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The aspartimide problem in Fmoc-based SPPS. Part III

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Abstract: A newly developed Fmoc-Asp derivative, Fmoc-Asp β-(2,3,4-trimethyl-pent-3-yl) ester, has been tried in the Fmoc-based SPPS of H-Val-Lys-Asp-Xaa-Tyr-Ile-OH, a well-established peptide model for studying base-catalysed aspartimide formation. When synthesizing the hexapeptide incorporating Gly, Arg(Pbf), Asn(Mtt), Asp(OtBu) or Cys(Acm) for Xaa, considerable amounts of aspartimide-related by-products were to be expected. The Asp^3 β-carboxy protecting group and the duration of exposure to bases were varied. By-product formation could be reduced by incorporation of the new Asp derivative more efficiently than by introducing the less bulky Asp(OMpe). Significant improvements were observed in cases of prolonged contact with piperidine or DBU. Both β-carboxy protecting groups were superior to the standard Asp(OtBu) which was also included in this study, but the additional stabilization gained by our new protecting group was valuable especially in syntheses of long peptides or difficult sequences. Copyright © 2005 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: aspartimide formation; Fmoc-solid phase synthesis; Asp β-carboxy protection; Asp (β-2,3,4-trimethyl-pent-3-yl) ester

INTRODUCTION

Base-catalysed aspartimide formation, which may occur during the removal of Fmoc N^{α}-protection, is one of the most notorious side reactions in Fmoc-based SPPS [1]. The extent of cyclization is determined by several considerations, such as the nature of the Asp β-carboxy protecting group and the conditions chosen for Fmoc cleavage. Data obtained from syntheses of Asp-containing peptides show convincingly that by increasing the bulk of the β-carboxy protecting group, aspartimide formation can be greatly reduced [2,3]. Up to now, the sterically demanding β-(3-methyl-pent-3-yl) ester (OMpe) [2] was the best commercially available Fmoc-Asp derivative for reducing this side reaction. It was hoped that a further increase in steric hindrance would prevent aspartimide formation even more effectively. For confirmation of this hypothesis the well-established model peptide Val-Lys-Asp-Xaa-Tyr-Ile-OH (Xaa = Gly, Cys(Acm), Arg, Asn, Asp) [4,5] was chosen. These sequences readily form aspartimide when treated with bases during their assembly via Fmoc-SPPS.

Additional methyl groups will increase the bulk of the β-carboxy protecting group. The structures of the Asp derivatives in question are displayed in Figure 1. The tertiary alcohols required to synthesize these derivatives are commercially available (2,3,4-trimethyl-3-pentanol = 1,1-di(isopropyl)ethanol (Die-OH) and tricyclohexylmethanol (Tcm-OH)), or they may be obtained from the corresponding ketones and Grignard reagents.

On the other hand, additional steric hindrance means that the acylation of the teriary alcohol becomes increasingly difficult. Furthermore, suitable Asp derivatives will suffer under the highly basic conditions described in the literature for the esterification of hindered tertiary alcohols [6].

MATERIALS AND METHODS

^{1}H-NMR measurements were performed on a Bruker Avance DRX 500 spectrometer, 500 MHz, employing tetramethylsilane as an internal standard. ESI-MS spectra were recorded in the positive mode with a Finnigan MAT LCQ mass spectrometer. Analytical HPLC chromatograms were obtained employing a Merck-Hitachi chromatograph consisting of a pump L-6200, UV-detector L-4000, integrator D-2500 and column thermostat L-5025. TLC-monitoring was performed applying silica gel plates Merck Kieselgel 60 F_{254} and the following systems for development: chloroform/EtOAc/AcOH 90 : 10 : 2 v/v/v (system A), chloroform/MeOH/32% AcOH 5 : 3 : 1 v/v/v (system B), chloroform/MeOH/AcOH/water 85 : 13 : 0.5 : 1.5 v/v/v/v (system C); for detection: UV or KI/2-tolidine after oxidation with chlorine for general detection; ninhydrin for the presence or absence of free amino groups.

Z-Asp(ODie)-OBzl

2,3,4-trimethylpentan-3-ol (Avocado Organics; 10.61 g, 81.4 mmol) was added to a solution of Z-Asp-OBzl (9.93 g, 27.8 mmol) in DCM (40 ml). After cooling in an ice-bath, 0.5 eq DCC (2.97 g, 14.3 mmol) was added in portions whilst stirring vigorously. The resulting thick suspension was kept at 0 °C for

Abbreviations: As recommended in *J. Pept. Sci.* 1999; **5**: 465–471, with the following additions and variations: tBu, t-butyl; ODie, 1,1-diisopropyethyl ester (2,3,4-trimethylpent-3-yl ester); OMpe, 3-methylpent-3-yl ester; OTcm, tricyclohexylmethyl ester; OTim, triisopropylmethyl ester; Pbf, 2,3,4,6,7-pentamethyldihydrobenzofurane-5-sulfonyl.

