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Peptide Thioester Preparation by Fmoc Solid Phase Peptide Synthesis for Use in Native Chemical Ligation

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Abstract: Established methodology for the preparation of peptide thioesters requires the use of *t*-butoxycarbonyl chemistry owing to the lability of thioester linkers to the nucleophilic reagents used in Fmoc solid phase peptide synthesis. Both the greater ease of use and the broad applicability of the method has led to the development of an Fmoc-based methodology for direct peptide thioester synthesis. It was found that successful preparation of a peptide thioester could be achieved when the non-nucleophilic base, 1,8-diazabicyclo[5.4.0]undec-7-ene, together with 1-hydroxybenzotriazole in dimethylformamide, were used as the N^{α}-Fmoc deprotection reagent. Native chemical ligation of the resulting thioester product to an *N*-terminal cysteine-containing peptide was successfully performed in aqueous solution to produce a fragment peptide of human α-synuclein. The formation of aspartimide (cyclic imide) in a base-sensitive hexapeptide fragment of scorpion toxin II was found to be significant under the deprotection conditions used. However, this could be controlled by the judicious protection of sensitive residues using the 2-hydroxy-4-methoxybenzyl group.

Keywords: aspartimide; 1,8-diazabicyclo[5.4.0]undec-7-ene; 9-fluorenylmethoxycarbonyl; 1-hydroxybenzotriazole; 2-hydroxy-4-methoxybenzyl; native chemical ligation; peptide thioesters; solid phase peptide synthesis

INTRODUCTION

The chemical synthesis of proteins by the traditional linear solid phase methodology is somewhat restricted by the difficulty of ensuring complete amino acid acylation and N^{α}-deprotection throughout a long peptide chain, especially when syntheses of over 60 amino acids are attempted [1]. An alternative synthetic approach to large peptides is native chemical ligation, which is based on the preparation, by solid phase peptide synthesis (SPPS), of small, well-characterized peptide fragments bearing appropriate reactive groups on their termini. The chemoselective formation of native peptide linkages between the fragments is then performed in aqueous solution under conditions that facilitate accurate monitoring of the reaction [2,3]. This allows a more ready acquisition of biomolecules and, unlike protein production by recombinant DNA methodology, the incorporation of non-natural amino acids as an aid for protein structure–activity studies is possible.

One established method involves the ligation of a *C*-terminally thioesterified peptide to a peptide bearing a free *N*-terminal cysteine as shown in Figure 1 [2]. In this approach, the peptide thioester is prepared in two steps. Peptide elongation is performed on a solid support derivatized with a 4-(α-mercaptobenzyl)phenoxyacetic acid linker, which yields a free peptide thioacid upon acidolysis by hydrogen fluoride (HF) [4]. This species is then thioesterified in aqueous solution [2]. An alternative method that bypasses the requirement for peptide

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thioacid thioesterification was described by Hojo and Aimoto [5] and further refined by Camarero *et al.* [6]. In this approach, *t*-butoxycarbonyl (Boc) SPPS was performed on a resin-bound 3-mercaptopropionic acid linker to yield a peptide thioester suitable for native chemical ligation directly upon acidolysis by HF. Furthermore, the linker was prepared directly on the solid support [6].

Thioester species undergo aminolysis when exposed to the nucleophilic N^{α}-9-fluorenylmethoxycarbonyl (Fmoc) deprotection reagents used in Fmoc-SPPS [7]. Peptide thioester synthesis is therefore performed principally using Boc-SPPS, necessitating trifluoroacetic acid (TFA) treatments for N^{α}-Boc deprotection and final side-chain deprotection and cleavage from the solid support using anhydrous HF. Many laboratories do not have facilities for performing HF cleavage, due both to the cost of the apparatus and the hazards inherent in its operation. Li and co-workers described the use of 25% 1-methylpyrrolidine together with 2% hexamethyleneimine and 2% 1-hydroxybenzotriazole (HOBt) (v/v/w) in 50:50 *N*-methylpyrrolidinone (NMP) and dimethylsulfoxide (DMSO) as a reagent for N^{α}-Fmoc removal, which did not significantly decompose a 3-mercapto-3-methyl-butanoic acid derived peptide thioester [7,8]. This led to the production of a peptide thioester via Fmoc-SPPS in good yield, although this deblock solution was observed to cause significant subsequent aminolysis of a primary peptide thioester species [7]. In addition, the tertiary thioester linker described is not commercially available at this time and its preparation requires a multi-step reaction in solution. Furthermore, the suitability of these tertiary peptide thioesters for chemical ligation has not yet been

