Ramli S, Gentle IR, Ross BP (2009) Efficient manual Fmoc solid-phase synthesis of the N-terminal segment of surfactant protein B (SP-B(1-25)). Protein and peptide letters 16: 810–814.

doi:10.2174/092986609788681706

PMID 19601911

Journal home page: http://www.benthamscience.com/ppl/index.htm

Efficient Manual Fmoc Solid-Phase Synthesis of the N-Terminal Segment of Surfactant Protein B (SP-B_{1-25})

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Abstract: The N-terminal 25 residue segment of human surfactant protein B (SP-B_{1-25}) was synthesised in 26% yield by manual Fmoc solid-phase peptide synthesis (Fmoc SPPS) using low-loading Fmoc-Gly-Wang resin. Substantial oxidation of Met^{21} occurred during the synthesis, and the addition of Bu_4NBr to a TFA/water/EDT/TIS cleavage cocktail enabled facile reduction of $Met(O)^{21}$-SP-B_{1-25} to SP-B_{1-25}. The methods described herein are generally applicable to the Fmoc SPPS of difficult sequences containing methionine.

Keywords: Surfactant protein B (SP-B), Fmoc solid-phase peptide synthesis, peptide aggregation, low-loading resin, methionine sulfoxide, tetrabutylammonium bromide.

INTRODUCTION

Surfactant protein B (SP-B) is a 79-residue 8-kDa protein found in mammalian lung surfactant predominantly as a disulfide linked homodimer [1]. SP-B is known to have extensive interaction with phospholipids to enhance their adsorption and dynamic film

behaviour. It increases the ability of surfactant phospholipids to adsorb to the air-liquid interface and facilitates the formation of surfactant monolayers by accelerating the spreading of phospholipids and stabilizing the pulmonary alveoli [2-7].

A great deal of attention has been focused on the N-terminal 25 residue segment of SP-B which faithfully reproduces the functional aspects of the full-length protein [8, 9]. The primary sequence of human SP-B_{1-25} is FPIPL^{5}PYCWL^{10}CRALI^{15}KRIQA^{20}MIPKG^{25}. The first six residues are highly hydrophobic and form an extended conformation (possibly β-sheet), residues 7-9 are irregularly structured, residues 10-22 form an amphipathic α-helix, and the last three residues are random structure [10, 11].

As part of our research program investigating the interactions between surfactant proteins and lipids, we undertook the preparation of human SP-B_{1-25} by manual solid-phase peptide synthesis (SPPS) using 9-fluorenylmethoxycarbonyl (Fmoc) amino acids [12]. Fmoc SPPS is more convenient than *tert*-butoxycarbonyl (Boc)-SPPS because the peptide can be cleaved from the resin using relatively mild reagents (e.g. a mixture of TFA/TIS/water), whereas Boc-SPPS requires hazardous hydrogen fluoride and associated specialised equipment for cleavage [12]. It was anticipated that the Fmoc synthesis of SP-B_{1-25} could be problematic because of hydrophobic domains and the presence of a readily oxidisable methionine in position 21 of the sequence. Although the synthesis of SP-B_{1-25} using Boc and Fmoc strategies has been reported elsewhere, automated peptide synthesizers were used and many important experimental details were not published [11, 13, 14]. Herein we provide a cogent description of the manual Fmoc SPPS of SP-B_{1-25}, an example of the synthesis of a difficult readily oxidised peptide sequence.

