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Tetrahedron Letters 47 (2006) 4121–4124
Tetrahedron Letters

Synthesis of 'difficult' peptides free of aspartimide and related products, using peptoid methodology

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Received 16 March 2006; revised 13 April 2006; accepted 18 April 2006

Abstract—We developed an efficient, cost effective strategy for Fmoc-based solid phase synthesis of 'difficult' peptides and/or peptides containing Asp/Asn-Gly sequences, free of aspartimide and related products, using a peptoid methodology for the preparation of N-substituted glycines.
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The formation of aspartimide or aminosuccinimide (3-amino-pyrrolidine-2,5-dione, Asu) is the first step of the well-known degradation of aspartic acid/asparagine containing peptides and proteins at alkaline, neutral and acidic pH, both in vitro and in vivo[1] (Scheme 1). The reaction is especially prevalent at Asp/Asn-Gly sites, and results in a variety of rearranged and racemized products of which the unnatural β-Asp is generally formed in the largest amount. Asu formation is especially problematic in Fmoc-*tert*-based SPSS because the strong base, such as piperidine, that is used for deprotection promotes Asu formation.

Asu formation can be diminished by the use of additives,[2] by decreasing the basicity[†] of the deprotection mixture[3] or by applying (instead of O*t*Bu) bulky, alkyl type protecting groups to the side chain of Asp.[4,5] It can be eliminated by solid phase immobilization via the amide bond[6] or via reversible amide backbone protection with Hmb[7] and analogues[8] (Scheme 1). However, coupling of residues with Hmb-backbone protection is inefficient, and is often accompanied by depsipeptide formation.[9,10] Hmb-analogues can only partially resolve these problems.

We recently found that the Dmb amide protecting group is efficient in preventing secondary structure formation at Gly-Gly sites, and is orthogonal with respect to standard Fmoc SPPS.[11] In this work we explore the use of Dmb, Tmb and Nbzl protecting groups (**Z**) for the synthesis of difficult/Asu-prone peptides, in three different strategies: (A) Fmoc-Asn/Asp-(**Z**)Gly-OH dipeptide building blocks; (B) Fmoc-(**Z**)Gly-OH monomer building blocks; and finally, (C) 'sub-monomeric' synthesis of H-(**Z**)Gly on the resin in which the intermediate products do not need to be isolated and purified. Strategy C (Scheme 2) is based on the principle of solid phase peptoid synthesis.[14] The first step is the acylation of an N-terminal amino group of peptide–resin with bromo- or chloroacetic acid in the presence of a coupling agent, which is followed by S_N2 displacement of the halogen with excess primary amine.

We tested the new methods on VK<u>DG</u>YI, a well known model for Asu-formation,[9,12] as well as on H-G[1]LFGAIAGFIE**NG**WEGMI<u>**DG**</u>[20] GRKKRRQRRR[30]-OH, a peptide containing $HA2_{1-20}$ and HIV-TAT_{48-57} sequences, designed to deliver molecules of

Keywords: Aspartimide; Backbone protection; Caged peptides; 'difficult' peptides; Peptoid.
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[†] Alternative solutions were proposed, such as (i) Nα-protecting group,[19] and (ii) linkers for SPPS of protected peptides, both cleavable under almost neutral conditions,[20] and finally, (iii) linkers cleavable with reagents less basic than PIP.[21]

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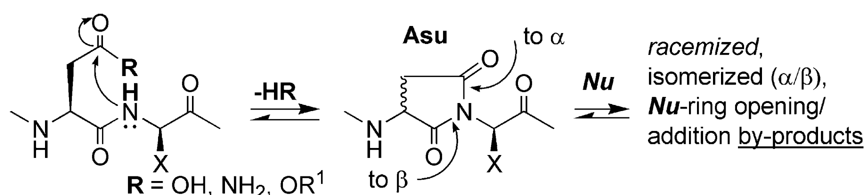
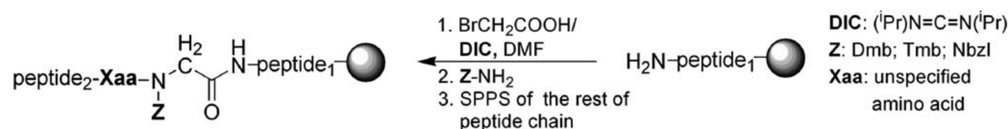
doi:10.1016/j.tetlet.2006.04.074



Scheme 1.