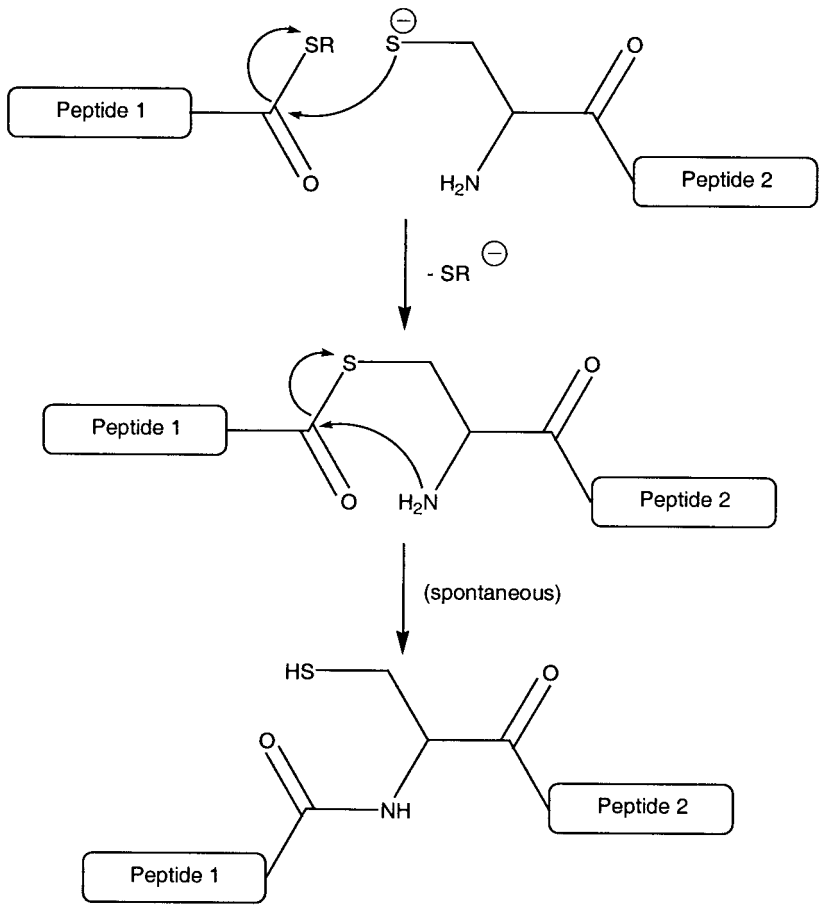

Figure 1 Schematic representation of native chemical ligation [2]. A peptide bearing a *C*-terminal thioester is attacked by the thiol moiety of a peptide bearing a free *N*-terminal cysteine forming a thioester likage between the peptide fragments in a chemoselective manner. The thioester then spontaneously rearranges to form a native amide bonded species yielding the target material.

$$V^{77}AQKTVEGA\underline{G^{86}S^{87}}IAAATGFVKKDQL^{100}$$

$$V^{77}AQKTVEGA\underline{G^{86}C^{87}}IAAATGFVKKDQL^{100}$$

Figure 2 The Gly[86]-Ser[87] bond in the sequence of human α-synuclein (top) was mutated (bottom) in order to enable its synthesis by native chemical ligation. Since the change of Ser to Cys only involves exchanging a hydroxyl for a thiol moiety, the mutation is conservative.

reported and their sterically hindered nature may significantly reduce the ligation rate. Clearly, an Fmoc-SPPS preparation of reactive peptide thioester species by exclusive use of solid phase conversions and commercially available reagents is highly desirable.

A non-nucleophilic base known to be amenable to solid phase peptide sythesis is 1,7-diazabicyclo[5.4.0]undec-7-ene (DBU) [9,10]. Owing to its sterically hindered nature, it is significantly less nucleophilic than piperidine despite being a stronger base. In our laboratories, continuous flow peptide synthesis with N^{α}-Fmoc deprotection by 1% DBU in dimethylformamide (DMF) (v/v) is used routinely. Here, we report the use of DBU together with HOBt in DMF for N^{α}-Fmoc deprotection enabling peptide thioester production via Fmoc-SPPS for native chemical ligation. An 18 amino acid test peptide and a 24 amino acid peptide corresponding to residues 77–100 of human α-synuclein (Figure 2), a peptide of interest in these laboratories, were each prepared via chemoselective ligation. The effect of this N^{α}-Fmoc deprotection reagent on an aspartimide-prone peptide was also assessed.

MATERIALS AND METHODS

Instrumentation

Automated Fmoc continuous flow SPPS was performed on a Pioneer Peptide Synthesis System (PerSeptive Biosystems, Framingham, USA) or a MilliGen 9050 automated peptide synthesizer (Millipore, Milford, USA). Manual continuous flow SPPS was performed using a CRB Fmoc peptide synthesizer equipped with UV monitoring as previously described [11]. Reverse-phase high-performance liquid chromatography (RP-HPLC) was performed using a Waters PDA instrument (Milford, USA) controlled by Millennium 32 software. Linear gradients of 0.1% TFA in acetonitrile (ACN) in 0.1% aqueous TFA (Buffer A) were used throughout. Vydac C18 RP-HPLC columns were used exclusively. Columns with dimensions 22 × 250 mm (preparative, 10 ml/min flow rate), 10 × 250 mm (semi-preparative, 4 ml/min) and 4.6 × 250 mm (analytical, 1.5 ml/min) were used. Matrix assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF-MS) data were acquired on a Bruker BIFLEX instrument (Franzen, Germany) in linear mode at 19.5 kV with delayed ion extraction. Capillary electrophoresis (CE) was performed using a Beckman P/ACE System 2100 CE (Fullerton, USA) equipped with a 50-cm glass capillary of 50 μm internal diameter. The potential difference used was 15 kV and the analysis was run at pH 2.5 in sodium citrate buffer. Quantitative amino acid analysis (AAA) was performed on Fmoc-derivatized hydrolysates using a GBC amino acid analysis system (Melbourne, Australia) equipped with an ODS HYPERSIL reverse-phase column (Runcorn, UK) and an LC1250 fluorescence detector.

