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The Aspartimide Problem in Fmoc-based SPPS. Part I†

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Abstract: A variety of Asp β-carboxy protecting groups, Hmb backbone protection and a range of Fmoc cleavage protocols have been employed in syntheses of the model hexapeptide H-VKDGYI-OH to investigate the aspartimide problem in more detail. The extent of formation of aspartimide and aspartimide-related by-products was determined by RP-HPLC. This study included three new Fmoc-Asp-OH derivatives: the β-(4-pyridyl-diphenylmethyl) and β-(9-phenyl-fluoren-9-yl) esters and also the orthoester Fmoc-β-(4-methyl-2,6,7-trioxabicyclo[2.2.2]-oct-1-yl)-alanine. 3-Methylpent-3-yl protection of the Asp side chain resulted in significant improvements with respect to aspartimide formation. Complete suppression was achieved using the combination OtBu side chain protection and Hmb backbone protection for the preceding Gly residue. Copyright © 2003 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: aspartimide formation; Fmoc-solid phase peptide synthesis; Asp β-carboxy protection; backbone protection; Asp-Gly motif; carboxy protection by orthoester formation

INTRODUCTION

Aspartimide formation is one of the best-documented side reactions in peptide synthesis. The sequence-dependent cyclization is catalysed by acids and by bases [1], and even bulky β-carboxy side-chain protecting groups such as O*t*Bu do not prevent completely its occurrence. During Fmoc/*t*Bu-based SPPS, the repetitive piperidine treatments needed for Fmoc removal lead to successive formation of aspartimide. Further by-products result from racemisation of the imide derivative, which may also be opened by nucleophiles [2] as shown in Figure 1.

In previous studies, the hexapeptide fragment Val-Lys-Asp-Gly-Tyr-Ile (**I**) derived from scorpion toxin II [3] has been applied to investigate the susceptibility of the Asp-Gly motif for aspartimide formation. In this study, different protecting groups and a variety of conditions using model peptide **I** were investigated systematically. To address the problem of incomplete Fmoc removal, harsher conditions (stronger bases) for Fmoc deblocking were applied to verify the effect on aspartimide formation. The aim of this project was to use the resulting optimized combination of protecting groups and bases for the SPPS of longer peptides to improve the quality of the crude product. In addition to these systematic experiments, our study includes information on the synthesis of the following new Asp derivatives: Fmoc-Asp(OPyBzh)-OH, Fmoc-Asp(OPhFl)-OH and Fmoc-Asp(OBO)-OH.

MATERIALS AND METHODS

^{1}H-NMR measurements were performed on a Bruker Avance DRX 500 spectrometer, 500 MHz, employing

Abbreviations: As recommended in *J. Peptide Sci.* 1999; **5**: 465–471, with the following additions and variations: LCMS, liquid chromatography coupled with mass spectrometry; *t*Bu, *t*-butyl; d, D-Asp; *i*PrOH, isopropanol; OMpe, 3-methylpent-3-yl ester; OPhFl, 9-phenyl-fluoren-9-yl ester; OPp, 2-phenylisopropyl ester; OPyBzh, 4-pyridyl-diphenylmethyl ester; Fmoc-Asp(OBO)-OH, Fmoc-β-(4-methyl-2,6,7-trioxabicyclo[2.2.2]-oct-1-yl)-alanine; TMG, 1,1,3,3-tetramethylguanidine.

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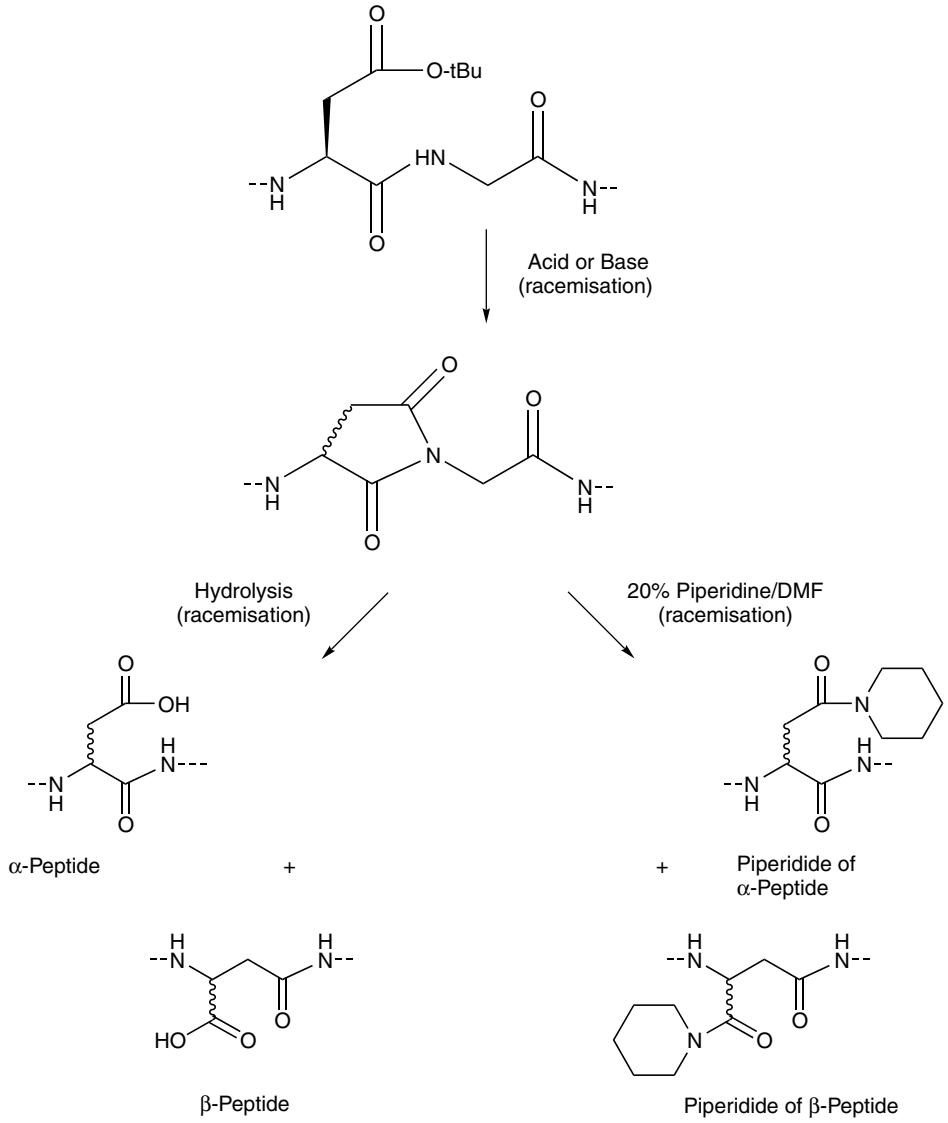