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For parts I and II, see references [3] and [5], respectively.

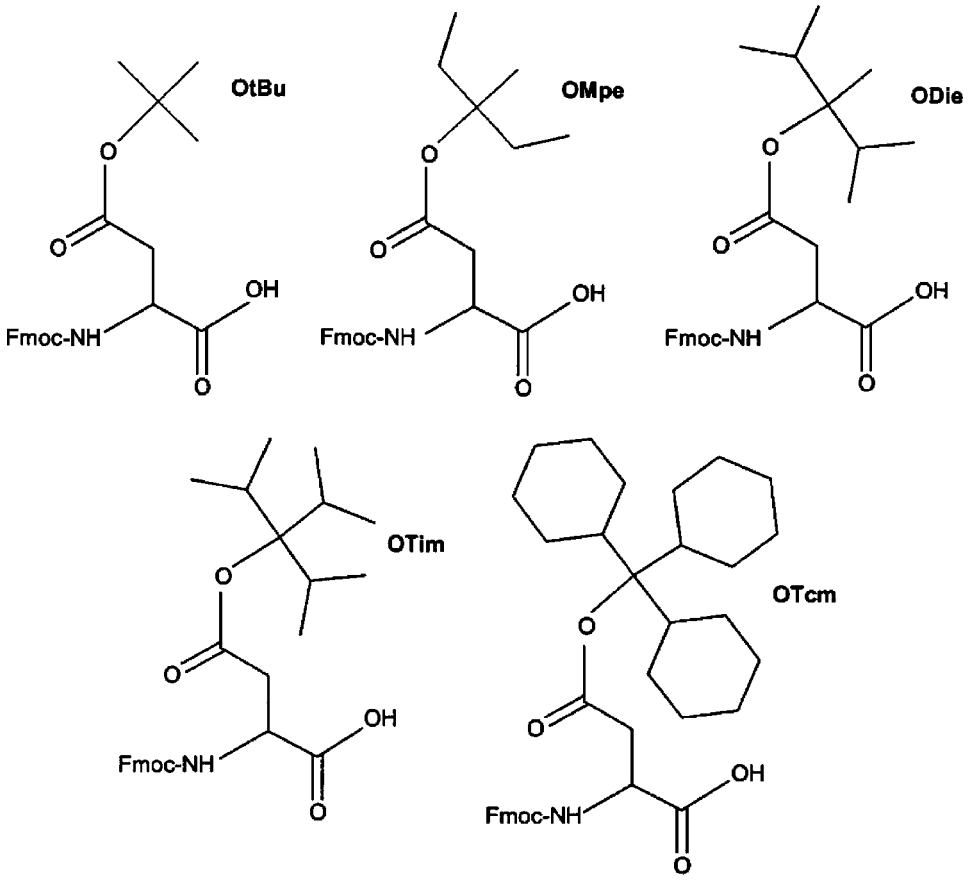

Figure 1 Structures of Fmoc-Asp derivatives with increasingly bulky β-carboxy protection.

15 min before adding 0.19 eq DMAP (661 mg, 5.4 mmol). Stirring was continued at ambient temperature. The reaction was monitored by TLC (system A). After 8 h 0.21 eq DCC (1.23 g, 5.9 mmol) was added to the yellowish suspension which then slowly darkened. After stirring overnight (23 h) a third portion of DCC (1.43 g, 6.9 mmol) was added. The TLC chromatograms showed that esterification was accompanied by the formation of small amounts of more polar by-products, amongst them the acylurea. By-product formation was also responsible for the darkening of the suspension. A further portion of DCC (0.86 g, 4.2 mmol) was added after 31 h and the reaction was allowed to proceed for a second night. AcOH (500 µl) was added and, after stirring for 15 min, the brown suspension was diluted with toluene/EtOAc 95 : 5 v/v (400 ml) and filtered over a plug of silica gel (30 g). The bulk of the solvent was evaporated *in vacuo* and the remaining dark syrup was applied to a column of silica gel (400 g). Elution with toluene/EtOAc 95 : 5 yielded the desired product, a colorless oil: 3.71 g (28%).

TLC (system A) 0.69; ESI-MS: 470.0 (MH^+), 491.9 (MNa^+) 960.8 (M_2Na^+); ^{1}H-NMR: δ 0.86–0.90 [12H, m, CH_3 (Die)], 1.33 [3H, s, CH_3-C (Die)], 2.17–2.26 [2H, m, CH (Die)], 2.78–3.03 [2H, m, β-CH_2 (Asp)], 4.59–4.63 [1H, m, α-CH (Asp)], 5.12 [2H, s, O-CH_2 (Bzl)], 5.17–5.18 [2H, d, $J = 3.1$ Hz, O-CH_2 (Z)], 5.82–5.84 [1H, d, $J = 8.6$ Hz, NH], 7.30–7.54 [10H, m, arom. (Bzl, Z)].