Reagents for Peptide Synthesis

Amino acids used were purchased from Auspep (Melbourne, Australia), PerSeptive Biosystems (Framingham, USA), Bachem (Bubendorf, Switzerland) and Peninsula (Belmont, USA). Chiral amino acids used were all of L-configuration. Side-chain protecting groups used were: Asp(O*t*Bu), Cys(Trt), Gln(Trt), Glu(O*t*Bu), Lys(Boc), and Thr(*t*Bu). Synthesis was performed using Pepsyn K resin (Calbiochem-Novabiochem, Läufelfingen, Switzerland) and PEG-PS-PAL resin (PerSeptive). 3-Bromopropionic acid and DBU were purchased from Sigma-Aldrich (Melbourne, Australia). *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluorophosphate (HBTU) was purchased from Calbiochem-Novabiochem, HOBt was purchased from Fluka (Buchs, Switzerland). All other reagents used were of the highest standard commercially available.

Pepsyn K Thioester Resin Preparation

Ethylene diamine-treated Pepsyn K resin (0.44 g, 0.11 mmol/g) was suspended in DMF (5 ml) in a polycarbonate filter syringe. 3-Bromopropionic acid (eight equivalents, 0.4 mmol) was added to the resin as its symmetric anhydride in dichloromethane (DCM; 10 ml) and allowed to stand for 30 min. The resin was then washed with DCM and the coupling repeated. A 2,4,6-trinitrobenzene sulfonic acid (TNBSA) test performed at this time was slightly

positive. After acetylation for 10 min using acetic anhydride:diisopropylethylamine (DIEA):DMF 15:15:70 (v:v:v) (2.86 ml total), a negative TNBSA test was obtained. The resin was washed with DMF and treated twice for 20 min with 10% thiolacetic acid and 10% DIEA in DMF (10 ml), with DMF washing between treatments. The resin was then washed with DMF and treated with 10% 2-mercaptoethanol and 10% DIEA in DMF (10 ml) for 2×20 min. After DMF washing, Fmoc-Gly-OH (eight equivalents, 0.4 mmol, 0.119 g), and HBTU (7.9 equivalents, 0.395 mmol, 0.150 g) were dissolved in DMF (ca 1 ml). DIEA (12 equivalents, 0.6 mmol, 102 μl) was added and the mixture pre-activated for 2 min, added to the resin (suspended in DMF (ca 5 ml)) and stirred occasionally for 1 h. The resin was washed with DMF and the coupling repeated, yielding a negative TNBSA test.

SPPS of Human α-Synuclein 77–86COSR (Syn77–86COSR) on Pepsyn K

Continuous flow Fmoc solid phase peptide synthesis methodology was used throughout. N^{α}-Fmoc deprotection was performed by a continuous flow of 1% DBU/1% HOBt/DMF (v/w/v) for 10 min. Acylation was performed using Fmoc-amino acid-OH (five equivalnets, 0.25 mmol), HBTU (4.9 equivalents, 0.245 mmol) and DIEA (7.5 equivalents, 0.375 mmol) in DMF (1 ml), pre-activated for 2 min and recirculated through the resin for 10 min.

Cleavage of Syn77–86COSBn from Pepsyn K

Peptide–resin (0.3g) was swollen for 30 min in DCM and treated with 10% benzylmercaptan (BnSH; 10 ml) and 10% DIEA in DMF (v/v/v) for 5×30 min in a polycarbonate filter syringe. The resin was washed with DCM (3×10 ml) and the combined solution was concentrated *in vacuo*. The clear, colourless oil obtained was treated with 95% aqueous TFA for 2 h. The solution was sparged with N_2 to a total volume of ca 10 ml and the product precipitated with cold diethyl ether (Et_2O). After additional trituration with Et_2O, the product was lyophilized from 10% aqueous ACN containing 0.1% TFA to yield 4.65 mg of crude Syn77–86 benzyl thioester (Syn77–86COSBn).

Purification of Syn77–86COSBn

Crude Syn77–86COSBn (4 mg) was dissolved in 0.1% aqueous TFA (1.5 ml) along with a few drops of ACN to aid dissolution, filtered (0.45 μm) and purified using semi-preparative RP-HPLC (15% B to 35% B in Buffer A over 30 min). The major product (R_t 18.0 min) was collected and lyophilized (yield 0.58 mg, 14.5% from crude). The product eluted as a single peak from analytical RP-HPLC (Figure 3), R_t 13.0 min. No impurities were detected by MALDI-TOF-MS (MH^+ found = 1064.9, calcd. = 1065.3).