Figure 1 Formation of aspartimide and related products.

tetramethylsilane as an internal standard. ESIMS and LCMS-spectra were recorded in the positive mode with a Finnigan MAT LCQ mass spectrometer coupled to a Waters Alliance HPLC system. 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. TLC-monitoring was performed applying silica gel plates Merck Kieselgel 60 F_{254} and the following systems for development: chloroform/MeOH/AcOH (90 : 8 : 2) (A), chloroform/32% aqueous AcOH/MeOH (15 : 4 : 1) (B), chloroform/AcOH/EtOAc (50 : 2 : 50) (C), chloroform/AcOH/EtOAc (90 : 2 : 10) (D); for detection: UV or KI/2-tolidine after oxidation with chlorine for general detection; ninhydrin for the presence or absence of free amino groups.

Fmoc-Asp(OPyBzh)-OBzl

Fmoc-Asp-OBzl (18.02 g, 40.4 mmol) was suspended in dry dichloroethane (100 ml) at room temperature yielding a sticky gel. PyBzh-Cl·HCl (10.45 g, 33.0 mmol), which was synthesized according to Coyle *et al.* [4], was added and the gel dissolved. A solution of DIPEA (12.6 ml, 73.6 mmol) in dichloroethane (13 ml) was added dropwise. The reaction was monitored by TLC (system A). The moderately basic solution slowly turned darker and slightly turbid. After stirring at room temperature

for 48 h, the solution was kept at 40 °C for 2 h, then the solvent was removed. The residue was taken up in water and extracted three times with EtOAc (500 ml each). Extraction removed only very polar impurities such as the DIPEA hydrochloride. Further aqueous washes did not remove excess Fmoc-Asp-OBzl, which crystallized after drying with Na_2SO_4. The solvent was evaporated and the residue was chromatographed on SiO_2 (873 g). Elution with EtOAc/hexane (1 : 1) and removal of the solvent rendered 14.77 g (65%) of Fmoc-Asp(OPyBzh)-OBzl. TLC (system A): r_f 0.65; ESIMS: 689.1 (MH^+), 1376.5 (M_2H^+); ^{1}H-NMR ($CDCl_3$): δ 3.03–3.25 (2H, m, β-CH_2), 4.11–4.17 [1H, t, $J = 7.1$ Hz, H9 (Fmoc)], 4.31–4.41 [2H, m, O-CH_2 (Fmoc)], 4.68–4.70 (1H, m, α-CH), 5.00–5.14 [2H, m, O-CH_2 (Bzl)], 5.72–5.74 (1H, d, $J = 8.4$ Hz, NH), 7.25–7.30 [19H, m, arom. (Bzl, Bzh, Fmoc)], 7.34–7.38 [2H, d, $J = 7.5$ Hz, H3/5 (pyridine)], 7.51–7.53 [2H, d, $J = 6.7$ Hz, H1/8 (Fmoc)], 7.74–7.76 [2H, d, $J = 7.6$ Hz, H4/5 (Fmoc)], 8.49–8.50 [2H, d, $J = 6.0$ Hz, H2/6 (pyridine)].

Fmoc-Asp(OPyBzh)-OH (collidine salt)

Fmoc-Asp(OPyBzh)-OBzl (3.01 g, 4.37 mmol) was dissolved in EtOH (350 ml) at ambient temperature under nitrogen. Pd/C (0.5 g) was added and the ester was hydrogenated under atmospheric pressure. The conversion was monitored by TLC (system B). After 1.5 h, the catalyst was filtered off and the solvent removed *in vacuo*, leaving a foam which was redissolved in EtOAc (500 ml). Water (300 ml) and collidine (0.6 ml, 4.5 mmol) were added for extraction, followed by a wash with brine (200 ml). The crude material resulting after removal of the solvent was applied for chromatography on SiO_2 (206 g). The product was eluted with EtOAc/*i*PrOH (2 : 3). Evaporation of the solvent yielded 2.31 g (73%) of Fmoc-Asp(OPyBzh)-OH (collidine salt). The free acid used for NMR-analysis was obtained by treating a sample of the collidine salt with 0.05 M HCl after uptake in EtOAc followed by three aqueous washes, drying and evaporation of the solvent. TLC (system B): r_f 0.51; ESIMS: 599.2 (MH^+), 1196.8 (M_2H^+), 244.3 ($PyBzh^+$); ^{1}H-NMR ($CDCl_3$): δ 2.91–3.45 (2H, m, β-CH_2), 4.19–4.22 [1H, t, $J = 7.1$ Hz, H9 (Fmoc)], 4.27–4.40 [2H, m, O-CH_2 (Fmoc)], 4.67–4.69 (1H, m, α-H), 5.88–5.89 (1H, d, $J = 8.1$ Hz, NH), 7.24–7.40 [14H, m, arom. (Fmoc, PyBzh)], 7.55–7.57 [2H, d, $J = 7.7$ Hz, H3/5 (pyridine)], 7.59–7.61 [2H, d, $J = 5.3$ Hz, H1/8 (Fmoc)], 7.73–7.75 [2H, d, $J = 8.0$ Hz, H4/5 (Fmoc)], 8.49–8.50 [2H, d, $J = 6.0$ Hz, H2/6 (pyridine)].

