7.2	10.3
2	Asp(OtBu)[a]	45	86.0	nd	nd
3	Asp(OtBu)	225	9.3	2.9	41.2
4	Asp(OtBu)[a]	225	34.0	2.7	22.0
5	Asn(Mtt)	45	80.8	10.7	6.8
6	Asn(Mtt)[a]	45	91.6	4.1	2.3
7	Asn(Mtt)	225	1.4	1.4	80.0
8	Asn(Mtt)[a]	225	25.9	5.3	56.9

Conditions of SPPS see Materials and Methods. Fmoc removal was performed with DBU/piperidine/DMF 1 : 20 : 79 (v/v/v), 5 and 10 min, at room temperature. Percentages of desired product and by-products were determined by RP-HPLC, as reported in Materials and Methods. nd, not detectable (<0.3%).

[a] Derivative for incorporation of Asp^3 : Fmoc-Asp(OMpe)-OH.

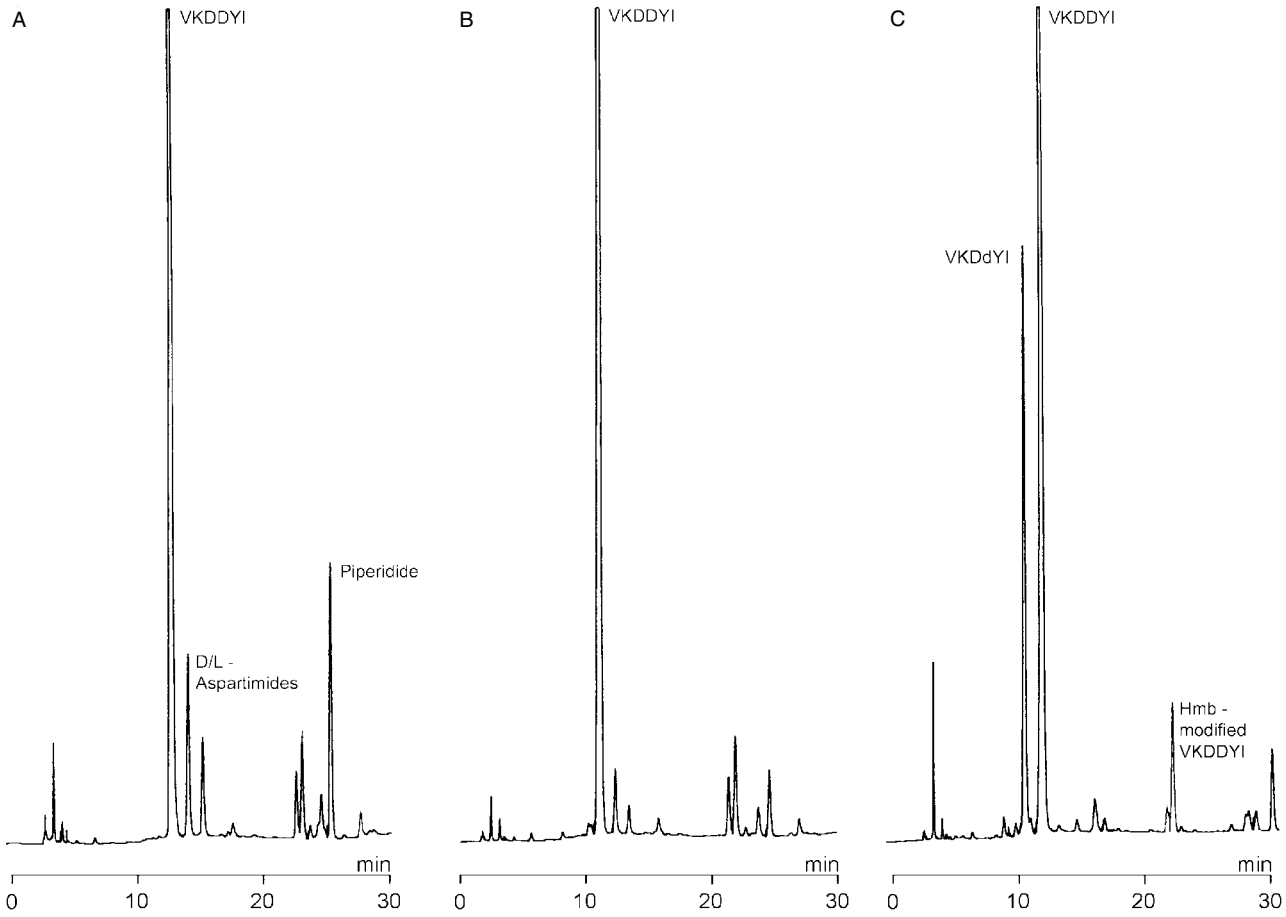

Figure 3 Analytical HPLC-profiles of crude products obtained after synthesis of VKDDYI using DBU/piperidine/DMF 1:20:79 for Fmoc cleavage. A: Asp^3 was incorporated as Fmoc-Asp(OtBu)-OH, B: Asp^3 was incorporated as Fmoc-Asp(OMpe)-OH, C: The motif Asp-Asp was introduced by coupling Fmoc-Asp(OtBu)-HmbAsp(OtBu)-OH activated with TATU/DIPEA, final cleavage from the resin was performed in 95% aq TFA, 6 h.

evaluation of this approach, the base-sensitive Asp-Asp motif was investigated in more detail. As a consequence, the dipeptide derivative Fmoc-Asp(OtBu)-HmbAsp(OtBu)-OH was synthesized and coupled to H-Tyr(tBu)-Ile-Wang resin. A variety of coupling conditions were studied (DEPBT, TATU, TBTU) and even optimized solutions achieved with DEPBT/DIPEA or TATU were unsatisfactory. Difficulties in coupling this very bulky derivative and incomplete conversions were expected [7]. However, considerable amounts of the side product H-Val-Lys-Asp^3-D-Asp^4-Tyr-Ile-OH were observed. In our hands, 13% of the target peptide was epimerized at Asp^4 following our elaborate DEPBT coupling protocol. In addition, the epimer H-Val-Lys-D-Asp^3-Asp^4-Tyr-Ile-OH could be detected. Furthermore, complete removal of the Hmb moiety could be achieved neither by very long treatment with 95% aq TFA nor by prolonged cleavage in the presence of EDT or TES, whereas backbone deprotection in case of the Asp-HmbGly motif never posed a problem. Figure 3C shows the impurity profile of the crude product still containing a small amount of the backbone-protected peptide after treatment with 95% aq TFA for 6 h.