Ligation of Syn77–86COSBn to a Test Peptide

The peptide CGRGYARA had been synthesized previously by automated Fmoc-SPPS. Syn77–86COSBn (0.58 mg, 0.54 μmol) and CGRGYARA (0.46 mg, 0.54 μmol) were dissolved in freshly degassed 6 M guanidine–HCl (GdHCl)/NaH_2PO_4 buffer (180 μl), pH 7.5, with the addition of 2% thiophenol (PhSH) and 1% BnSH (v/v). The mixture was vortexed occasionally and the progress of reaction monitored by analytical RP-HPLC (10% B to 90% B in A over 30 min, 20 μl per injection). Samples were taken for analysis at 0, 1, 2 (Figure 4), 3, 4, and 22 h, respectively, after which the reaction was 97% complete.

PEG-PS-PAL Thioester Resin Preparation

Fmoc-PEG-PS-PAL resin (1.0 g, 0.15 mmol/g) was swollen in DMF. The Fmoc group was removed using 1% DBU/DMF, the resin was washed with DMF for 10 min and a positive TNBSA test was obtained. 3-Bromopropionic acid (ten equivalents, 1.5 mmol, 0.229 g) and HBTU (9.8 equivalents, 1.47 mmol,

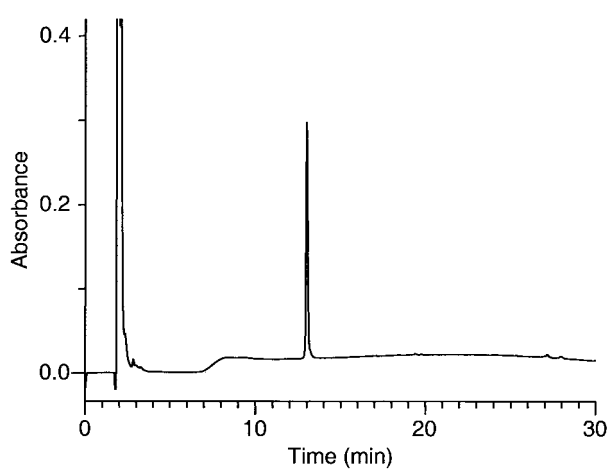

Figure 3 Analytical RP-HPLC profile of purified Syn77–86COSBn. The required product eluted at 13.0 min using a linear gradient of 10% Buffer B to 90% Buffer B in Buffer A over 30 min. MALDI-TOF-MS detected a single species with MH^+ = 1064.9 (calcd. = 1065.3).

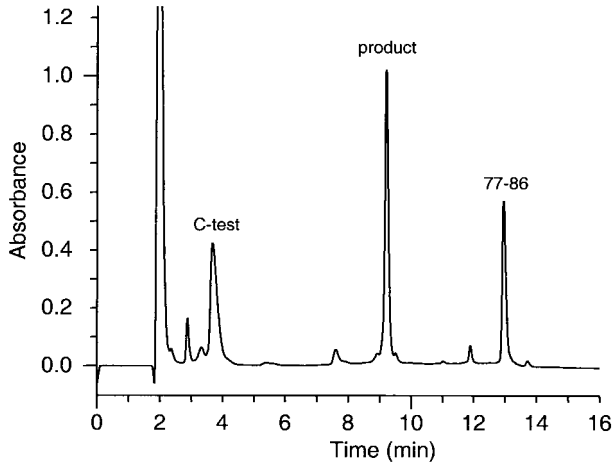

Figure 4 RP-HPLC profile showing progress of Syn77–86COSBn (77–86) ligation to the test sequence CGRGYARA (C-test) after 2 h. Peak identities were confirmed using MALDI-TOF-MS ($R_t = 3.7$ min, MH^+ found = 853.4, calcd. for C-test = 854.0; $R_t = 9.2$ min, MH^+ found = 1795.0, calcd. for product = 1794.1; $R_t = 13.0$ min, MH^+ found = 1066.3, calcd. for 77–86 = 1065.3).

0.558 g) were dissolved in DMF (2 ml). DIEA (15 equivalents, 2.25 mmol, 382 μl) was added, the mixture pre-activated for 2 min and recirculated through the resin for 2 h. The resin was washed with DMF and the coupling repeated overnight. After flushing with DMF for 10 min, a negative TNBSA test was obtained. The resin was then acetylated and converted to the free thiol form as described for Pepsyn K, except that the thiolacetic acid/DIEA treatments and the thiolysis reactions were each performed for 30 min. After DMF washing, Fmoc-Gly-OH (eight equivalents, 1.2 mmol, 0.357 g), HBTU (7.9 equivalents, 1.18 mmol, 0.449 g) and DIEA (12 equivalents, 1.8 mmol, 306 μl) were dissolved in DMF (2.5 ml) and recirculated through the resin for 1 h. The resin was washed again with DMF and the coupling repeated yielding a negative TNBSA test. The derivatized resin (0.966 g) was recovered and a quantitative Fmoc test gave an average resin substitution of 0.087 mmol/g.

SPPS of Syn77–86COSR on PEG-PS-PAL

Synthesis was performed as for Syn77–86COSR on Pepsyn K, except that N^{α}-Fmoc deprotection of G^{86}, A^{85}, G^{84} and E^{83} were performed by 4×2 min flow treatments with 1% DBU/1% HOBt/DMF interspersed with 10 min DMF flushes. Amino acid acylations were performed for 30 min, and V^{77} was coupled as Boc-Val-OH due to difficulties previously experienced with N^{α}-Fmoc deprotection of this residue.