MATERIALS AND METHODS

Materials

All reagents were AR grade unless stated otherwise. Amino acids, Rink amide 4-methylbenzhydrylamine (MBHA) resins, *O*-(benzotriazol-1-yl)-*N*, *N*, *N*', *N*'-tetramethyluronium-hexafluorophosphate (HBTU) and *O*-(7-azabenzotriazol-1-yl)-*N*, *N*, *N*', *N*'-tetramethyluronium-hexafluorophosphate (HATU) were purchased from Novabiochem (Laufelfingen, Switzerland). Piperidine (99.5%) was purchased from Auspep (Melbourne, Australia). *N*, *N*-Dimethylformamide (DMF) (Anhydroscan grade) was purchased from Labscan (Bangkok, Thailand). Trifluoroacetic acid (TFA) (> 99.9%) was purchased from SDS (Peypin, France). *N*, *N*-Diisopropylethylamine (DIEA) (99.5% redistilled grade), *N*, *N*'-diisopropylcarbodiimide (DIC), triisopropylsilane (TIS), and tetrabutylammonium bromide were purchased from Sigma-Aldrich (St. Louis, MO). 1, 2-Ethanedithiol (EDT) was purchased from SAFC (St. Louis, MO). Dichloromethane (DCM) was purchased from Merck (Kilsyth, Victoria, Australia). Methanol was purchased from Ajax Finechem (Seven Hills, NSW, Australia). *N*-(2-Hydroxy-4-methoxybenzyl)-L-leucine [(Hmb)Leu-OH] was prepared according to the method of Nicholás *et al*. [15] and was converted to Fmoc-(FmocHmb)Leu-OH using the method of Johnson *et al*. [16].

Peptide synthesis

(a) General methods. Peptide syntheses were accomplished manually by a stepwise solid-phase procedure in 20 mL glass reaction vessels with teflon-lined screw caps. The following protocol was applied (cycle, reagent, time/mode): (i) deprotection, 20% v/v piperidine in DMF, 1 × 5 min shaking; (ii) deprotection, 20% v/v piperidine in DMF, 1 × 15

min shaking; (iii) flow wash, DMF, 30 s; (iv) coupling, activated amino acid, ~ 60 min shaking; (v) wash, DMF. *N*-α-Fmoc protected amino acids were used with the following side-chain protecting groups: Arg, Pbf; Cys, Trt; Gln, Trt; Lys, Boc; Trp, Boc; Tyr, *t*-Bu. Prior to attachment of the first residue, the resin was swelled with DCM for 1 h then washed thoroughly with DMF. The Fmoc group was removed from the resin by treatment with 20% v/v piperidine in DMF (2 × 30 min), and then the resin was washed thoroughly with DMF. Most amino acids were attached using HBTU/DIEA activation: amino acid (4 eq), HBTU (4 eq; 0.5 M HBTU in DMF), DIEA (5.3 eq). To minimise racemisation, the symmetric anhydride (4 eq) was used to couple Fmoc-Cys(Trt)-OH [17]: amino acid (8 eq) was dissolved in DCM (20 mL) and DMF (~ 15 drops) was added to ensure complete dissolution; the solution was cooled to 0 °C, DIC (4 eq) was added, and the solution was stirred for 30 min; the DCM was removed *in vacuo* and the residue was dissolved in DMF (~ 5 mL) and added to the resin; the mixture was typically shaken overnight (~ 18 h). The coupling efficiency for each amino acid was determined by the quantitative ninhydrin test [18] except for the residue immediately following a proline, which was routinely recoupled. Recoupling (a maximum of two times) was performed if the initial coupling efficiency was < 99.5%. After coupling of the final residue, the resin was washed successively with DMF, DCM, and methanol, then flushed with nitrogen gas. The resin was dried *in vacuo* over self-indicating silica dessicant (5-10 mesh). Unless stated otherwise, solvent A was 0.1% TFA in water; and solvent B was 0.1% TFA in 90% acetonitrile/10% water. **(b) Synthesis A.** The peptide was assembled on a 0.25 mmol scale using Fmoc-Gly-Wang resin (100-200 mesh, 0.85 mmol/g resin loading). Recouplings afforded an ultimate coupling efficiency of ≥ 97.6%. The dried resin was transferred to a round-bottomed flask and the peptide cleaved from the resin by