Fmoc-Asp(ODie)-OH

Z-Asp(ODie)-OBzl (3.6 g, 7.6 mmol) was dissolved in dioxane (30 ml) at ambient temperature under nitrogen. After Pd/C (0.58 g) was added, the ester was hydrogenated under atmospheric pressure. The conversion was monitored by TLC (system B). Precipitating material was redissolved by addition of 4 ml of 5% aqueous Na_2CO_3, which had to be repeated twice, as precipitation continued. After 6 h, the catalyst was filtered off. The pH of the filtrate was adjusted to 8 by adding solid $NaHCO_3$ (632 mg, 7.5 mmol), then a solution of Fmoc-OSu (2.69 g, 7.9 mmol) in dioxane (15 ml) was added dropwise with vigorous stirring. The concomitantly forming precipitate was slowly redissolved, the pH had to be readjusted to >7 by addition of 5% aqueous $NaHCO_3$. The conversion was followed by TLC (system C). After 2 h H-Asp(ODie)-OH had disappeared practically completely. The solution of the desired product was diluted with water up to 200 ml. After adjustment to pH 8 the slightly turbid solution was extracted with hexane and tBuOMe (75 ml each). After addition of tBuOMe (250 ml), the pH was adjusted to 2 using solid $KHSO_4$ with vigorous stirring. The phases were separated, the upper phase was washed with water (150 ml) and brine (100 ml) followed by drying with Na_2SO_4. Removal of the solvent left an oily residue, which was dried in a desiccator: 3.50 g (97%).

TLC (system B) 0.69, (system C) 0.41; ESI-MS: 489.9 (MNa^+), 505.9 (MK^+); ^{1}H-NMR: δ 0.90–0.94 [12H, m, CH_3 (Die)], 1.38 [3H, s, CH_3-C (Die)], 2.21–2.29 [2H, m, CH (Die)], 2.82–3.06 [2H, m, β-CH_2 (Asp)], 4.20–4.23 [1H, t, $J = 7.2$ Hz, α-CH (Asp)], 4.32–4.42 [2H, m, CH_2 (Fmoc)], 4.63–4.67 [1H, m, H9 (Fmoc)], 5.95–5.97 [1H, d, $J = 8.7$ Hz, NH], 7.26–7.73 [8H, m, arom. (Fmoc)].

Attempted Synthesis of Z-Asp(OTcm)-OBzl

Several attempts to synthesize Z-Asp(OTcm)-OBzl under the conditions as above failed. Further experiments using Fmoc-Asp(Cl)-OBzl, Z-Asp(Cl)-OBzl or the mixed anhydride of Z-Asp-OBzl and pivalic acid were unsuccessful as well.

Solid Phase Synthesis of VKDXaaYI

Solid-phase syntheses were performed on Wang resin. Fmoc was used for N^{α}-protection, and the following groups were chosen for protecting side-chain functionalities: Boc for Lys, tBu for Tyr, OtBu, OMpe and ODie for Asp^3, OtBu for Asp^4, Pbf for Arg, Mtt for Asn and Acm for Cys. 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 Asp^3 derivative. Subsequently the conditions for Fmoc cleavage were varied (see Tables). All couplings were performed with threefold excesses 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 tBuOMe. The crude products were characterized by analytical RP-HPLC and ESI-MS. 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; 5 min at 0% B, then a linear gradient from 0% B to 30% B in 45 min (Xaa = Gly) or buffer A: 5% CH_3CN, buffer B 60% CH_3CN; linear gradient: 5% to 35% B in 45 min (Xaa = Asn, Asp, Arg, Cys(Acm)); flow 1 ml/min; detection at 220 nm.

RESULTS AND DISCUSSION

Synthesis of the Asp Derivatives

The acylation of 3-methyl-3-pentanol proceeds quite sluggishly [2]. Nevertheless, good yields of the Asp β-(3-methylpent-3-yl ester) could be obtained in our laboratories by a modified esterification protocol. As the first esterification experiments with the more hindered alcohols 2,3,4-trimethyl-3-pentanol and tricyclohexylmethanol applying Fmoc(or Z)-Asp(Cl)-OBzl or the mixed anhydride of Z-Asp-OBzl and pivalic acid had failed, this protocol was eventually chosen. It is known that acylations of tertiary alcohols may proceed more readily with the symmetrical anhydride in the presence of catalytic amounts of DMAP than with the acid chloride, even though the latter is the more reactive, less hindered derivative [7]. DCM is the solvent of choice yielding the purest product, but high conversions have also been achieved in moderately polar solvents such as THF. Esterification at elevated temperatures promoted the formations of by-products such as the acylurea generated by rearrangement of the DCC-activated Z-Asp-OBzl. As Fmoc may be cleaved prematurely even by catalytic amounts of DMAP [8], the Z-derivative, Z-Asp-OBzl, had to be used exclusively. Thus, esterification is followed by hydrogenolysis and introduction of the Fmoc group.