Cleavage of Syn77–86COS$(CH_2)_2$CONH$_2$ from PEG-PS-PAL

The peptide–resin (1.044 g) was stirred for 2 h with 95% aqueous TFA along with sufficient triethylsilane (TES) to suppress yellow colouration. The resin was filtered and washed with 10×2 ml of TFA. The solution was then sparged with N_2 to a total volume of 5 ml and isolated by cold Et_2O precipitation. The product was then lyophilized from 20% aqueous ACN (yield 32 mg, hydroscopic).

Purification of Syn77–86COS$(CH_2)_2$CONH$_2$

Crude Syn77–86COS$(CH_2)_2$CONH$_2$ (32 mg) was dissolved in 0.1% TFA (aq.; 6 ml) along with a few drops of ACN, filtered (0.2 μm) and purified using preparative RP-HPLC (5% B to 15% B in A over 1 h). The major peak (R_t 29 min) was collected and lyophilized to yield 3.83 mg (12% from crude) of purified Syn77–86COS$(CH_2)_2$CONH$_2$ (hydroscopic). The yield of purified thioester based on both the calculated resin substitution and AAA data was 4%. Analytical RP-HPLC (5% B to 15% B in A over 30 min) $R_t = 17.1$ min, purity = 91% by area. MALDI-TOF-MS (Figure 5). CE $R_t = 10.8$ min, purity = 88% by area. AAA gave the following amino acid ratios (theory in parentheses): Glu/Gln 1.99 (2), Gly 2.17 (2), Thr 0.83 (1), Ala 2.37 (2), Val 1.97 (2), Lys 0.81 (1).

Synthesis of Human α-Synuclein 87–100 (S87C mutant) (Syn87–100 S87C)

Human α-synuclein 87–100 (S87C mutant) was synthesized by automated Fmoc-SPPS and purified by preperative RP-HPLC (10% B to 50% B in A over 30 min). Analytical RP-HPLC: (10% B to 50% B in A over 30 min), $R_t = 16.1$ min, purity 91% by peak area. MALDI-TOF-MS: MH^+ found = 1464.9, MH^+ calcd. = 1464.8. CE: $R_t = 12.3$ min, purity 92% by peak area. AAA ratios (theory in parentheses): Asp/Asn 1.06 (1), Glu/Gln 1.10 (1), Gly 1.17 (1), Thr 1.06 (1), Ala 3.16 (3), Val 0.97 (1), Ile 0.78 (1), Leu 0.93 (1), Phe 1.31 (1), Lys 1.65 (2), Cys not detected (1).

Synthesis of Human α-Synuclein 77–100 (S87C Mutant) (Syn77–100 S87C)

Purified Syn77–86COS$(CH_2)_2$CONH$_2$ (3.83 mg, 3.66 μmol) and Syn87–100 S87C (5.37 mg, 3.66 μmol) were dissolved in freshly degassed 6 M GdHCl/

NaH_2PO_4 (610 μl), pH 7.5 along with 2% PhSH and 1% BnSH (v/v). The mixture was vortexed occasionally for 23 h. Monitoring by RP-HPLC was performed (Vydac C18, 5% B to 90% B in A over 30 min, 10 μl per injection) at 0, 1, 2 (Figure 6), 3, 19 and 23 h, respectively. The reaction was complete after 23 h. Syn77–100 S87C was collected by preperative RP-HPLC (10% B to 60% B in A over 30 min) and lyophilized to yield the required product (3.94 mg), in a yield by mass of 45% for the ligation step. Analytical RP-HPLC (Figure 7), $R_t = 16.3$ min, purity = 95% by area. MALDI-TOF-MS (Figure 7). CE $R_t = 11.8$ min, purity = 86% by area. AAA gave the following ratios (theory in parentheses): Asp/Asn 0.93 (1), Glu/Gln 2.85 (3), Gly 3.51 (3), Thr 1.81 (2), Ala 5.96 (5), Val 2.94 (3), Ile 0.93 (1), Leu 1.06 (1), Phe 2.15 (1), Lys 2.65 (3), Cys not detected (1). The ratios for Ala and Gly are higher than expected, presumably due to minor impurities, and the ratio for Phe is high due to an analysis artifact. All other ratios are in good agreement with theory.