Fmoc-Asp(OPhFl)-OAll

Fmoc-Asp-OAll (2.37 g, 6 mmol) was dissolved in dry DCM (15 ml). PhFl-Br (1.97 g, 6.1 mmol) and DIPEA (1.03 ml, 6 mmol) were added. The reaction was monitored by TLC (system D) and stopped after 19 h. After evaporation of the solvent, the residue was taken up in EtOAc/water (110 ml each) to remove DIPEA·HBr. Extraction with H_2O removed most of the unreacted Fmoc-Asp-OAll. After washing with brine (50 ml each), drying with Na_2SO_4 and evaporation of EtOAc, the crude Fmoc-Asp(OPhFl)-OAll was obtained as a white foam. Chromatography on SiO_2 eluting with hexane/EtOAc (4 : 1) afforded the desired product (3.09 g, 81%). TLC (system D): r_f 0.66; ESIMS: 658.1 (MNa^+), 674.1 (MK^+), 241.3 ($PhFl^+$); ^{1}H-NMR ($CDCl_3$): δ 2.92–3.20 (2H, m, β-CH_2), 4.17–4.20 [H, t, $J = 7.2$ Hz, H9 (Fmoc)], 4.27–4.41 [2H, m, O-CH_2 (Fmoc)], 4.46–4.55 [2H, m, O-CH_2 (All)], 4.63–4.67 (1H, m, α-CH), 5.14–5.17 [1H, m, CH=(All)], 5.20–5.24 [1H, m, CH=(All)], 5.71–5.77 (2H, m, =CH(All) and NH), 7.25–7.40 (15H, m, arom. (Fmoc, PhFl), 7.53–7.55 (2H, d, $J = 7.3$ Hz, arom.), 7.69–7.71 (2H, d, $J = 7.5$ Hz, arom.), 7.73–7.76 (2H, d, $J = 7.6$, arom.).

Fmoc-Asp(OPhFl)-OH (collidine salt)

Fmoc-Asp(OPhFl)-OAll (3.24 g, 5.1 mmol) was dissolved in dry DCM (50 ml) under nitrogen. $Pd(PPh_3)_4$ (0.12 g, 0.1 mmol), triphenylsilane (1.3 ml, 10 mmol) and collidine (0.7 ml, 5.28 mmol) were added consecutively. The homogeneous solution rapidly darkened, with the concomitant evolution of gas. TLC-monitoring (system B) indicated smooth allyl cleavage. The solvent was removed after 35 min and the amorphous residue was dissolved in EtOAc (150 ml). The resulting solution was washed with water and brine (2 × 80 ml each). The EtOAc solution yielded a dark foam upon evaporation. Redissolution in EtOAc and treatment with carbopal P1 removed the colour. Evaporation of the solvent yielded an off-white foam, which was applied to a column containing SiO_2 (124 g). Elution with EtOAc and EtOAc/*i*PrOH (1 : 1) afforded 2.17 g (61%) of Fmoc-Asp(OPhFl)-OH, collidine salt. The free acid used for NMR-analysis was obtained by treating a sample of the collidine salt in EtOAc with 2% aqueous citric acid followed by washes with water and brine, drying and evaporation. TLC

(system B): r_f 0.64; ESIMS (negative mode): 593.9 (M-H), 1189.7 (M_2-H); ^{1}H-NMR ($CDCl_3$): δ 2.92–3.17 (2H, m, β-CH_2, 4.16–4.19 [1H, t, $J = 7.0$ Hz, H9 (Fmoc)], 4.30–4.41 [2H, m, O-CH_2 (Fmoc)], 4.62–4.65 (1H, m, α-CH), 5.67–5.69 (1H, d, $J =$ 8.6 Hz, NH), 7.16–7.39 (15H, m, arom.), 7.51–7.53 (2H, d, $J = 7.6$ Hz, arom.), 7.66–7.68 (2H, m, arom.), 7.73–7.75 (2H, d, $J = 7.4$ Hz, arom.).

Fmoc-Asp(O(3-methyloxetan-3-ylmethyl))-OBzl

Fmoc-Asp-OBzl (22.39 g, 50.2 mmol) was dissolved in dry THF (28 ml) followed by slow addition of oxalyl chloride (4.9 ml, 57.9 mmol). The evolution of gas was markedly enhanced when adding 2 drops of DMF. Evaporation after 4.5 h yielded a yellow solid, which was redissolved in a mixture of dry DCM (50 ml) and THF (10 ml). The solution of the acid chloride was added dropwise within 1 h to a mixture of 3-methyl-3-hydroxymethyl-oxetane (5.17 g, 50.6 mmol), dry DCM (50 ml) and pyridine (5 ml) at 0 °C. Ester formation was monitored by TLC (system C). The ice-bath was removed after 2 h, and stirring was continued at room temperature overnight. EtOAc (750 ml) and water (250 ml) were added to extract the pyridinium salts. A further aqueous extraction was followed by treatment with 2% Na_2CO_3 (250 ml) to remove unreacted Fmoc-Asp-OBzl. After an additional aqueous wash, a brine extraction, treatment with Na_2SO_4 and evaporation of the solvent, 25.0 g (94%) of the desired ester was isolated. TLC: r_f 0.65 (system A), r_f 0.51 (system C).