The new derivative Fmoc-Asp(ODie)-OH readily reacted applying standard coupling conditions (cf. Material and Methods). After completion of the SPPS the side chain protecting group was smoothly removed by treatment with 95% aq TFA.

Esterification of the sterically even more demanding tricyclohexylmethanol failed. Neither Z-Asp(OTcm)-OBzl could be obtained via DCC/DMAP activation in analogy to Z-Asp(ODie)-OBzl. Nor did the acid chloride Fmoc-Asp(Cl)-OBzl acylate Tcm-OH in the presence of pyridine.

Synthesis of VKDXaaYI Applying OtBu, OMpe and ODie for β-carboxy Protection of Asp^3

All Asp-Xaa motifs included in this study were chosen due to their marked propensity towards aspartimide formation [5]. For constructing long peptides containing such motifs close to the *C*-terminus by Fmoc-based SPPS a reliable, robust protecting group for the β-carboxy function is a mandatory prerequisite. Then the 'standard' OtBu group may have to be replaced by bulkier moieties, e.g. OMpe, due to inadequate stability of the former towards bases.

Aspartimide formation is promoted by applying harsher, i.e. more basic Fmoc cleavage conditions: DBU/piperidine/DMF 1 : 20 : 79 v/v/v (DBU) instead of piperidine/DMF 1 : 4 v/v (piperidine). Prolonged exposure of the peptide resins to piperidine or DBU following completion of peptide assembly served to mimic the numerous Fmoc cleavage cycles during the SPPS of longer peptides. The results assembled in Tables 1–10 show the influence of the type of the Fmoc cleavage cocktail (piperidine or DBU) and contact time (45 min, 225 min, 405 min) as well as the impact of the nature of the preceding amino acid Xaa (Arg, Asn, Asp, Cys(Acm), Gly) and the choice of the β-carboxy protecting group (OtBu, OMpe, ODie). The beneficial effect of additional steric hindrance due to the OMpe group is convincingly demonstrated again by our data, whereas the data of the new, even bulkier ODie moiety showed a less dramatic positive effect if compared with OMpe. But nevertheless, the additional stabilization gained by ODie side chain protection became more pronounced the longer the duration of exposure to bases.

***Xaa* = Gly.** The data acquired from HPLC-chromatograms of VKDGYI obtained by varying Asp β-carboxy protection and type of Fmoc cleavage cocktail are assembled in Tables 1 and 2. Closer inspection of the results compiled in Table 1 shows that the proportion of desired product rapidly decreased during extended contact with piperidine. The stabilizing effect of ODie is evident, it becomes more pronounced when prolonging the

Table 1 Extent of Formation of Aspartimide and Piperidide during Syntheses of H-Val-Lys-Asp-**Gly**-Tyr-Ile-OH varying the Asp β-carboxy protecting group. Focus on Piperidine Treatment

Entry no	Asp(OR) R =	Time (min)	Desired product (%)	L/D-Aspartimide (%)	α-Piperidide (%)	β-Piperidide (%)	D-Piperidides[a] (%)
1	tBu	45	91.8	2.2	0.2	<0.2	<0.2
2		225	70.1	14.4	6.4	0.8	1.4/0.5
3		405	52.6	15.9	18.2	1.6	5.7/1.3
4	Mpe	45	94.0	1.1	<0.2	<0.2	<0.2
5		225	75.8	10.7	5.0	1.0	1.0/0.3
6		405	64.1	11.8	12.5	1.9	3.6/0.8
7	Die	45	92.4	0.8	<0.2	<0.2	<0.2
8		225	80.4	8.5	3.7	0.4	0.7/0.2
9		405	76.1	7.6	7.3	0.9	2.1/0.6

Conditions of SPPS see Materials and Methods. Fmoc cleavage applying 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.
[a] α/β-Regioisomers not assigned.

Table 2 Extent of Formation of Aspartimide and Piperidide during Syntheses of H-Val-Lys-Asp-**Gly**-Tyr-Ile-OH varying the Asp β-carboxy protecting group. Focus on DBU Treatment

Entry no	Asp(OR) R =	Time (min)	Desired product (%)	L/D-Aspartimide (%)	α-Piperidide (%)	β-Piperidide (%)	D-Piperidides[a] (%)
1	tBu	45	61.5	20.6	6.4	1.3	0.8/0.3
2		225	0.2	5.8	57.0	2.6	22.5/5.0
3	Mpe	45	82.8	8.9	2.0	<0.2	0.3/<0.2
4		225	10.6	11.8	46.7	2.5	17.5/3.9
5	Die	45	87.0	3.9	0.8	<0.2	<0.2
6		225	33.2	12.2	30.8	1.6	10.7/2.5

Conditions of SPPS see Materials and Methods. Fmoc cleavage applying DBU/piperidine/DMF (1 : 20 : 79), 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.
[a] α/β-Regioisomers not assigned

duration of treatment with this base (see, e.g. entries 3 and 9). Exposure to DBU accelerates aspartimide formation and further side reactions, as the corresponding data listed in Table 2 clearly demonstrate. Entry 6 shows that even stabilization by ODie does not suffice, even though it is superior to OMpe (cf. entry 4). The best approach to avoid formation of aspartimide in the Asp-Gly motif is, and will very probably remain, Hmb protection as described in part I of this series [3]. Attempts to introduce Hmb by on-resin reductive alkylation with 2-hydroxy-4-methoxybenzaldehyde/$NaBH(OAc)_3$ or $NaBH_3CN$, which would be very helpful with other Asp-Xaa sequences since the corresponding building blocks are not easily accessible and cause additional problems during coupling, cf. [5], were positive but not fully satisfactory and would not be applicable in routine Fmoc-based SPPS (details not shown).