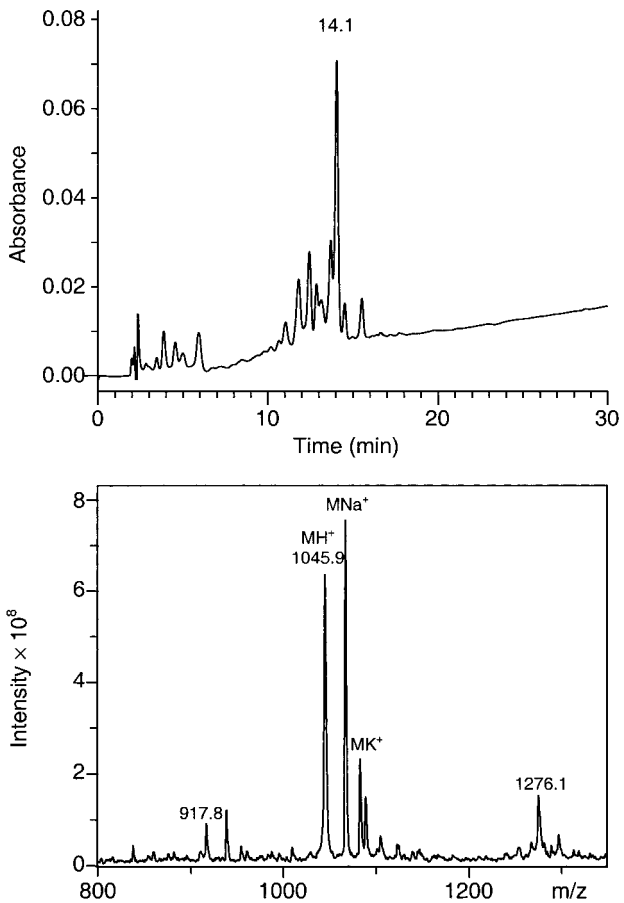

Figure 5 Crude RP-HPLC profile of Syn77–86COS$(CH_2)_2CONH_2$ (top), and MALDI-TOF-MS data for the purified major component (bottom) corresponding to the required material (MH^+ calcd. = 1046.2). Minor impurities at $m/z = 917.8$ (Ala + Gly deletion) and $m/z = 1276.1$ (required material + 230 Da, unidentified) were also detected.

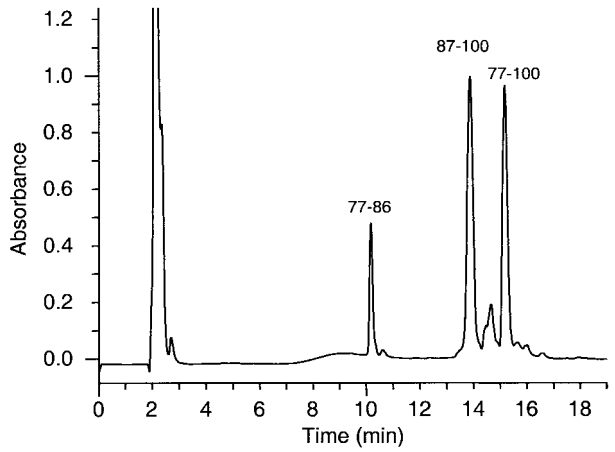

Figure 6 RP-HPLC profile showing progress of Syn77–86COS$(CH_2)_2CONH_2$ (77–86) ligation to Syn87–100 S87C after 2 h. Peak identities were confirmed using MALDI-TOF-MS ($R_t = 10.2$ min, MH^+ found = 1046.8, calcd. for 77–86 thioester = 1046.2; $R_t = 13.9$ min, MH^+ found = 1465.2, calcd. for 87–100 = 1465.8; $R_t = 14.7$ min, MH^+ found = 1065.4, calcd. for 77–86 Bn thioester intermediate = 1065.3; $R_t = 15.2$, MH^+ found = 2404.1, calcd. for Syn77–100 S87C = 2405.9).

Aspartimide Formation Study

The aspartimide-prone hexapeptide fragment of scorpion toxin II (VKDGYI) [12] was synthesized on Pepsyn K resin using automated Fmoc-SPPS. A synthesis in which the base labile Asp-Gly bond was protected by the incorporation of *N,O*-BisFmoc-Hmb-Gly-OPfp was also performed [13]. The peptide–resin samples were treated with either 1% DBU/DMF (v/v) or 1% DBU/1% HOBt/DMF (v/w/v) for 24 h, as shown in Table 1, washed with DMF, methanol and Et_2O and dried *in vacuo*. Cleavage was performed using 92.5% TFA/5% H_2O/2.5% TES, the products isolated by cold Et_2O precipitation and lyophilized from 0.1% aqueous TFA. Each sample was then dissolved in 0.1% TFA (2 ml), and analysed by analytical RP-HPLC (10% B to 40% B in A over 30 min) to determine the extent of side-product formation.

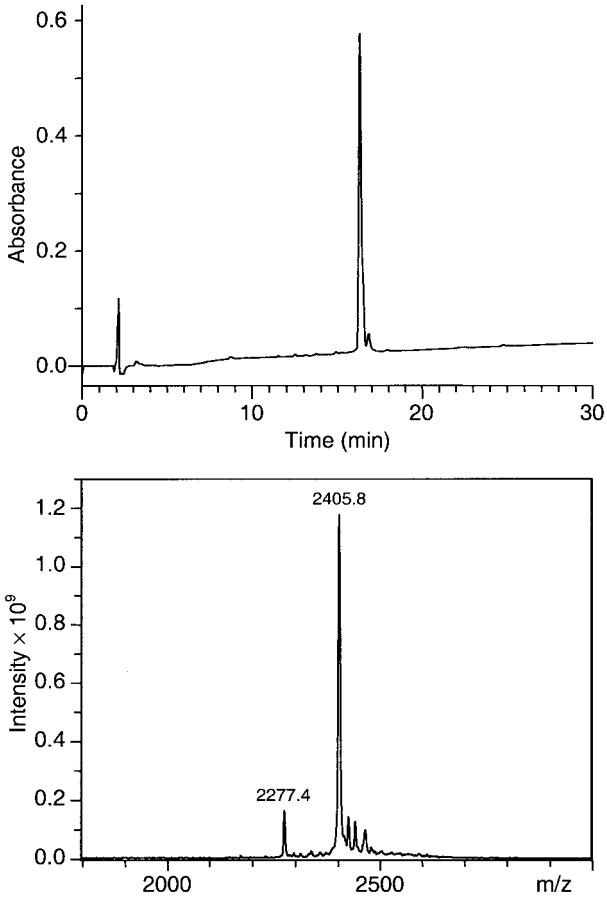

Figure 7 RP-HPLC profile (top) and MALDI-TOF-MS (bottom) of Syn77–100 S87C from ligation. A minor Gly-Ala deletion ($MH^+ - 128$ Da) was detected.