Fmoc-Asp(OBO)-OBzl

After dissolution in dry DCM (100 ml), crude Fmoc-Asp[O(3-methyloxetan-3-ylmethyl)]-OBzl (24.87 g, max. 47.0 mmol) was subjected to ortho ester rearrangement [5]. Under Ar atmosphere, the solution was cooled with ice and BF_3OEt_2 (0.3 ml, 2.39 mmol) was added under vigorous stirring, then the ice-bath was removed. According to TLC (system C), the rearrangement proceeded rapidly, though a range of by-products could be detected. After 5 h, the reaction was quenched by addition of triethylamine (0.33 ml, 2.3 mmol). After evaporation, the crude product was purified by column chromatography on SiO_2 (1.1 kg, prewashed with collidine in EtOAc/hexane (1 : 2)). Upon removal of the solvent, Fmoc-Asp(OBO)-OBzl (13.0 g, 52%) crystallized. Mp: 136°–138 °C. TLC: r_f 0.73 (system A), r_f 0.59 (system C); ESIMS: 552.2 (MNa^+), 1080.8 (M_2Na^+); ^{1}H-NMR: δ 0.78 [3H, s, CH_3 (OBO)], 2.19–2.41 (2H, m, β-CH_2), 3.83 [6H, s, O-CH_2 (OBO)], 4.25–4.28 [1H, t, $J = 7.2$ Hz, H9 (Fmoc)], 4.32–4.38 [2H, m, CH_2-O (Fmoc)], 4.54–4.56 (1H, m, α-CH_2), 5.16 [2H, s, O-CH_2 (Bzl)], 6.07–6.08 (1H, d, $J = 9.3$ Hz, NH), 7.25–7.41 [9H, m, arom. (Fmoc, Bzl)], 7.60–7.63 (2H, m, arom.), 7.75–7.77 (2H, d, $J = 7.5$, arom.).

Fmoc-Asp(OBO)-OH (collidine salt)

Fmoc-Asp(OBO)-OBzl (1.0 g, 1.88 mmol) was dissolved under an Ar atmosphere in dry THF (50 ml) and collidine (0.26 ml, 1.96 mmol) and Pd/C (60 mg) were added. Hydrogenation was carried out under atmospheric pressure. TLC (system B) indicated a smooth reaction. Hydrolysis of the OBO group was not observed. After 2.2 h the catalyst was removed followed by evaporation of the solvent. The product was obtained in quantitative yield (1.06 g) and it was used without further purification in SPPS. TLC (system B) r_f (orthoester) 0.53 (diol resulting from hydrolysis, r_f 0.28). ESIMS: 440.0 (MH^+), 462.1 (MNa^+), 458.2 (MH_3O^+ , produced by hydrolysis of OBO).

Assessment of the Acid-lability of OPyBzh and OPhFl Esters

To determine the stability of Fmoc-Asp(OPyBzh)-OBzl and Fmoc-Asp(OPhFl)-OAll in 1% TFA/DCM,

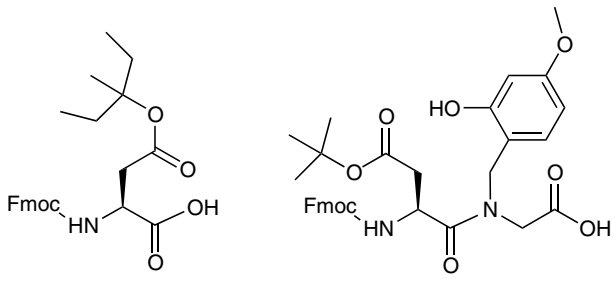

Figure 2 Structure of Fmoc-Asp(OMpe)-OH and Fmoc-Asp(OtBu)-HmbGly-OH.

the derivative (ca. 50 mg) was dissolved in DCM (1 ml). Then 2% TFA/DCM (1 ml) was added with vigorous stirring. The progression of the cleavage was followed by TLC (PyBzh: TLC system A, PhFl: TLC system D).

Solid Phase Synthesis of VKDGYI (I)

Solid phase synthesis was performed on the Wang resin and on Sasrin™ (see Results and Discussion). Fmoc was used for N^{α}-protection, *t*Bu and Boc were employed for side-chain protection of Tyr and Lys, respectively. The various β−carboxy protecting groups used for Asp are listed in Table 1. Piperidine/DMF (1 : 4) was the standard cleavage cocktail used for Fmoc removal up to the incorporation of the Fmoc-Asp derivative. Subsequent variations of Fmoc cleavage conditions are summarized in Table 2. In all cases, the resin was treated twice, for 5 and 10 min, with the base of choice. All couplings were performed using a threefold excess of Fmoc amino acid derivative, TBTU

Table 1 Extent of Formation of Aspartimide and Other By-products during Syntheses of H-Val-Lys-Asp-Gly-Tyr-Ile-OH.

Protection	Desired product	Desired product(%)	Aspartimide (D and L) (%)	α-Piperidide (%)	β-Piperidide (%)	By-product[a] (%)
O*t*Bu	VKDGYI	91.1	2.3	1.5	nd	nd
OAll	VKD(OAll)GYI	nd	49.6	12.0	2.9	25.0
OPp	VKDGYI	80.7	9.0	1.1	0.3	1.5
OBzl	VKD(OBzl)GYI	1.5	63.6	12.3	2.0	14.2
OPyBzh	VKDGYI	1.0	55.8	12.2	2.6	14.8
OPhFl	VKDGYI	7.0	65.3	8.9	1.6	7.8
OBO	VKD(X)GYI[b]	85.3	6.1	0.9	nd	1.7
OMpe	VKDGYI	93.9	0.7	nd	nd	nd
O*t*Bu/Hmb	VKDGYI	94.0	nd	nd	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
[a] See Results and Discussion.
[b] X = $OCH_2C(CH_3)(CH_2OH)_2$.
nd = not detectable (<0.3%).