stirring in a solution of TFA/water/EDT/TIS 94:2.5:2.5:1 v/v (15 mL/g resin) for 2 h. The resin was removed by filtration and washed with TFA (2 × 5 mL). The filtrate was removed *in vacuo* to afford the crude product which was triturated with cold diethyl ether, dissolved in a mixture of solvent A and solvent B (1:1 v/v) and lyophilized. **(c) Synthesis B.** The peptide was assembled on a 0.125 mmol scale using Fmoc-Gly-Wang resin (100-200 mesh, 0.27 mmol/g resin loading). The initial coupling efficiency was ≥ 99.5% for all residues. The dried resin was transferred to a round-bottomed flask and the peptide cleaved from the resin by stirring in a solution of TFA/water/EDT/TIS 94:2.5:2.5:1 v/v (25 mL/g resin). After 2 h, Bu_4NBr (1151 mg) was added and the mixture was stirred for a further 15 min. The resin was removed by filtration and washed with TFA (2 × 5 mL). The filtrate was removed *in vacuo* to afford the crude product which was triturated with cold diethyl ether, dissolved in a mixture of solvent A and solvent B (1:1 v/v) and lyophilized. **(d) Synthesis C.** The peptide was prepared as per Synthesis B, except Fmoc-(FmocHmb)Leu-OH[14] was used in place of Fmoc-Leu-OH[14]. Fmoc-(FmocHmb)Leu-OH[14] was coupled using HATU/DIEA activation: amino acid (3 eq), HATU (3 eq; 0.5 M HATU in DMF), DIEA (4.3 eq). The next residue (Fmoc-Ala-OH[13]) was coupled to the sterically hindered (Hmb)Leu[14] as the symmetric anhydride (10 eq) using the same protocol used for Fmoc-Cys(Trt)-OH (above) except 20 eq of amino acid was used with 10 eq of DIC. **(e) Purification. (i) Preparative RP-HPLC.** The crude peptide was purified on a Waters HPLC system (Series 600 controller, 600F pump, 2996 PDA detector; Waters, Milford, MA) using a Gemini® C18 column (10 μm, 110 Å, 250 × 21.1 mm; Phenomenex, Torrence, CA) fitted with a guard cartridge (Phenomenex SecurityGuard™, Gemini® C18, 15 × 21.2 mm) with detection at 230 nm. The flow rate was 10 mL/min and the eluent composition comprised a linear gradient from 10% to 100%

solvent B over 30 min, then maintained at 100% solvent B for a further 20 minutes. Fractions (~ 8 mL) of the eluent were collected and examined for the desired peptide by electrospray ionisation mass spectrometry (ESIMS). **(ii) ESIMS.** Mass spectra (ESIMS) were recorded on a 3200 Q Trap® hybrid triple quadrupole/linear ion trap mass spectrometer (Applied Biosystems/MDS Sciex, Toronto, Canada) operating in positive-ion electrospray mode. The mobile phase for mass spectrometry was solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). A sample (50 μL) of a fraction from the preparative RP-HPLC was dissolved in a mixture of solvent A and solvent B (1:1 v/v, 1 mL) and this solution was infused into the mass spectrometer source at a flow rate of 20 μL/min. The mass spectrum was recorded by scanning the mass range 150-1700 by 0.1 amu, and fractions containing the desired peptide were identified. **(iii) Analytical RP-HPLC.** The purity of fractions containing the desired peptide was examined on a Shimadzu HPLC system (two LC-10AD pumps, DGU-20A3 degasser, SPD-M20A diode array detector, SIL-10AXL autoinjector, CBM-20A communications bus module; Shimadzu, Japan) using a Gemini® C18 column (5 μm, 110 Å, 250 × 4.6 mm; Phenomenex, Torrence, CA) fitted with a guard cartridge (Phenomenex SecurityGuard™, Gemini® C18, 4 × 3.0 mm) with detection at 214 nm. The flow rate was 1 mL/min with an eluent composition comprising a linear gradient from 25% to 100% solvent B over 15 min, then maintained at 100% solvent B for a further 10 minutes. A sample (50 μL) of each fraction was injected onto the system and pure fractions of the desired peptide were combined and lyophilized. **(f) Peptide characterisation.** The purified peptide was characterised by ESIMS, high resolution mass spectrometry (HRMS) and analytical RP-HPLC using two systems. **(i) ESIMS.** The mass spectrum was determined as describe in section (e) (ii) above except that the sample was