***Xaa* = Asn.** The same phenomena may be observed when substituting Gly^4 by Asn. The corresponding data acquired from treatment with piperidine are listed in Table 3. The results listed in Table 4 show that the Asp(OR)-Asn(Mtt) motif is especially labile towards contact with DBU. Hence, as the comparison between entries 5, 6, 8 and 9 demonstrates, a remarkable degree of stabilization could be achieved by replacing OMpe by ODie side chain protection. Chromatograms of VKDNYI obtained after prolonged piperidine treatment are presented in Figure 2. A slight increase of purity was observed following prolonged exposure of the Asp(ODie)-containing hexapeptide to this base. But the additional stabilization gained by applying ODie β-carboxy protection showed far more impressively after treatment with DBU (see Figure 3), albeit formation of by-products could not be totally suppressed in the presence of the bulkier protecting group.

***Xaa* = Asp.** The Asp^4-analog is slightly less sensitive towards piperidine than the Asn^4-analog, as can be

Table 3 Extent of Formation of Aspartimide and Piperidide during Syntheses of H-Val-Lys-Asp-**Asn**-Tyr-Ile-OH varying the Asp β-carboxy protecting group. Focus on Piperidine Treatment

Entry no	Asp(OR) R =	Time (min)	Desired product (%)	Aspartimide (%)	Piperidide (%)
1	tBu	45	91.4	2.1	0.2
2		225	74.7	8.6	11.3
3		405	73.0	7.5	14.5
4	Mpe	45	93.1	1.2	0.5
5		225	89.3	3.2	3.4
6		405	86.7	3.0	5.0
7	Die	45	93.7	1.0	0.5
8		225	93.2	1.3	0.9
9		405	92.2	1.2	1.5

Conditions of SPPS see Materials and Methods. Fmoc cleavage applying 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.

Table 4 Extent of Formation of Aspartimide and Piperidide during Syntheses of H-Val-Lys-Asp-**Asn**-Tyr-Ile-OH varying the Asp β-carboxy protecting group. Focus on DBU Treatment

Entry no	Asp(OR) R =	Time (min)	Desired product (%)	Aspartimide (%)	Piperidide (%)
1	tBu	45	75.3	12.6	6.2
2		225	4.6	5.3	69.3
3		405	—	—	—
4	Mpe	45	84.2	4.3	2.3
5		225	26.8	8.5	54.5
6		405	18.3	4.7	66.4
7	Die	45	91.9	1.4	0.6
8		225	80.3	3.4	11.0
9		405	61.4	3.0	27.6

Conditions of SPPS see Materials and Methods. Fmoc cleavage applying DBU/piperidine/DMF (1 : 20 : 79), 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.

seen in Table 5. Moreover, as the results assembled in Table 6 demonstrate, the Asp-Asp motif readily cyclizes in the presence of DBU. But, as entries 8 and 9 show, the stabilizing effect of ODie β-carboxy protection is not as distinct as in the case of the Asp-Asn motif (cf. Table 4). It should be kept in mind when discussing the data of Tables 5 and 6 that aspartimide-related by-products may also be generated from Asp^4(OtBu)-Tyr^5(tBu). Such products can be formed especially during prolonged exposure to DBU (and apparently accumulate if aspartimide formation from Asp^3-Asp^4 is effectively suppressed), albeit the Asp-Tyr motif is rather stable [5]. Moreover, mass spectra of entries 2, 5 and 8 show the formation of bis-piperidides and aspartimide-piperidides.

***Xaa* = Arg.** The data obtained from the hexapeptides containing Arg in place of Gly^4 after piperidine treatment are assembled in Table 7. They show a moderate stabilizing effect of the new protecting group in the vicinity of Arg. The effect is demonstrated as well by the HPLC-chromatograms of products obtained from peptide resins exposed to piperidine displayed in Figure 4. The substitution of Gly^4 by Arg generates a distinctly DBU-labile sequence, as demonstrated by