Table 2 Extent of Formation of Aspartimide and Other By-products during Syntheses of H-Val-Lys-Asp-Gly-Tyr-Ile-OH.

Protection	Desired product	Desired product(%)	Aspartimide (D and L) (%)	α-Piperidide (%)	β-Piperidide (%)	By-product[a] (%)
O*t*Bu	VKDGYI	91.1	2.3	1.5	nd	nd
O*t*Bu[b]	VKDGYI	81.9	7.2	1.2	nd	1.8
OMpe	VKDGYI	93.9	0.7	nd	nd	nd
OMpe[b]	VKDGYI	91.0	3.3	0.5	nd	0.7
O*t*Bu/Hmb	VKDGYI	94.0	nd	nd	nd	nd
O*t*Bu/Hmb[b]	VKDGYI	94.4	nd	nd	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 or at 45 °C. Percentages of desired product and by-products were determined by RP-HPLC, as reported in Materials and Methods
[a] See Results and Discussion.
[b] Fmoc removal at 45 °C.
nd = not detectable (<0.3%).

and collidine in DMF. The coupling reaction was carried out for 1 h at ambient temperature. No additional collidine was applied for the coupling reaction in the cases of Fmoc-Asp derivatives that were already prepared as collidine salts. The pH was adjusted to pH 7 with collidine during the synthesis of the Asp(OPhFl)- or Asp(OBO)-containing peptides due to the acid-sensitivity of the side-chain protection. The syntheses were monitored by the Kaiser test and the 2,4,6-trinitrobenzenesulfonic acid test. Cleavage from the resin was performed with 95% aqueous TFA at room temperature for 1 h, followed by precipitation of the peptide with ice-cold *t*BuOMe. Alternatively, in the case of the Sasrin™, the protected peptides were obtained by repetitive short treatments (2 to 3 min) of the peptidyl resin with 1% TFA/DCM. The peptide-containing filtrates were neutralized immediately with pyridine, checked by TLC (system B) and evaporated. Conditions of analytical HPLC chromatography were: column: Baker-bond C_{18} 300Å; buffer system: 0.095 M phosphoric acid and 0.09 M triethylamine in water (pH 2.3), A: 5% CH_3CN, 95% buffer; B: 60% CH_3CN, 40% buffer; gradient: 5 to 35% B in 45 min; flow 1 ml/min; detection at 220 nm.

LC-MS identification of the most important products formed: 694.4 (MH^+, VKDGYI), 676.3 (MH^+, aspartimide formation), 761.3 (MH^+, piperidide formation), 577.3 (MH^+, further unidentified by-product, see Results and Discussion).

RESULTS AND DISCUSSION

An absolutely reliable protocol for the removal of the Fmoc group is a prerequisite for the SPPS of long peptides. Harsher cleavage methods which may ensure complete deblocking for larger peptides, enhance the risk of aspartimide formation and other base-induced side reactions. The extent of base-catalysed aspartimide formation varies considerably, depending on the type of base and the β-carboxy protecting group. However, Fmoc cleavage procedures known to be more efficient in Fmoc removal were also included in this study. In the case of high sensitivity towards bases, extensive cyclization followed by considerable amounts of further by-products resulting from the opening of the D/L-aspartimide ring were observed (see Figure 1). To be able to trace the following compounds, independent syntheses were performed: VKdGYI, VKd(GYI), VKD(GYI), VKD(piperidide)GYI and VKD(GYI)-piperidide. We did not synthesize the isomeric D-piperidides, although they may also be formed via the racemized aspartimide. With these derivatives in hand, HPLC conditions were optimized to obtain a satisfactory chromatographic separation of potential contaminants. A further by-product related to aspartimide formation could not be identified. MS suggests the loss of the *N*-terminal valine from the aspartimide-containing peptide.

Established Asp β-protecting Groups

The OtBu side-chain protection of Asp for the synthesis of **I** using 20% piperidine/DMF for Fmoc-cleavage at ambient temperature was the standard for the comparison against other derivatives. As indicated in Table 1, in the case of **I** by-products are easily generated. As expected, elevated temperatures, e.g. 45 degrees (see Table 2), promote the side reaction and also the stronger bases such as DBU or TMG give rise to a considerable amount of D/L-aspartimide and subsequent products. The latter results are listed in Table 3. Figure 7A shows a typical HPLC chromatogram if

Table 3 Fmoc Cleavage using Stronger Bases

Fmoc-Asp derivative	Desired product (%)	Aspartimide (D/L) (%)	α-Piperidide (%)	β-Piperidide (%)	By-product (%)
O*t*Bu[a]	52.1	21.8	9.4	0.6	5.8
O*t*Bu/Hmb[a]	94.1	nd	nd	nd	nd
OMpe[a]	83.0	7.8	1.9	nd	1.1
O*t*Bu[b]	66.9	17.6	4.4	0.4	3.2

[a] DBU/piperidine/DMF (1 : 20 : 79).
[b] TMG/piperidine/DMF (2 : 20 : 78).
nd, not detectable (<0.3%).

1% DBU is applied for Fmoc removal after incorporation of the Asp residue applying OtBu protection. In addition, prolonged treatments with base, to mimic the conditions of the synthesis of long peptides, further increased the amount of D/L-aspartimide and related products. In particular, an extended contact with piperidine/DMF (an additional 3 h treatment of the peptide resin) indeed generated a remarkable amount of by-products (HPLC: VKDGYI 71.9%, L/D-aspartimide 11.4%, α-piperidide 6.3%).