prepared by diluting the lyophilised peptide to a concentration of 5 μg/mL using a mixture of solvent A and solvent B (1:1 v/v). **(ii) HRMS.** High resolution mass spectra were recorded via loop injection on a micrOTOF-Q^{TM} ESI-Qq-TOF mass spectrometer with a multimode source (Bruker Daltonics, Bremen, Germany). **(ii) Analytical RP-HPLC.** The purity of the peptides was determined by analytical RP-HPLC using the Shimadzu HPLC system with detection at 214 nm. An aliquot (50 μL) of a solution of the peptide (1 mg/mL in solvent A/solvent B 1:1 v/v) was injected into the system and the purity was calculated by percent area. **System 1:** Gemini® C18 column (5 μm, 110 Å, 250 × 4.6 mm; Phenomenex, Torrence, CA) fitted with a guard cartridge (Phenomenex SecurityGuard^{TM}, Gemini® C18, 4 × 3.0 mm). The mobile phase was solvent A (0.1% TFA in water) and solvent B (0.1% TFA in 90% acetonitrile / 10% water). Samples were eluted at a flow rate of 1 mL/min with a linear gradient of 20-100% solvent B over 40 min then a further 10 min at 100% solvent B. **System 2:** Protein C4 (214TP54) column (5 μm, 300 Å, 250 × 4.6 mm; Vydac, Hesperia, CA) fitted with a guard cartridge (Phenomenex SecurityGuard^{TM}, widepore C4, 4 × 3.0 mm). The mobile phase was solvent A (0.1% TFA in water) and solvent B (0.1% TFA in 90% methanol / 10% water). Samples were eluted at a flow rate of 1 mL/min with a linear gradient of 20-100% solvent B over 40 min then a further 10 min at 100% solvent B.

RESULTS & DISCUSSION

SP-B_{1-25} was synthesized by three methods designated Synthesis A, Synthesis B and Synthesis C. All syntheses utilized manual Fmoc SPPS with primarily HBTU/DIEA activation and 20% v/v piperidine in DMF for removal of the N-terminal Fmoc group. SP-B_{1-25} was initially synthesized (Synthesis A) on 0.85 mmol/g Fmoc-Gly-Wang resin and the efficiency of the *first* coupling for each residue is illustrated in Figure **1-**Synthesis A. This

coupling efficiency was > 99.3% for residues 24 to 13 [Fmoc-Lys(Boc)-OH[24] to Fmoc-Ala-OH[13]], and recouplings ensured that the ultimate efficiency was always ≥ 99.5%. The initial coupling efficiency of the next residue, Fmoc-Arg(Pbf)-OH[12], was very poor at 84.9% and initial efficiency remained poor (≤ 98.5%; $average_{12\text{-}7}$ 92.9%) through to residue 7 [Fmoc-Tyr(*t*-Bu)-OH[7]]. Recouplings of residues 12 through 7 enabled an ultimate efficiency of ≥ 97.6%, however this was deemed unsatisfactory, especially when to achieve this value the coupling reaction sometimes had to be repeated three times, which greatly increased the financial cost and time required for the process. When this method (Synthesis A) was repeated the coupling efficiencies were almost identical to those illustrated in Figure **1-**Synthesis A (data not shown).

The poor coupling efficiency of residues 12 to 7 is probably due to aggregation resulting in poor solvation of the peptide-polymer matrix and consequently inaccessibility of the reactants and reagents to the N-terminal amino group. Although the precise nature of the aggregation is unclear, this phenomenon is often caused by self-association of the polymer-bound peptide through the formation of intermolecular hydrogen bonds resulting in a beta-sheet secondary structure [19]. For the initial synthesis of SP-$B_{1\text{-}25}$, the onset of aggregation occurred immediately following a sequence of hydrophobic amino acids, specifically Ala[13]–Leu[14]–Ile[15]–, and was ten residues after the addition of the last beta-sheet inhibiting proline residue (Pro[23]). It is known that hydrophobic sequences have a high propensity to aggregate, and that tertiary amide residues such as proline inhibit such aggregation for at least six residues [20].