In the case of Hmb protection, Asp related side reactions were completely inhibited also in the Asp-Asp case. However, in the course of this study, the problems related to the incorporation of Fmoc-Asp(OtBu)-HmbAsp(OtBu)-OH during stepwise SPPS were not resolved and the complete removal of the Hmb group has also not been adequately addressed. In an earlier report Hmb protection for an Asp-Asn motif was applied by a stepwise approach [8]. In this contribution, coupling of an Asp building block to Hmb-protected Asn was described.

These results are not completely in line with our observations on Hmb cleavage in the Asp-Asp case. In addition, in the past, incomplete conversion was detected even for the coupling of Asp-derivatives to Hmb protected Gly.

Xaa = Thr. When applying TBTU or similar coupling reagents, side-chain protection of Fmoc-Thr-OH is normally not strictly required and in some instances, the coupling of this derivative may be desired. In fact, for sequences with a high propensity for β-sheet formation the use of unprotected Thr and, thus, the more hydrophilic character of the polymer supported semi-protected peptide chain may represent a strategic advantage. This holds definitely true for the case of on-resin post-synthetic modifications on the secondary hydroxyl function, e.g. phosphorylation, for which the incorporation of an unprotected Thr side chain represents the preferred choice. Moreover, partially protected fragments have a tendency for increased solubility compared with their fully protected counterparts.

However, in the vicinity of an Asp residue, protection of the β-hydroxyl is mandatory to avoid Asp related side-reactions during synthesis. The motif Asp(OtBu)-Thr readily supports cyclization in the presence of bases (see Table 7, Entries No 3 and 4), followed by the reopening of the cycle by nucleophiles such as piperidine or water (see Figure 4A). On the other hand, Asp(OtBu)-Thr(tBu) withstood even prolonged treatment with DBU (see Table 8, Entry No 2).

Table 7 Extent of Formation of Aspartimide and Piperidide during Syntheses of H-Val-Lys-Asp[3]-Thr[4]-Tyr-Ile-OH. Focus on Piperidine Treatment

Entry no	Fmoc-Thr derivative	Time (min)	Desired product (%)	Aspartimide (%)	Piperidide (%)
1	Thr(tBu)	45	95.9	1.0	1.0
2	Thr(tBu)	225	95.5	0.8	1.0
3	Thr	45	86.3	8.0	3.1
4	Thr	225	18.6	7.3	57.7

Conditions of SPPS see Materials and Methods. Fmoc removal was performed with piperidine/DMF (1 : 4), 5 and 10 min, at room temperature. Percentages of desired product and by-products were determined by RP-HPLC, as reported in Materials and Methods. nd, not detectable (<0.3%).