Peptides containing an Asp β-allyl ester are rather sensitive towards base treatment during SPPS. Total conversion into aspartimide has been reported [6]. The SPPS of **I** employing Fmoc-Asp(OAll)-OH and 1% DBU/DMF for Fmoc cleavage [6] yielded aspartimide (HPLC: 84.8%) as the main product. In the presence of piperidine, piperidides and further by-products (see Table 1) were observed. In case of the derivative Fmoc-Asp(OBzl)-OH, a similar pattern of by-products appeared.

Aspartimide formation is impeded by bulky β-carboxy protecting groups and, in most cases, the β-O*t*Bu-group provides sufficient steric hindrance. Therefore, the OPp protected Asp derivative [7] bearing the very acid-labile phenylisopropyl side-chain protecting group was included in our study. Surprisingly, this more bulky protecting group was somewhat more susceptible towards aspartimide formation in comparison to standard OtBu protection (see Table 1).

Synthesis and Application of new Asp Derivatives

Based on these observations, we decided to design even bulkier side-chain protecting groups and to test their potential to inhibit aspartimide formation. As a first example, the 4-pyridyl-diphenylmethyl ester (OPyBzh) protecting group was evaluated. This bulky moiety was expected to increase the base stability of **I** during synthesis while maintaining a reasonable sensitivity towards diluted TFA to facilitate postsynthetic cleavage. Thus, the derivative Fmoc-Asp(OPyBzh)-OH was synthesized starting from Fmoc-Asp-OBzl as outlined in Figure 3. An alternative synthesis starting from Z-Asp-OBzl failed due to the instability of the pyridylbenzhydryl ester under the conditions of hydrogenation. Consequently, prolonged hydrogenation of the corresponding Fmoc derivative had to be avoided. The first experiment was related to studies on the lability under acidic conditions. No significant cleavage of the PyBzh moiety was observed within a 5 h treatment with 1% TFA/DCM. By contrast, the PyBzh group was split off in 2% TFA/DCM within 30 min. Thus, the properties of this protecting group corresponded to our expectations and the Asp derivative was applied for the synthesis of **I**.

In the case of the PyBzh derivative, **I** could not be obtained by standard synthesis on Wang resin. Acidolytic cleavage yielded mostly aspartimide and the piperidides (see Table 1). Thus, the fully protected fragment was produced by synthesis on Sasrin™. Unexpectedly, the fully protected fragment Fmoc-Asp(OPyBzh)-Gly-Tyr(*t*Bu)-Ile-OH instantaneously yielded the aspartimide upon treatment with only 2% piperidine/DMF. We reasoned that the pyridyl nitrogen may assist in aspartimide formation by a kind of neighbouring group effect.

Therefore, another trityl analogue for β-carboxy protection of Asp was synthesized, the 9-phenylfluorenyl ester(OPhFl). The PhFl ester has been recently described [9], albeit the corresponding Fmoc-Asp

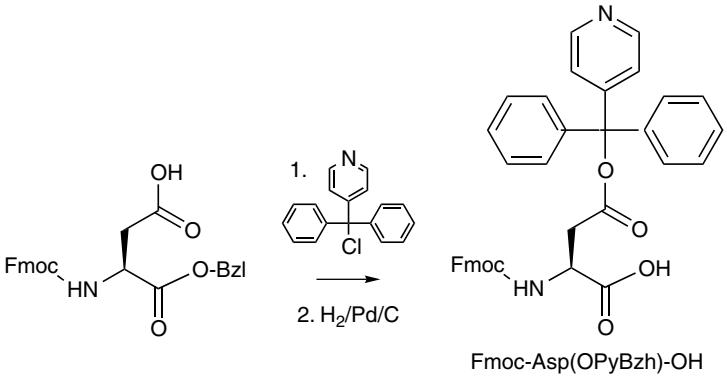

Figure 3 Synthesis of Fmoc-Asp(OPyBzh)-OH.

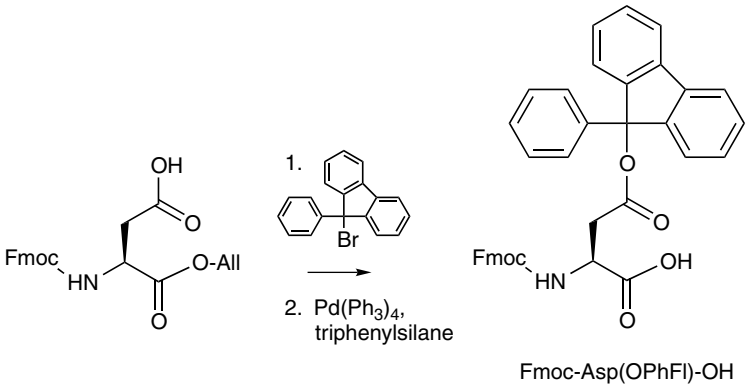

Figure 4 Synthesis of Fmoc-Asp(OPhFl)-OH.

derivative has not been produced so far. Fmoc-Asp(OPhFl)-OH was obtained starting from Fmoc-Asp-OAll as indicated in Figure 4. In our hands, several hours were required to completely cleave the PhFl group with 0.5% TFA/DCM and also removal by hydrogenation could be easily achieved. Since the conditions for removal agreed with the reported properties of the PhFl group, this derivative was applied for synthesis of the test peptide. Surprisingly, synthesis of **I** on Wang resin using the derivative Fmoc-Asp(OPhFl)-OH (collidine salt) afforded only the aspartimide and the piperidides (see Table 1). For studying the behaviour of the OPhFl-group in more detail another synthesis of **I** was started on Sasrin™. Mild acidolysis of a sample taken after the coupling of Fmoc-Asp(OPhFl)-OH yielded the expected intermediate Fmoc-Asp-Gly-Tyr(*t*Bu)-Ile-OH. Cleavage of a sample taken after piperidine treatment afforded a mixture of the desired product H-Asp-Gly-Tyr(*t*Bu)-Ile-OH, aspartimide and piperidide as detected by MS. Further cleavages following stepwise elongation showed that the fraction of the desired product rapidly decreased. According to HPLC analysis, a mixture containing D/L-aspartimide (63.0%), α-piperidide (12.8%), β-piperidide (2.3%), and only a trace of the desired product **I** was obtained after final cleavage. There is evidence that only a few piperidine treatments are sufficient to significantly decrease the quality of an Asp-Gly containing peptide via concomitant aspartimide formation. This finding is more difficult to interpret, but it is in accordance with the observation of Kunz *et al.* [9] on the instability of the PhFl group under basic conditions. In the latter publication, the formation of pyroglutamic acid from *N*-terminal Glu(OPhFl) during prolonged treatment with morpholine was described.