The peptide was cleaved from the resin and the crude product was analysed by analytical RP-HPLC and ESIMS (Figure **2A**). The crude product contained predominantly an oxidised form of SP-B_{1-25}, in which methionine^{21} was oxidised to methionine sulfoxide [Met(O)]. The thioether side-chain of methionine is highly susceptible to air oxidation [21], and therefore effort was made during the synthesis to minimise exposure to air. Nevertheless, partial oxidation occurred and the ratio of Met(O)^{21}-SP-B_{1-25} to SP-B_{1-25} was ~ 2:1 (Figure **2A**).

Undoubtedly, the higher substitution value of the resin (0.85 mmol/g) exacerbated aggregation during the synthesis, and the ensuing necessity for multiple recouplings meant that exposure to air and subsequent oxidation of Met^{21} was promoted. Therefore, low-loading resin was considered as a solution to these problems. A second method (Synthesis B) for the synthesis of SP-B_{1-25} was trialled, utilising a low-loading Fmoc-Gly-Wang resin (0.27 mmol/g). The efficiency of the *first* coupling for each residue is illustrated in Figure **1**-Synthesis B and initial coupling efficiency was ≥ 99.5% for *all* residues. Upon completion of the synthesis, a small sample of the resin (50 mg) was subjected to cleavage conditions (identical to those used for Synthesis A) and the crude product was analysed by analytical RP-HPLC and ESIMS (Figure **2B**). This revealed that despite excellent coupling efficiencies, the crude product contained oxidised peptide with a ratio of Met(O)^{21}-SP-B_{1-25} to SP-B_{1-25} of ~ 1:1 (Figure **2B**). Met^{21} is close to the C-terminus of SP-B_{1-25} and accordingly it is introduced early in the synthesis making significant exposure to air oxygen and some degree of oxidation inevitable during manual SPPS. Consequently, a method to reduce the oxidised peptide was sought.

Two reagents that are suitable for the rapid reduction of methionine sulfoxide-containing peptides are: (a) a mixture of TMSBr/EDT in anhydrous solvent [22]; and (b) a mixture of Bu_4NBr/thioanisole/EDT/anisole in TFA [23]. In these mixtures, TMSBr and Bu_4NBr are reducing agents, and EDT, thioanisole, and anisole are scavengers that react with bromine generated during the redox reaction thus preventing bromine from reacting with aromatic amino acids or mediating cysteine oxidation. If a reductive cleavage is performed, as opposed to the reduction of a free peptide, then the scavengers also fulfil the normal role of trapping reactive carbocations generated during cleavage. We chose to execute a one-pot peptide cleavage and reduction, and our preferred cleavage cocktail was TFA/water/EDT/TIS which prevented the inclusion of TMSBr because it is sensitive to hydrolysis. Hence, resin from Synthesis B (896 mg) was suspended in a mixture of TFA/water/EDT/TIS 94:2.5:2.5:1 v/v (22 mL) and stirred. After two hours, Bu_4NBr (1151 mg) was added and the mixture was stirred for a further 15 min, followed by filtration, evaporation of the filtrate *in vacuo*, trituration of the crude product with cold diethyl ether, and lyophilisation. Analysis of the lyophilised powder by analytical RP-HPLC and ESIMS (Figure **2C**) indicated complete reduction of $Met(O)^{21}$-SP-B_{1-25} to SP-B_{1-25} with no apparent formation of by-products such as homodimer. Thus Bu_4NBr is a convenient reagent for the reduction of Met(O) to Met, and compared with previous reports which utilised the Reagent R cleavage cocktail (thioanisole/EDT/anisole in TFA) on a small scale (5-30 mg peptidyl resin) [23], the current study proves that Bu_4NBr is compatible with the popular TFA/water/EDT/TIS cleavage cocktail on a larger scale (896 mg peptidyl resin, ~ 0.1 mmol).