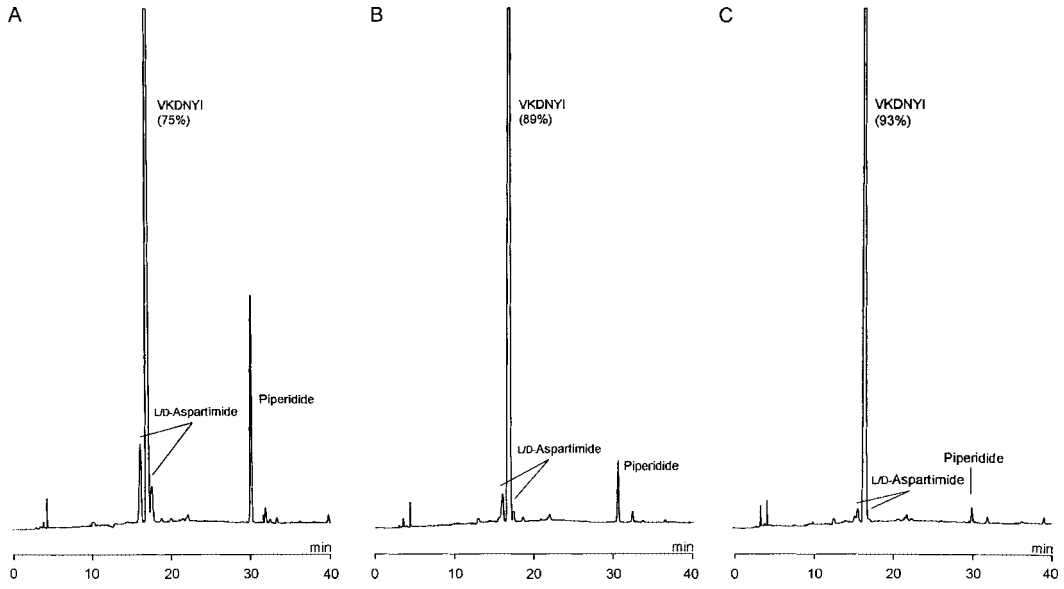

Figure 2 Analytical HPLC-profiles of crude products obtained after synthesis of VKDNYI followed by an additional treatment with piperidine/DMF (1 : 4) for 3 h. Asp was introduced as Fmoc-Asp(OtBu)-OH (A), Fmoc-Asp(OMpe)-OH (B), Fmoc-Asp(ODie)-OH (C).

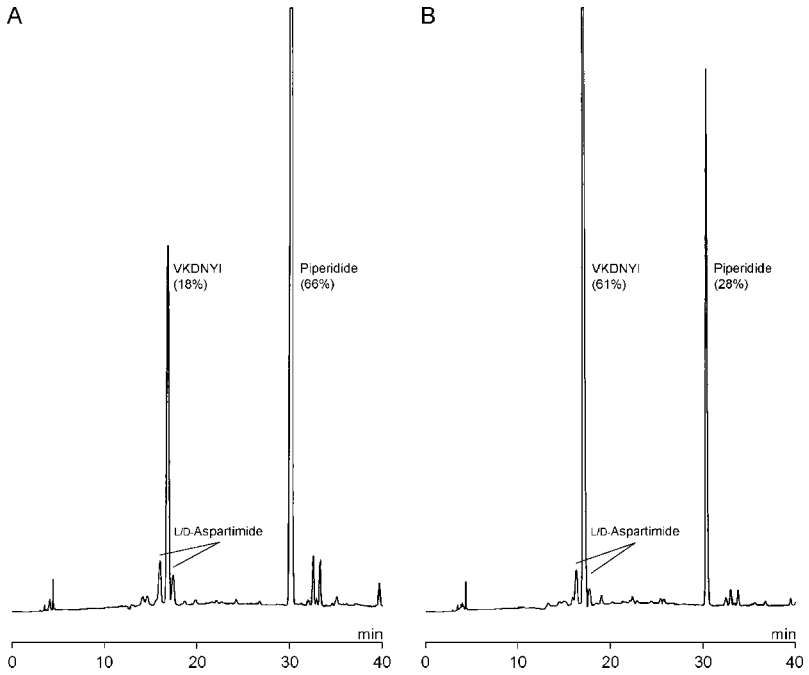

Figure 3 Analytical HPLC-profiles of crude products obtained after SPPS of VKDNYI using DBU/piperidine/DMF 1 : 20 : 79 for removal of Fmoc followed by an additional 6 h treatment with this cocktail. Asp was incorporated as Fmoc-Asp(OMpe)-OH (A), Fmoc-Asp(ODie)-OH (B).

Table 5 Extent of Formation of Aspartimide and Piperidide during Syntheses of H-Val-Lys-Asp^3-**Asp**^{4}-Tyr-Ile-OH varying the Asp^3 β-carboxy protecting group (Asp^4: OtBu). Focus on Piperidine Treatment

Entry no	Asp^3(OR) R =	Time (min)	Desired product (%)	Asp^3-Aspartimide (%)	Asp^3-Piperidide (%)
1	tBu	45	91.6	1.4	0.2
2		225	87.1	3.8	3.9
3		405	81.9	3.9	8.0
4	Mpe	45	92.7	1.1	0.3
5		225	89.6	2.1	1.6
6		405	87.9	1.6	3.2
7	Die	45	91.6	1.0	<0.2
8		225	90.0	1.1	1.1
9		405	88.9	0.7	1.7

Conditions of SPPS see Materials and Methods. Fmoc cleavage applying 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.