The results on trityl protection are somehow in line with the observation of increased amounts of side products observed in case of OPp protection. Obviously, aromatic residues cannot sufficiently suppress the attack of the amide nitrogen and, furthermore, due to the enhanced lability, they promote the leaving group character of these protecting groups which result in a higher propensity to form the aspartimide. Due to these disappointing results, trityl-type protecting groups were not further investigated.

The transformation of the β-carboxy group was thought to be an alternative approach to ensure complete suppression of aspartimide formation. We anticipated that the bicyclic OBO orthoester [10] would be optimal Asp β-carboxy protection during Fmoc/tBu assisted SPPS. Fmoc-Asp(OBO)-OH (isolated as the collidine salt) was difficult to synthesize (see Figure 5), but with the derivative in hand, a synthesis of **I** was performed. Following TFA treatment, the desired product H-Val-Lys-Asp[O-2,2-di(hydroxymethyl)propyl]-Gly-Tyr-Ile-OH was obtained. However, a small amount of the α-piperidide was detected (see Table 1). This finding can only be explained assuming slow hydrolysis of the orthoester moiety during SPPS (see Figure 6), aspartimide formation from the resulting Asp β-[2,2-di(hydroxymethyl)propyl] ester, and subsequent cleavage by piperidine. Furthermore, large quantities of aspartimide are formed during the second stage of OBO removal, which involves the saponification of the 2,2-di(hydroxymethyl)propyl ester (see Figure 6). The mild conditions, as developed by Ramage *et al.* [11] for the saponification of the 2,2-di(hydroxymethyl)-2-nitro-ethoxycarbonyl group, failed in our case. In summary, conditions for basic hydrolysis could not be adjusted to avoid the known side reactions.

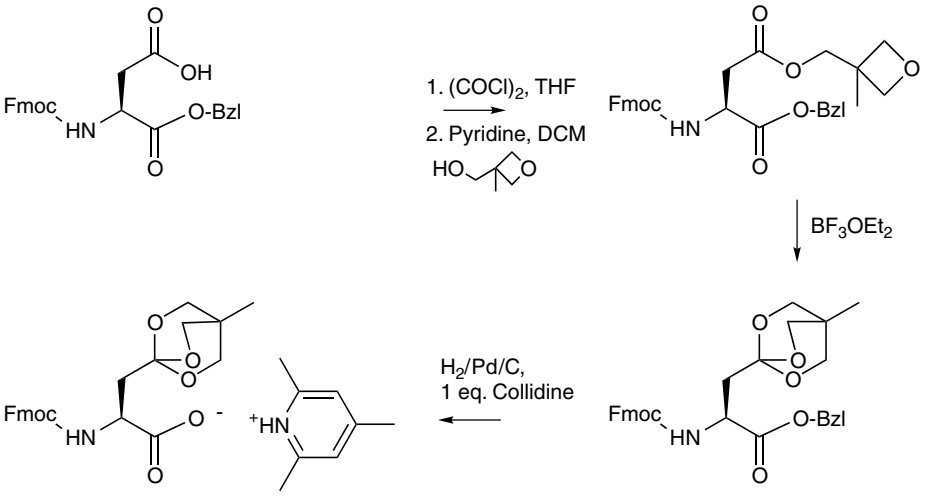

Figure 5 Synthesis of Fmoc-Asp(OBO)-OH.

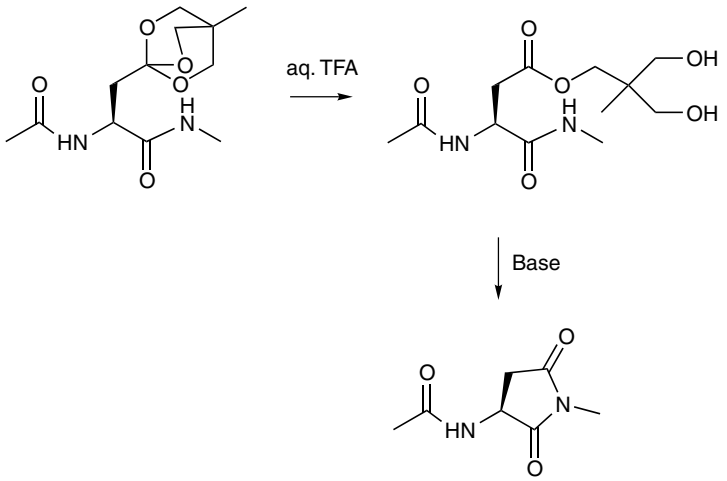

Figure 6 Removal of the OBO protecting group.

Hence, standard OtBu protection was reconsidered and possibilities for a systematic improvement were sought. Firstly, the synthesis of **I** incorporating an Fmoc-Asp derivative having the more bulky 3-methylpent-3-yl ester (OMpe) [8] (see Figure 2) attached to the β-carboxy functionality was studied. Especially the results obtained on synthesis of **I** under harsher conditions (1% DBU), clearly showed an improvement compared with OtBu protection (see Table 3 and Figure 7B). These results were corroborated by the finding that elevated temperatures (45 degrees) have a more pronounced effect on aspartimide formation in the case of standard OtBu protection (see Table 2).