Purification of the crude peptide by preparative RP-HPLC afforded 94 mg of SP-B_{1-25} (26% yield). Figure **3** contains the analytical RP-HPLC chromatograms obtained from two systems, the ESIMS, and accurate mass data, for the purified peptide.

A third synthesis (Synthesis C) of SP-B_{1-25} was completed using the low-loading Fmoc-Gly-Wang resin (0.27 mmol/g) and incorporating (Hmb)Leu^{14} into the resin-bound peptide using Fmoc-(FmocHmb)Leu-OH. Hmb-amino acid residues inhibit aggregation by reversibly protecting the peptide backbone secondary amide bond as a tertiary amide thus preventing hydrogen bonding [24]. However, in this case no improvement to the purity of the crude product was observed when compared with Synthesis B (data not shown). Thus, the coupling of Fmoc-(FmocHmb)Leu-OH^{14} in place of Fmoc-Leu-OH^{14} is unnecessary when a low-loading resin (~ 0.27 mmol/g) is used.

SP-B_{1-25} was previously obtained using automated peptide synthesizers applying the Boc and Fmoc strategies, however significant experimental details were unclear. The manual Fmoc SPPS of SP-B_{1-25} described above permits the preparation of SP-B_{1-25} in good yield without the need for expensive automated synthesisers, hazardous hydrogen fluoride and associated specialised equipment for cleavage. The methods described in this paper are also generally applicable to the synthesis of other difficult methionine-containing peptides by Fmoc SPPS. Hydrophobic sequences have a high propensity to aggregate resulting in diminished coupling efficiency, and this synthesis highlights the effectiveness of a low-loading resin in averting such aggregation. Methionine is highly susceptible to air oxidation, however, Bu_4NBr can be incorporated into the familiar TFA/water/EDT/TIS cleavage cocktail to enable facile reduction of Met(O) to Met.

CONCLUSION

SP-B_{1-25} was synthesised in good yield by manual Fmoc SPPS using low-loading resin. Substantial oxidation of Met^{21} occurred during the synthesis, and a reductive cleavage incorporating Bu_4NBr into the cleavage cocktail enabled facile reduction of $Met(O)^{21}$-SP-B_{1-25} to SP-B_{1-25}. The use of a higher-loading resin resulted in aggregation upon deprotection of Fmoc-Ala^{13} and subsequent poor coupling efficiencies for residues 12 to 7. When a low-loading resin was used, no benefit was gained from the coupling of Fmoc-(FmocHmb)Leu-OH^{14} in place of Fmoc-Leu-OH^{14}.

ACKNOWLEDGEMENTS

We thank Mr Graham MacFarlane (The University of Queensland, School of Molecular and Microbial Sciences) for accurate mass measurements. This work was supported by the Ministry of Higher Education Malaysia (KPTM) and the National University of Malaysia (UKM).