RESULTS AND DISCUSSION

Model Thioester Synthesis and Ligation

To determine the viability of the proposed peptide thioester synthesis methodology, Syn77–86COSR was prepared on Pepsyn K resin. Model studies had shown the addition of 1% HOBt to the 1% DBU/DMF Fmoc-deprotection solution not only suppressed aminolysis of the thioester linker as shown previously [7], but also significantly reduced the rate of N^{α}-Fmoc deprotection (data not shown), which may have been problematic during SPPS. The crude synthetic product was found by RP-HPLC to contain a major product, which was identified by MALDI-TOF-MS as the required material. Several minor products were also present, including Gly and Gly-Ala *C*-terminally truncated species. These were attributed to cleavage of the resin bound amino acid during Fmoc deprotection by aminolysis followed by acylation of the resulting free thiol support by the incoming activated amino acid. No products with intact *N*-terminal Fmoc groups were detected, indicating complete Fmoc deprotection was achieved throughout. After purification, the target material was obtained in good purity as determined by RP-HPLC (Figure 3) and MALDI-TOF-MS.

Interestingly, the only aminolysis derived deletion sequences detected were in the first two residues coupled. No Gly-Ala-Gly or longer deletions were observed. It appears that the thioester linker was protected from nucleophilic attack by steric hindrance caused by the growing peptide chain. Therefore, although highly optimized Fmoc deprotection conditions are required for the *C*-terminal two or three amino acids in order to limit deletion sequence formation, the protective effect of the growing peptide chain should enable more stringent Fmoc removal conditions to be applied after this early stage.

Ligation of synthetic Syn77–86COSBn to the test peptide CGRGYARA occurred rapidly under standard solution conditions [6]. The addition of BnSH and PhSH to the ligation solution has been found to increase the rate of native chemical ligation by the formation of reactive aromatic thioester species,

Table 1 Study of Aspartimide Formation in Scorpion Toxin II Fragment Induced by 1% DBU/1% HOBt/DMF (v/w/v)

Peptide sample treated on Pepsyn K resin	Solution used for base treatment	Target peptide obtained (% of total area)
VKDGYI	1% DBU/DMF (v/v)	19.5
VKDGYI	1% DBU/1% HOBt/DMF (v/w/v)	50.1
VKD(Hmb)GYI	1% DBU/1% HOBt/DMF (v/w/v)	97.0

An improved yield of target peptide was obtained by including 1% HOBt in the 1% DBU/DMF solution, but significant by-product formation still occurred. Hmb backbone amide protection completely inhibited by-product formation for both 1% DBU/1% HOBt/DMF and 1% DBU/DMF (not shown).

which are then more rapidly displaced by incoming thiols than the parent aliphatic thioesters [6,14]. In this system, any rate enhancement obtained would have been negligible since a benzyl thioester species had been prepared initially. However, the presence of these thiols in the ligation buffer would aid in the prevention of oxidation, and subsequent dimerization of the free cysteine containing test peptide. Alternatively, the addition of trialkylphosphines to ligation buffers has also been shown to effectively prevent disulfide formation and enhance ligation yield [15].

Preparation of Syn77–86COS$(CH_2)_2$CONH$_2$

A limitation of the peptide thioester cleavage method used in the model study was that it required cleavage of a fully protected peptide thioester followed by side-chain deprotection. Poor solubility is an inherent characteristic of many fully protected peptides and would limit the broad applicability of this approach due to the handling difficulties associated with these species. Furthermore, ensuring complete thiolysis to liberate the fully protected peptide from the resin could be problematic. Therefore, a method enabling peptide thioester resin cleavage and side-chain deprotection in a single step was required. A well-defined approach was the direct production of an alkyl peptide thioester derived from 3-mercaptopropionic acid [5,6] as either a *C*-terminal peptide acid or amide. Esterification of 3-bromopropionic acid onto a 4-hydroxymethylphenoxyacetic acid (HMPA) linker for peptide acid synthesis proved troublesome with only low yields being obtained. Therefore, the peptide thioester was prepared with an amide *C*-terminal on PEG-PS-PAL resin.