Table 6 Extent of Formation of Aspartimide and Piperidide during Syntheses of H-Val-Lys-Asp^3-**Asp**^{4}-Tyr-Ile-OH varying the Asp^3 β-carboxy protecting group (Asp^4: OtBu). Focus on DBU Treatment

Entry no	Asp^3(OR) R =	Time (min)	Desired product (%)	Asp^3-Aspartimide (%)	Asp^3-Piperidide (%)
1	tBu	45	63.7	10.4	9.6
2		225	12.7	3.7	51.7
3		405	—	—	—
4	Mpe	45	80.8	3.7	2.3
5		225	37.5	3.4	23.7
6		405	19.3	1.9	36.9
7	Die	45	86.2	1.8	1.0
8		225	52.6	1.6	8.4
9		405	32.3	1.0	13.3

Conditions of SPPS see Materials and Methods. Fmoc cleavage applying DBU/piperidine/DMF (1 : 20 : 79), 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.

the data compiled in Table 8. Again, much better results were obtained by replacing OMpe with ODie. A remarkable stabilizing effect is shown by entries 8 and 9, it compares well with the corresponding data of the Asp-Asn motif compiled in Table 4.

***Xaa* = Cys(Acm).** Besides the Gly^4-peptide, the hexapeptide containing the Asp-Cys(Acm) combination turned out to be the most base-sensitive sequence studied during this work. Even though, regardless of the chosen combination of side-chain protecting group and base for Fmoc removal, further by-products are formed, the tendencies observed with the other analogs are repeated. The data resulting from exposure to piperidine are presented in Table 9. The values

Table 7 Extent of Formation of Aspartimide and Piperidide during Syntheses of H-Val-Lys-Asp-**Arg**-Tyr-Ile-OH varying the Asp β-carboxy protecting group. Focus on Piperidine Treatment

Entry no	Asp(OR) R =	Time (min)	Desired product (%)	Aspartimide (%)	Piperidide (%)
1	tBu	45	94.9	0.6	<0.2
2		225	70.8	19.1	4.7
3		405	66.3	20.1	7.4
4	Mpe	45	94.9	0.3	<0.2
5		225	91.4	4.1	0.2
6		405	83.6	7.2	2.8
7	Die	45	94.9	0.2	<0.2
8		225	96.0	0.4	0.4
9		405	93.1	1.4	0.6

Conditions of SPPS see Materials and Methods. Fmoc cleavage applying 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.

Table 8 Extent of Formation of Aspartimide and Piperidide during Syntheses of H-Val-Lys-Asp-**Arg**-Tyr-Ile-OH varying the Asp β-carboxy protecting group. Focus on DBU Treatment

Entry no	Asp(OR) R =	Time (min)	Desired product (%)	Aspartimide (%)	Piperidide (%)
1	tBu	45	73.6	15.3	1.1
2		225	5.6	29.6	49.9
3		405	—	—	—
4	Mpe	45	91.9	3.4	<0.2
5		225	35.8	26.1	27.6
6		405	15.5	16.3	52.4
7	Die	45	93.8	1.3	<0.2
8		225	77.6	6.0	6.6
9		405	64.6	7.2	16.9

Conditions of SPPS see Materials and Methods. Fmoc cleavage applying DBU/piperidine/DMF (1 : 20 : 79), 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.

assembled in Table 10 show a distinct lability in the presence of DBU as well as an impressive stabilizing effect of the ODie ester (cf. entry 6). As recently stated in Part II of this series [5], Cys preceding Asp should not be protected by Acm. But, as the entries 8 and 9 in Table 9 show such a significant reduction of aspartimide formation due to the bulkier ODie group, Acm protection may become a feasible alternative nevertheless. So, whenever in the course of a synthesis involving the consecutive formation of two or more disulfide bridges orthogonal S-protection is required and Acm in the vicinity of Asp cannot be avoided, ODie β-carboxy protection in combination with piperidine for Fmoc cleavage should be preferred.

CONCLUSION

Generating additional steric hindrance when introducing the Asp β-carboxy protecting group reduces base-catalysed aspartimide formation efficiently during