Scheme 2.

Table 1. Analytical data of backbone protected dipeptide Fmoc-Asx(Y)-(**Z**)Gly-OH (entries **1**–**9**) and Fmoc-(**Dmb**)Gly-OH (entry **10**, see *General procedure*)[17]

Entry	Asx	Y	Z	Retention time[a] (min)	Purity (%) crude/purified	MW calculated/ found $[M+H]^+$
1	Asp	O*t*Bu	Dmb	11.62	92/98.7	618.67/619.6
2	Asp	OAll	Dmb	11.05	95–97/99.0	602.65/603.5
3	Asp	O*t*Bu	Tmob	11.64	94/99.1	648.70/649.6
4	Asp	OAll	Tmob	11.15	88–93/99.1	632.66/633.5
5	Asp	O*t*Bu	Nbzl	11.48	96/99.2	603.62/604.4
6	Asp	OAll	Nbzl	10.63	92/99.1	587.19/588.5
7	Asn	Trt	Dmb	12.61	91/99.0	803.90/804.8
8	Asn	Trt	Nbzl	12.65	92/98.2	788.84/789.6
9	Asn	Trt	Tmob	13.30	88/98.5	833.92/834.7
10	—	—	Dmb	10.55	89–96/99.0	447.17/448.4

Dmb: 2,4-Dimethoxybenzyl; Hmb: 2-hydoxy-4-methoxybenzyl; Tmb: 2,4,6-trimethoxybenzyl; Nbzl: 2-nitrobenzyl.
[a] *RPHPLC separation conditions*: Zorbax 300 SB 5C18 (150 × 4.6 mm I.D.) column; linear gradient from A = 0.1% TFA in water to B = 0.1% TFA in MeCN at flow rate 1.5 mL min^{-1} in 15 min. Detection was at 214 nm or ESI-MS.

Table 2. Analytical data of H-Val-Lys-**Asx**-Gly-Tyr-Leu-NH_2/OH peptides prepared by parallel SPPS[18] using preformed building blocks (Table 1, entries **1** or **10**) or 'sub-monomer route' for the synthesis of aryl substituted *N*-benzylglycines

Entry	Peptide sequence	MW calculated/$[M+H]^+$	Building block or amine used	Desired peptide[a] (purity, %)
1	H-VK**D**GYI-NH_2	692.4/693.4	—	45[b]
2	H-VK**D**(OAll)GYI-NH_2	732.4/—	—	nd[b]
3	H-VK**D**GYI-NH_2	692.4/693.4	Fmoc-(**Dmb**)G-OH	94[b]
4	H-VK**D**GYI-NH_2	692.4/693.4	Fmoc-D(O*t*Bu)-(**Hmb**)G-OH	92[b]
5	H-VK**D**GYI-NH_2	692.4/693.4	Fmoc-D(O*t*Bu)-(**Dmb**)G-OH	92[b]
6	H-VK**D**GYI-NH_2	692.4/693.4	**Dmb**-NH_2	90[b]
7	H-VK**D**(OAll)GYI-NH_2	732.4/733.3	**Dmb**-NH_2	92[b]
8	H-VK**N**GYI-NH_2	691.4/692.4	**Dmb**-NH_2	90[b]
9a	H-VK(Boc)-**D**(OAll)-(Dmb)G-Y(*t*Bu)-I-OH	1039.6/1040.3	**Dmb**-NH_2	94[c]
9	H-VK**D**(OAll)-GYI-OH	733.4/734.4	**Dmb**-NH_2	92[c]
10a	H-VK(Boc)-**D**(OAll)-(Tmb)G-Y(*t*Bu)-I-OH	1069.6/1070.8	**Tmb**-NH_2	93[c]
10	H-VK**D**(OAll)-GYI-OH	733.4/734.4	**Tmb**-NH_2	91[c]
11a	H-VK(Boc)-**D**(O*t*Bu)-(Nbzl)GY(*t*Bu)-I-OH	1040.9/1041.8	**Nbzl**-NH_2	94[c]
11	H-VK**D**-(Nbzl)GYI-OH	828.4/829.3	**Nbzl**-NH_2	88[c]
12a	H-VK(Boc)-**N**(Trt)-(Nbzl)GY(*t*Bu)-I-OH	1225.6/1226.5	**Nbzl**-NH_2	98[c,d]
12	H-VK**N**-(Nbzl)GYI-OH	827.4/828.4	**Nbzl**-NH_2	95[c]

nd—(<1%).
[a] By RPHPLC after treatment with 20% piperidine in DMF at room temperature for 24 h.[12]
[b] *Tentagel R RAM resin*, Rapp Polymere, 90 μm, 0.22 mmol/g.
[c] 2-chlorotrityl resin, NovaBiochem, 200–400 μm, 0.25 mmol/g.
[d] Separation on Zorbax 300 SB 3.5CN column, all other peptides—on Zorbax 300 SB 5C18 column (for more details see Supplementary data).