Alternative Conditions for Fmoc Cleavage

Due to the sensitivity of the system, the Asp(OtBu)-Thr case was applied to study alternative Fmoc cleavage conditions aimed at suppression of base-induced aspartimide formation. Milder Fmoc cleavage conditions kept the degree of aspartimide formation remarkably low (see Table 8). Application of the Fmoc cleavage cocktail (Cocktail I) consisting of hexamethyleneimine/N-methylpyrrolidine/HOBt/NMP/DMSO 4 : 50 : 4 : 71 : 71 (v/v/w/v/v), which had originally been developed for the Fmoc-SPPS

Table 8 Extent of Formation of Aspartimide and Piperidide during Syntheses of H-Val-Lys-Asp[3]-Thr[4]-Tyr-Ile-OH. Focus on Alternative Deprotection Methods

Entry no	Fmoc-Thr derivative	Base, time (min)		Desired product (%)	Aspartimide (%)	Piperidide/Hexamethyleneimide
1	Thr(tBu)	DBU	45	96.0	0.9	1.0
2	Thr(tBu)	DBU	225	95.7	0.7	1.2
3	Thr	DBU	45	14.0	12.9	42.1
4	Thr	DBU	225	12.7	nd	64.6
5	Thr	Cocktail I	45	95.0	1.4	1.1
6	Thr	Cocktail I	225	93.4	3.3	1.3
7	Thr	Cocktail II	45	87.2	5.1	nd
8	Thr	Cocktail II	225	65.0	11.6	nd

Conditions of SPPS see Materials and Methods. Fmoc removal was performed in the presence of various bases. DBU: DBU/piperidine/DMF 1 : 20 : 79 (v/v/v), 5 and 10 min, at room temperature, Cocktail I: hexamethyleneimine/N-methylpyrrolidine/HOBt/NMP/DMSO 4 : 50 : 4 : 71 : 71 (v/v/w/v/v), 3 and 17 min [9], at room temperature, Cocktail II: 80 mM DBU/74 mM HOBt in DMF, 4 × 1 min [10], at room temperature. Percentages of desired product and by-products were determined by RP-HPLC, as reported in Materials and Methods. nd, not detectable (<0.3%).