A quantitative Fmoc test indicated that the substitution of the thioester resin obtained after derivatization was only 58% of the initial resin substitution. The 50% piperidine in DCM solution used for the test would attack the thioester linker possibly affecting the result of this test. However, piperidine should have completely cleaved the Fmoc group, whether bound to the solid phase, or in solution, within the 30 min treatment, so this is unlikely to reduce the accuracy of the result. Recoupling of Fmoc-Gly failed to further increase resin substitution and after double coupling of 3-bromopropionic acid to PEG-PS-PAL, a TNBSA test indicated complete coupling. It seems, therefore, that either reaction with thiolacetic acid/DIEA or the thiolysis with 2-mercaptoethanol/DIEA failed to go to completion. Use of a pre-formed linker would prevent this problem, but use of more rigorous on-resin derivatization conditions should also give satisfactory yields.

To limit aminolysis-derived *C*-terminal deletion sequence formation during peptide thioester preparation, further optimization of the N^{α}-Fmoc deprotection conditions used was required. It was decided to deprotect the peptide–resin using multiple short (2 min) exposures with 1 % DBU/1% HOBt/DMF in order to ensure complete N^{α}-Fmoc removal while limiting nucleophilic breakdown by reducing the time the peptide resin was exposed to the deprotection solution [16]. This precaution was only used for the first four residues as the model study indicated susceptibility to aminolysis decreased with increasing peptide chain length. The crude Syn77–86COS$(CH_2)_2$CONH$_2$ synthesized contained Gly and Gly-Ala deletion sequences as seen previously, but the products RP-HPLC profile (Figure 5, top) was significantly improved compared with the crude profile obtained in the model study (not shown). After purification, the target thioester was obtained in 4% overall yield. Clearly, further optimization of the deprotection conditions used early in the synthesis is required as aminolysis-derived products were still observed in small but significant amounts in this synthesis and their formation is reflected in the low yield of target peptide. Under the conditions used, it appears that N^{α}-Fmoc deprotection occurs more rapidly than aminolysis-derived by-product formation, since a major product of the required structure has been obtained in each synthesis. It is possible that further limitation of by-product formation may be achieved by reducing the number of treatments with 1% DBU/1% HOBt/DMF solution from 4 to 2–3 2-min treatments per residue. The synthesis may also benefit from an increased flow rate allowing the peptide–resin to be exposed to a similar volume of deblock reagent while exposure time could be further reduced as per the method used to reduce diketopiperazine formation during SPPS of sensitive sequences [17]. The presence of amine impurities derived from degradation of DMF may also cause thioester breakdown. Reduced by-product formation and improved overall yield may result from the use of a different solvent, such as DMSO [7].

Synthesis of Syn77–100 S87C

The ligation of the peptide thioester with Syn87–100 S87C was complete after approximately 19 h.

Interestingly, the benzyl thioester intermediate species was observed in the RP-HPLC profiles obtained during the ligation reaction (Figure 6) along with Syn77–86COS$(CH_2)_2$CONH$_2$, Syn87–100 S87C and the product, Syn77–100 S87C. No thiophenol derived thioester species was detected. Yield for the ligation was 45% by mass. Purity was found to be 95% by RP-HPLC (Figure 7, top) and 86% by capillary electrophoresis (not shown). MALDI-TOF-MS indicated the required product had been formed (Figure 7, bottom) together with a minor Gly-Ala deletion peptide. Syn77–100 S87C was obtained in 45% yield by mass.

Aspartimide Formation Study

The results of this study are shown in Table 1. Treatment of the resin bound scorpion toxin II peptide with 1% DBU/DMF caused significant side-product formation, with only 19% of the target peptide being obtained. Furthermore, this product eluted slightly earlier (R_t observed = 11.6 min) than the target peptide obtained in the remainder of the study (R_t approximate = 11.8 min). This indicates that the 'target' peptide obtained in this test may in fact have been a β-peptide by-product, the formation of which DBU is known to induce [18]. An increased yield of target peptide was obtained when 1% HOBt was added to the 1% DBU/DMF solution, but by-product formation was still significant. This is in agreement with previous studies, indicating that the addition of HOBt to Fmoc deprotection solutions can, in most cases, reduce aspartimide formation [16,19]. Backbone amide protection of the sensitive Asp-Gly bond using the Hmb group [20–22] attenuated by-product formation when the protected peptide–resin was treated with 1% DBU/DMF with or without 1% HOBt. The base lability of the scorpion toxin II hexapeptide is extreme [12,16], and is mirrored in only a minority of target sequences. The improved degree of protection against aspartimide formation conferred by HOBt in the deblock solution may, in many cases, be sufficient to prevent significant amounts of such by-products being formed. In sequences with significant base lability, however, the use of Hmb backbone amide protection is required if the Fmoc native chemical ligation methodology developed in this study is to be successfully applied.

CONCLUSION

The methodology described in this study provides an entirely Fmoc-SPPS based route to peptide thioesters and describes their use in native chemical ligation, thereby bringing this technique within the grasp of laboratories lacking facilities or expertise in Boc-SPPS. Although the Fmoc deprotection conditions used are not yet fully optimized, they are effective and provide an excellent foundation for future refinement. The observation that aminolysis of the thioester linker is suppressed by steric hindrance from the growing peptide chain should enable preparation of significantly longer peptide chains than described in this work. The use of 1% DBU/1% HOBt/DMF for Fmoc deprotection offers improved protection against aspartimide formation compared with 1% DBU/DMF, but if sequences known to be highly base labile are to be prepared using this approach then Hmb backbone amide protection of the offending residues will be required.

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