interest into cells.[13] When prepared with standard Fmoc-SPPS, both peptides showed extensive formation of Asu (delta mass – 18 Da) and piperidide (delta mass + 67 Da) as well as the presence of various race-

mized and isomerized products that makes product purification difficult and often impossible.

Table 1 shows the data of preformed building blocks prepared for Strategy A (**1**–**9**) and for Strategy B (**10**). Table 2 summarizes the characteristics of VKDGYI as prepared with the three strategies described above. In general, the yield and purity of the products reach, and even exceed, the level obtained with Hmb protection. We also noted that the removal of the Dmb protecting group from the dipeptide building blocks by TFA is ~30% faster than that of Hmb (for more details see Supplementary data). In addition, ESI-MS showed that the peptides were free of Asu and piperidide.

Finally, we prepared the above mentioned HA2-TAT peptide[‡] using Strategies A and C. The yield/purity of the products were comparable with those obtained with the Hmb-protected dipeptide building block, and both were found to be free of Asu/piperidides. Both the preformed building blocks (Strategies A and B) and the sub-monomeric approach (Strategy C) introduce an open chain, proline-like structure, which can disrupt unwanted H-bonds during the synthesis of difficult peptides. Derivative **10** (Strategy B) is useful for the synthesis of Xaa-Gly sequences (including Asn/Asp-Gly) and potentially allows the incorporation of substituted *N*-benzylglycine at any point of the synthesis of 'difficult' peptides. The 'sub-monomeric route' (Strategy C) is simple and flexible (different amines can be used for halogen-displacement), does not require separate or additional synthetic steps and, at the same time, is more efficient and ~30 times more cost effective than the use of premade building blocks. With this procedure, the yields of aryl substituted *N*-benzylglycine were nearly quantitative. The new backbone protecting groups used here were in many respects superior to the commercial reagents (shelf stable, easily TFA- or photo-cleavable, orthogonal) and applicable for the synthesis of both peptide acids and peptide amides.

The use of 2-nitrobenzylamine for bromine displacement in Strategy C is a new method for the preparation of backbone-caged peptides (Table 2, entry 11–12). It can also be applied for the synthesis of cyclic peptides and—combined with orthogonal side chain protected aspartic acid—for the synthesis of Asp/Asn-derivatives, for example, glycopeptides[15] or side- to side-chain cyclic peptides.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2006.04.074.

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[‡] *Automated peptide synthesis of* $HA2_{1\text{-}20}$*-HIV-TAT*$_{48\text{-}57}$:
First synthesis of peptide H-GLFGAIAGFIENGWEG-MI**DG**GRKKRRQRRR-OH containing the sequences of hemagglutinin $HA2_{1\text{-}20}$ and HIV-$TAT_{48\text{-}57}$ was assembled on TentaGel S Trt-Arg(Pbf)-resin (90 μm, substitution 0.2 mmol g^{-1}, 0.05 mmol scales, Fluka) on automated peptide synthesizer SP3 (Protein Technology), using standard coupling/deprotection cycle.
The second synthesis was carried out in an identical manner, except that the commercially available building block Fmoc-Asp (O*t*Bu)-(Hmb)Gly-OH was used to introduce the DG-sequence.
The third synthesis was carried out in an identical manner, except that the synthesis of H-(Dmb)Gly_{20}-peptide-Ile-resin was prepared by a two step sub-monomer solid-phase synthesis, using Dmb-NH_2 for bromine displacement[11] (see above and the text). Double coupling was performed from Met_{17} to N-terminal Gly_1 including.
The DCM washed peptide resins were cleaved/deprotected with TFA/TIPS/H_2O (95/2.5/2.5, v/v/v) for 2.5 h, and the peptides were isolated in the usual manner. The crude peptides were characterized by RPHPLC (10–55% MeCN in 60 min, 1.5 mL min^{-1}) and LC/ESI-MS (for more details see Supplementary data), as described.[11]