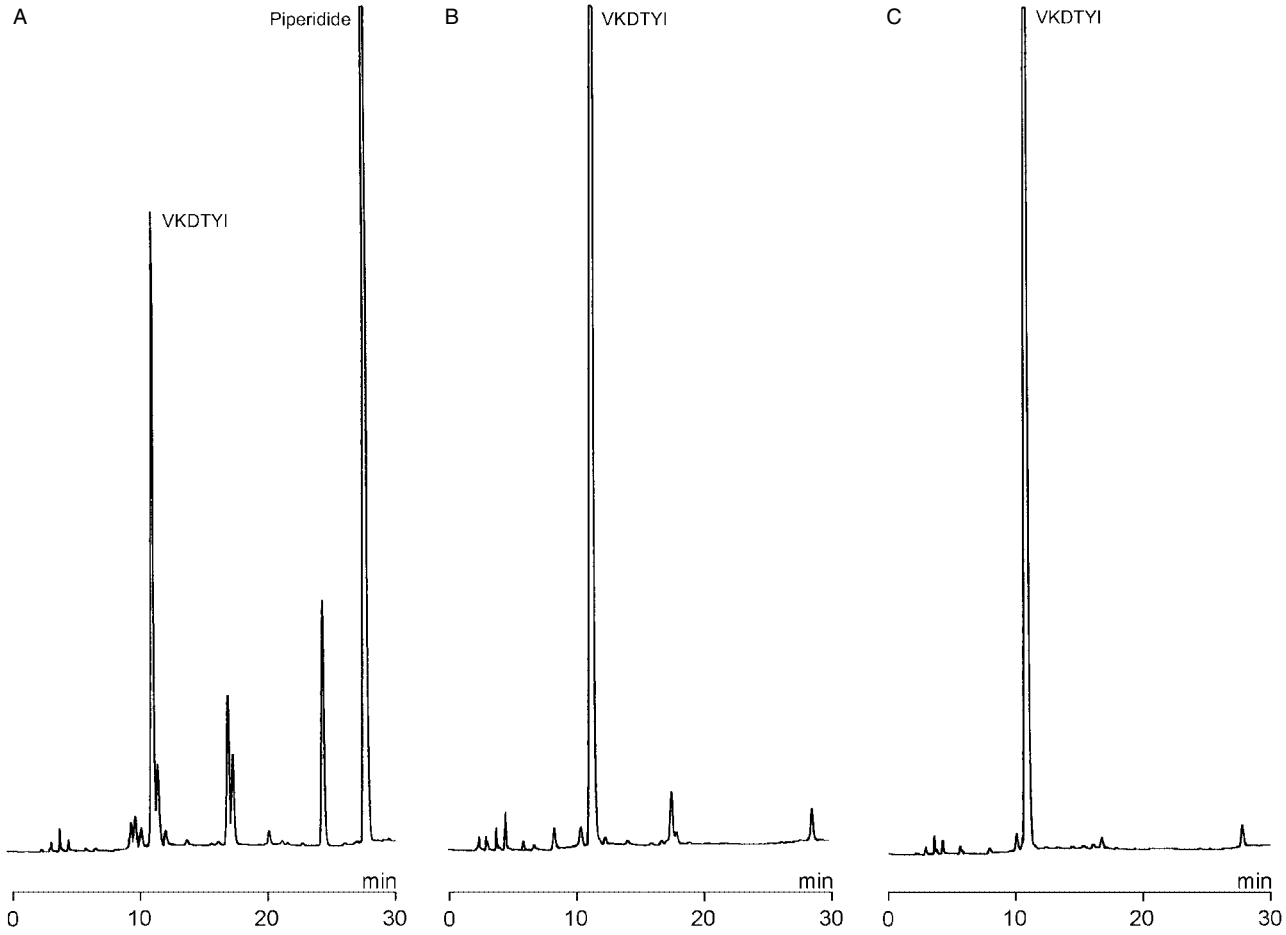

Figure 4 Analytical HPLC- profiles of crude products obtained after synthesis of VKDTYI. A: Thr was incorporated using Fmoc-Thr-OH, the total contact time with piperidine/DMF 1 : 4 was 225 min, B: Thr was introduced as Fmoc-Thr(tBu)-OH, the total contact time with piperidine/DMF 1 : 4 was 225 min, C: Thr was incorporated as Fmoc-Thr-OH, the total contact time with Cocktail I was 225 min.

of peptide thioesters [9], resulted in an excellent quality of crude product (see Figure 4C). Under these conditions for Fmoc deprotection, the sensitive combination Asp(OtBu)-Thr seemed to be rather stable. Another Fmoc cleavage reagent (Cocktail II), a 'buffered' DBU-cocktail, also compatible with the base-labile thioester linkage, 80 mM DBU/74 mM HOBt in DMF [10], led to less satisfactory results (see Table 8).

CONCLUSION

The Hmb protected dipeptide Fmoc-Asp(OtBu)-HmbAsp(OtBu)-OH, which represented the derivative of choice according to our studies on the Asp-Gly case, did completely inhibit Asp mediated side reactions. However, problems with respect to racemization and, less severe, the incomplete deprotection could not be solved in the course of these studies. Although, protection of the phenolic hydroxyl function and/or a stepwise approach could help to overcome the problem of racemization, earlier studies indicated additional hurdles. Therefore, significant development is required to overcome the problems of incorporation and deprotection for Hmb derivatives to come up with a generally applicable Hmb-based strategy.

On the basis of our current understanding, a complete solution of the aspartimide problem will be hard to identify. Nevertheless, the amount of by-products generated from this side-reaction may be reduced by applying the derivative Fmoc-Asp(OMpe)-OH instead of Fmoc-Asp(OtBu)-OH. In addition, milder conditions for

Fmoc-deblocking such as hexamethyleneimine/N-methylpyrrolidine/HOBt/NMP/DMSO (4 : 50 : 4 : 71 : 71) can be recommended for suppression of undesired side reactions.

A step further in this direction would represent an alternative N^{α}-protecting group which would require less basic conditions for Fmoc removal, but at the same time conferring stability towards coupling conditions (tertiary base, amino group to be acylated), i.e. the next synthetic step. Alternatively, the development of a suitable Asp side chain protection could lead to resolve the problem of aspartimide formation.

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