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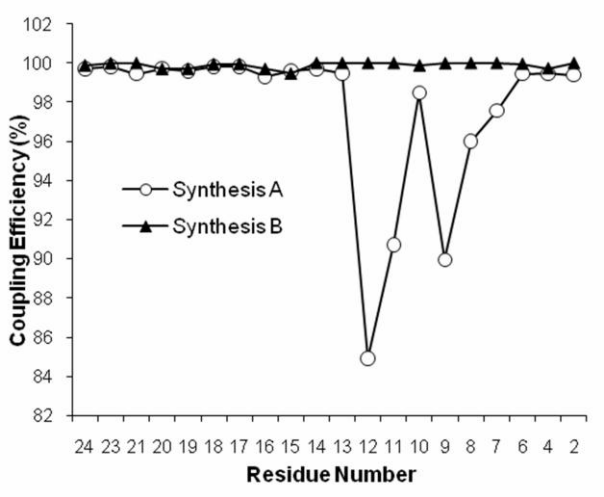
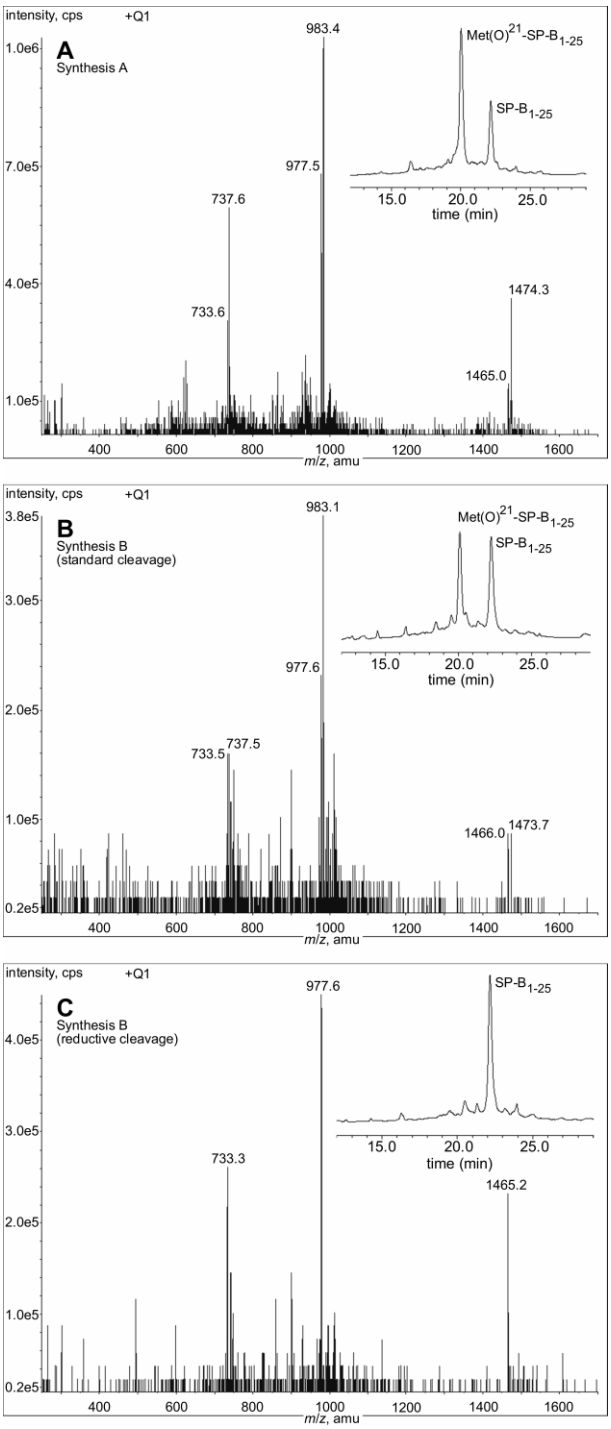
FIGURE LEGENDS:

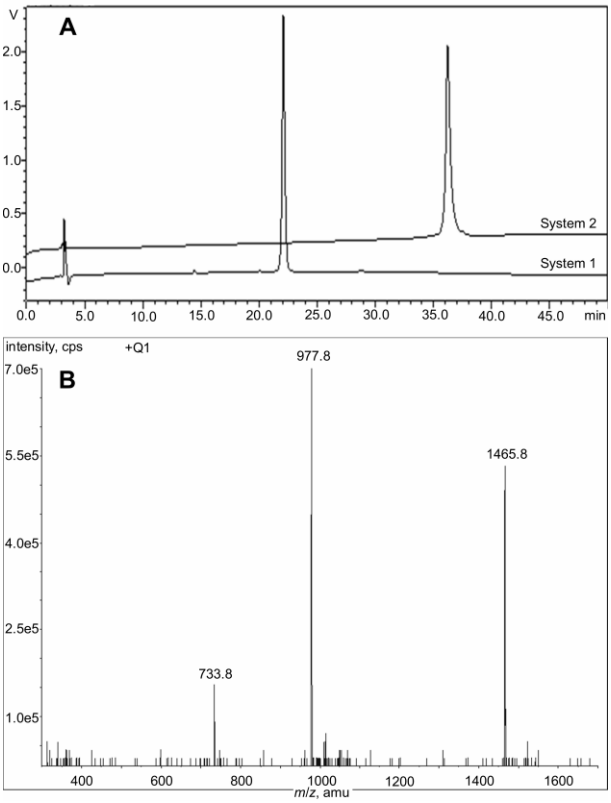
Figure 1. The efficiency of the *first* coupling for each residue in the synthesis of FPIPL^{5}PYCWL^{10}CRALI^{15}KRIQA^{20}MIPKG^{25}, as determined by the quantitative ninhydrin test. Data is absent for residues 22, 5, 3, and 1 because these residues are coupled to proline and the ninhydrin test cannot be performed on a secondary amine.