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17. *General procedure*: Preparation of backbone protected dipeptides (Table 2, entries 1–9) and Fmoc-(Dmb)Gly-OH, (Table 2, entry 10): All building blocks were prepared by modification of the published procedure.[11] Briefly, 1.2 mmol H-(Z)Gly-OH (for more details see Supplementary data) in 1 mL DCM were stirred with 2.5–3.0 mmol *N*,*O*-bis(trimethylsilyl)acetamide until dissolved (6–10 min), then the solution was added to 1 mmol dry Fmoc-Asx(Y)-OSu (entries 1–9) or Fmoc-OSu (entry 10), followed by 1 mL DMF. The mixture was stirred until activated ester (0.5–2 h) was no more detected (TLC, $CHCl_3$/MeOH/AcOH = 90/9/1). DCM (10 mL) and 10% citric acid (6 mL) were added and then stirred for 0.5 h. The bottom phase was separated, and the upper water/citric acid phase was reextracted with DCM (2×5 mL). The collected DCM phases were washed with water (6×5 mL), dried (Na_2SO_4), filtered, washed on the filter with DCM (2×3 mL), and the collected solvent was evaporated to dryness. Crude products (entries 1–10, Table 1) were purified to homogeneity >98% (RPHPLC) using column chromatography on silica gel 60 in $CHCl_3$ to 4% MeOH in $CHCl_3$ (for more details see Supplementary data). The yields of purified products were >75%.
18. *The semi-automatic parallel solid-phase organic synthesis of peptide acids/amides [Table 2]* was performed on a 0.025 mmol scale using liquid distribution manifold (Domino block).[16] Twelve 3 mL polypropylene syringes, equipped with porous polypropylene discs at the bottom, were used as reactors. All common coupling/deprotection steps for the preparation of H-Y(*t*Bu)-I-resins were performed as described.[13] *Table 2, entries 1–5:* Standard SPPS was performed in reactors 1–5, including the coupling step of Fmoc-Asp(O*t*Bu/OAll)-OH or selected building blocks, washed with DMF, DCM, stopped and kept in refrigerator for the next common step. *Table 2, entries 6 to 12:* Freshly prepared 2 M solution of bromoacetic acid in DMF (1.75 mL) was mixed with 2 M solution of DIC in DMF (1.75 mL) containing DIEA (0.05 mL) and then 0.5 mL of the above preactivated bromoacetic acid was rapidly added to each of H-Tyr(*t*Bu)-Ile-resins in reactors from entries 6–12. The peptide resins were mixed for 0.5 h at room temperature, filtered and washed with DMF (6×). These steps were repeated one more time. The reactors were charged with 0.5 mL 1 M solution of the corresponding benzylamine and closed, and the reaction was allowed to proceed at 40 °C for 4 h. After filtration and standard washing (DMF, 6×), the reactors were disconnected from the domino block and then to each one was added Fmoc-Asx(Y)OH/PyBop/HOBt (5 equiv) as a 0.3 M solution in DMF, containing DIEA (10 equiv). At this point reactors 1–5 were added to the domino block, washed, deprotected (PIP/DMF, 2×5 min) and washed again. The last two common coupling/deprotection steps were performed using Fmoc-Lys(Boc)-OH and Fmoc-Val-OH. The resins were washed with DMF (2×), PrOH-2, DCM, MeCN and finally with DCM (2×). Single bead ATR Spectromicroscopy of peptide-resins confirms the peptide structures (for more details see Supplementary data). Portions (∼20–25 mg) from resin 9–12 were deprotected with HFIP (4×0.5 mL, 10 min each one), evaporated to dryness, dissolved in MeCN (200 μL, each one) and analyzed (Table 2, entries 9a–12a). Portions (∼20–25 mg) from resin 1–12 were deprotected with TFA/water/TIPS (95/2.5/2.5, v/v/, 2 mL per each one) for 1 h at room temperature. The volatile solvents were evaporated at room temperature and precipitated with ice-cold Et_2O, redissolved in 1 M NaOAc buffer pH 6.6/MeCN (1/1), evaporated (SpeedVac concentrator) and analyzed (Table 2, entries 1–12) (for more details see Supplementary data).
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