Next, the effect of backbone protection in our test system should be established clearly. Backbone protection (Hmb) was reported in the literature [2,12] and the alkylation of the Asp-Xaa amide bonds was thought to be an efficient method for preventing aspartimide formation. The dipeptide Fmoc-Asp(O*t*Bu)-HmbGly-OH was produced and applied for the synthesis of **I**. The derivative coupled smoothly to the resin after activation with the more efficient coupling reagent TATU. During acidolysis, the Hmb group was readily removed. Even under harsher conditions, e.g. stronger base or elevated temperature, no aspartimide-related by-products could be detected (see Tables 1–3, Figure 7C). Thus, the combination of Asp(OtBu) side chain protection with Hmb backbone protection on the carboxy side is an efficient combination to completely suppress aspartimide formation. However, it remains

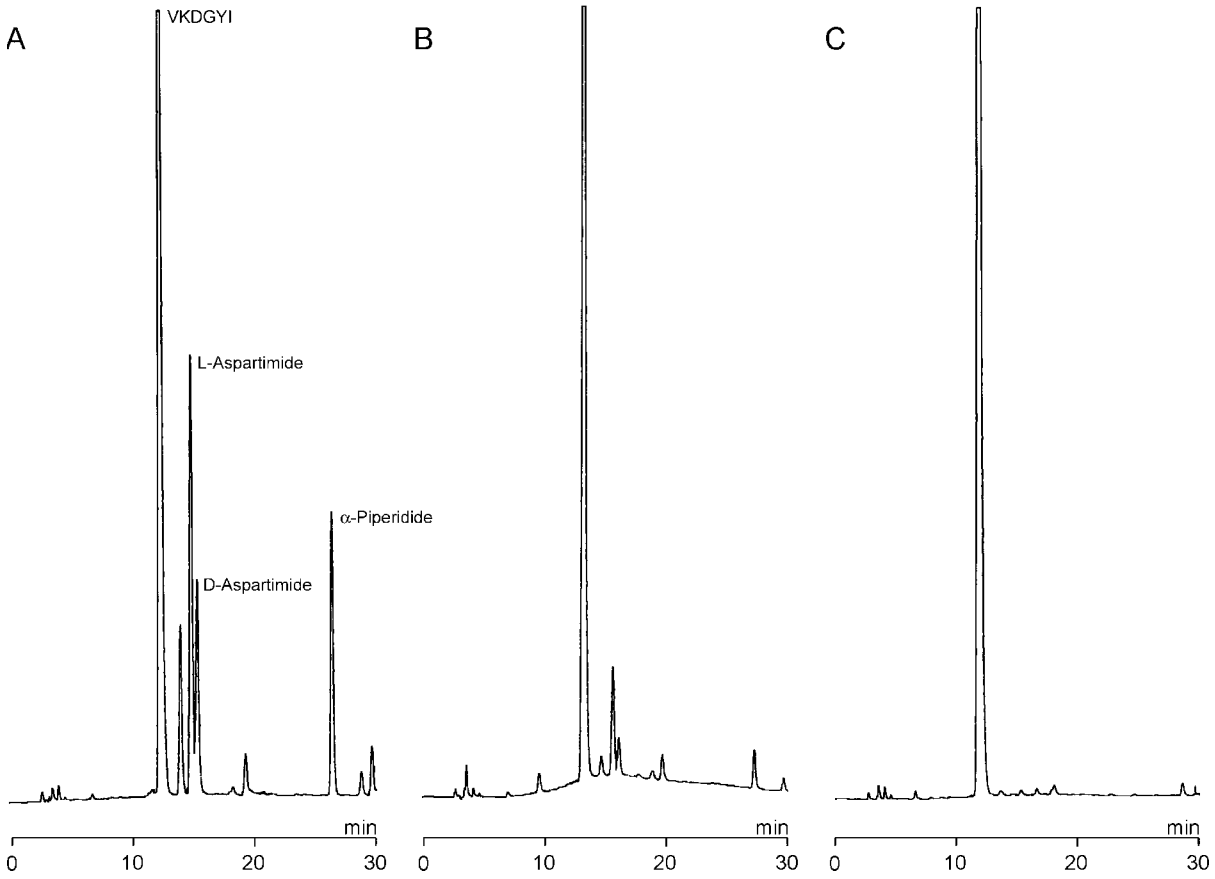

Figure 7 Analytical HPLC-profiles of crude products obtained after syntheses of peptide **I** using DBU/piperidine/DMF (1 : 20 : 79) for Fmoc cleavage. A: Synthesis performed with Fmoc-Asp(OtBu)-OH, B: Synthesis performed with Fmoc-Asp(OMpe)-OH, C: Synthesis performed with Fmoc-Asp(OtBu)-HmbGly-OH.

to be determined whether the accessibility of the dipeptide building block and its racemization-free incorporation can be realised for amino acids other than Gly.

CONCLUSION

This investigation clearly showed that the introduction of backbone protection reliably prevents aspartimide formation in the case of the sensitive Asp-Gly sequence. Among the numerous Asp side-chain protecting groups evaluated in the course of this study, only the slightly more bulky OMpe-group turned out to be superior to the standard O*t*Bu protection with respect to suppression of aspartimide-related side reactions. Alternative protecting groups which might have been expected to prevent aspartimide formation, such as trityl ester and orthoester derivatives, turned out to rather promote this undesired reaction. In continuation of this work, we plan to include other amino acids in place of Gly, and this will be the subject of future reports.

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