Figure 2. ESIMS and analytical RP-HPLC (System 1) data. (**A**) Synthesis A crude product containing Met(O)^{21}-SP-B_{1-25} and SP-B_{1-25}. ESIMS, *m*/*z*: 1474.3 $[M+O+2H]^{2+}$, 1465.0 $[M+2H]^{2+}$, 983.4 $[M+O+3H]^{3+}$, 977.5 $[M+3H]^{3+}$, 737.6 $[M+O+4H]^{4+}$ and 733.6 $[M+4H]^{4+}$. (**B**) Synthesis B crude product from standard cleavage of a small aliquot of resin, containing Met(O)^{21}-SP-B_{1-25} and SP-B_{1-25}. ESIMS, *m*/*z*: 1473.7 $[M+O+2H]^{2+}$, 1466.0 $[M+2H]^{2+}$, 983.1 $[M+O+3H]^{3+}$, 977.6 $[M+3H]^{3+}$, 737.5 $[M+O+4H]^{4+}$ and 733.5 $[M+4H]^{4+}$. (**C**) Synthesis B crude product from reductive cleavage, containing SP-B_{1-25} and no apparent Met(O)^{21}-SP-B_{1-25}. ESIMS, *m*/*z*: 1465.2 $[M+2H]^{2+}$, 977.6 $[M+3H]^{3+}$, and 733.3 $[M+4H]^{4+}$.

Figure 3. Analytical data for purified SP-B_{1-25} prepared by Synthesis B with reductive cleavage. (**A**) Analytical RP-HPLC: System 1, t_R 22.1 min, purity > 99%; System 2, t_R 36.2 min, purity > 99%. (**B**) ESIMS, *m*/*z*: 1465.8 $[M+2H]^{2+}$, 977.8 $[M+3H]^{3+}$, and 733.8 $[M+4H]^{4+}$. HRMS, *m*/*z* cacld for $C_{139}H_{226}N_{35}O_{28}S_3$ $[M+3H]^{3+}$ 976.5494, found 976.5481.


Coupling Efficiency (%)
102
100
98
96
94
92
90
88
86
84
82
Synthesis A
Synthesis B
24 23 21 20 19 18 17 16 15 14 13 12 11 10 9 8 7 6 4 2
Residue Number


intensity, cps
+Q1
A
Synthesis A
983.4
977.5
737.6
733.6
1474.3
1465.0
Met(O)21-SP-B1-25
SP-B1-25
time (min)
m/z, amu
B
Synthesis B
(standard cleavage)
983.1
977.6
737.5
733.5
1473.7
1466.0
C
Synthesis B
(reductive cleavage)
977.6
733.3
1465.2


A
V
2.0
1.5
1.0
0.5
0.0
System 2
System 1
0.0
5.0
10.0
15.0
20.0
25.0
30.0
35.0
40.0
45.0
min
B
intensity, cps
+Q1
7.0e5
5.5e5
4.0e5
2.5e5
1.0e5
977.8
1465.8
733.8
400
600
800
1000
1200
1400
1600
m/z, amu