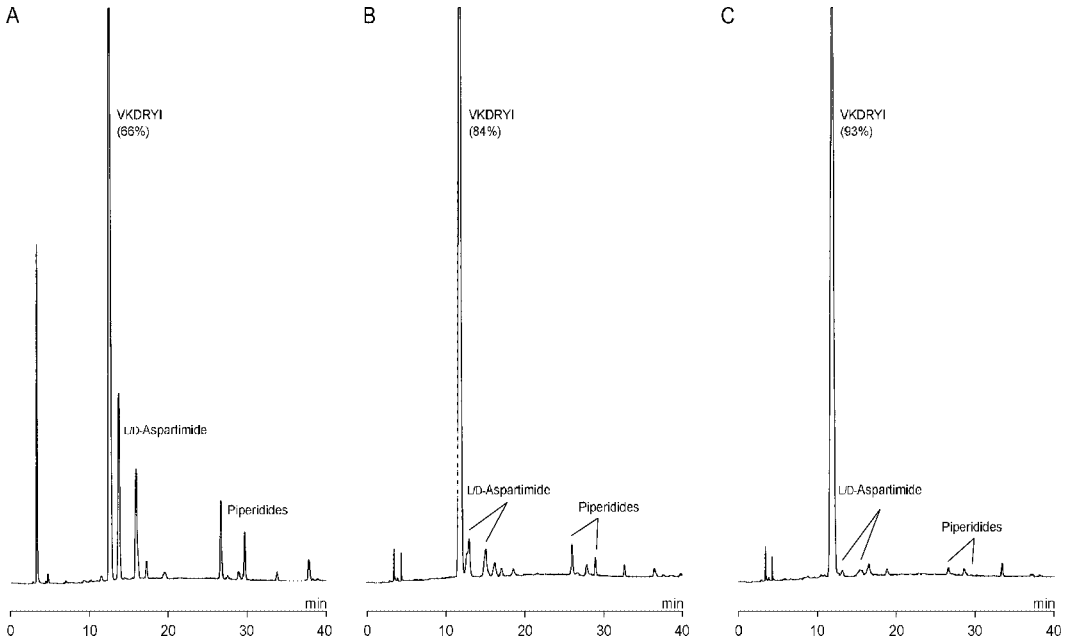

Figure 4 Analytical HPLC-profiles of crude peptides obtained after synthesis of VKDRYI followed by an additional exposure to piperidine/DMF 1 : 4 for 6 h. Asp was introduced as Fmoc-Asp(OtBu)-OH (A), Fmoc-Asp(OMpe)-OH (B), Fmoc-Asp(ODie)-OH (C).

Table 9 Extent of Formation of Aspartimide and Piperidide during Syntheses of H-Val-Lys-Asp-**Cys(Acm)**-Tyr-Ile-OH varying the Asp β-carboxy protecting group. Focus on Piperidine Treatment

Entry no	Asp(OR) R =	Time (min)	Desired product (%)	Aspartimide (%)	Piperidide (%)
1	tBu	45	89.1	2.3	0.3
2		225	53.4	20.1	15.7
3		405	39.4	15.6	27.2
4	Mpe	45	92.8	0.6	0.3
5		225	78.6	8.2	6.4
6		405	70.9	7.4	12.2
7	Die	45	91.0	0.4	0.7
8		225	88.9	1.5	1.9
9		405	88.5	1.4	3.0

Conditions of SPPS see Materials and Methods. Fmoc cleavage applying 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.

Table 10 Extent of Formation of Aspartimide and Piperidide during Syntheses of H-Val-Lys-Asp-**Cys(Acm)**-Tyr-Ile-OH varying the Asp β-carboxy protecting group. Focus on DBU Treatment

Entry no	Asp(OR) R =	Time (min)	Desired product (%)	Aspartimide (%)	Piperidide (%)
1	tBu	45	54.4	27.6	6.6
2		225	1.2	1.0	65.2
3	Mpe	45	85.8	6.0	1.2
4		225	9.4	8.3	56.7
5	Die	45	88.3	1.6	0.7
6		225	57.5	5.7	25.4

Conditions of SPPS see Materials and Methods. Fmoc cleavage applying DBU/piperidine/DMF (1 : 20 : 79), 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.

Fmoc-based SPPS. But total suppression of this side reaction cannot be achieved by simply increasing the bulk of the Asp side chain protection. The synthesis of the corresponding derivatives becomes more and more difficult if not impracticable. On the other hand, less stabilization per added methyl group is gained when increasing the number of replacements of t-butyl hydrogen atoms by methyl groups, cf. Figure 1. Nevertheless, the improved suppression of aspartimide formation by ODie (in comparison with OMpe) matters, when synthesizing long peptides. Then, the side chain protecting group of Asp has to withstand many deprotection cycles or even prolonged contact with DBU, as a stronger base may have to be introduced to reliably achieve complete removal of Fmoc. When synthesizing long peptides or difficult sequences necessitating harsher conditions for Fmoc removal β-carboxy protection by the bulkier ODie helps to increase the yield of desired product in the crude material. Additional prevention of aspartimide formation by our new protecting group can decide between failure or success of a synthesis.

As already discussed, prospects for a further increase in steric hindrance of Asp β-carboxy protecting groups do not look very promising. Syntheses will become extremely difficult, as can be seen in the case of Fmoc-Asp(OTcm)-OH, and improved methods will have to be found for generating the ester. Nevertheless, we believe that the results of our extended studies on the Asp problem will be beneficial to the peptide community involved in Fmoc-SPPS.

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